A Multivalent Clade C HIV-1 Env Trimer Cocktail Elicits a Higher Magnitude of Neutralizing Antibodies than Any Individual Component

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ABSTRACT

The sequence diversity of human immunodeficiency virus type 1 (HIV-1) presents a formidable challenge to the generation of an HIV-1 vaccine. One strategy to address such sequence diversity and to improve the magnitude of neutralizing antibodies (NAbs) is to utilize multivalent mixtures of HIV-1 envelope (Env) immunogens. Here we report the generation and characterization of three novel, acute clade C HIV-1 Env gp140 trimers (459C, 405C, and 939C), each with unique antigenic properties. Among the single trimers tested, 459C elicited the most potent NAb responses in vaccinated guinea pigs. We evaluated the immunogenicity of various mixtures of clade C Env trimers and found that a quadrivalent cocktail of clade C trimers elicited a greater magnitude of NAbs against a panel of tier 1A and 1B viruses than any single clade C trimer alone, demonstrating that the mixture had an advantage over all individual components of the cocktail. These data suggest that vaccination with a mixture of clade C Env trimers represents a promising strategy to augment vaccine-elicited NAb responses.

IMPORTANCE

It is currently not known how to generate potent NAbs to the diverse circulating HIV-1 Envs by vaccination. One strategy to address this diversity is to utilize mixtures of different soluble HIV-1 envelope proteins. In this study, we generated and characterized three distinct, novel, acute clade C soluble trimers. We vaccinated guinea pigs with single trimers as well as mixtures of trimers, and we found that a mixture of four trimers elicited a greater magnitude of NAbs than any single trimer within the mixture. The results of this study suggest that further development of Env trimer cocktails is warranted.

Protection afforded by most currently licensed vaccines is correlated with the generation of neutralizing antibodies (NAbs) (1–3). However, no HIV-1 vaccine to date has been capable of eliciting broad and potent NAbs (4–7). Difficulties in generating broadly neutralizing antibodies (bNAbs) arise from the extensive sequence diversity of circulating strains of HIV-1 (8) and from the unusual characteristics of antibodies associated with the development of breadth (9). However, 15% of HIV-1 infected individuals develop bNAbs with substantial breadth, while over 50% of people make antibodies with at least moderate breadth, typically several years into chronic infection (10–13). Moreover, multiple broadly neutralizing monoclonal antibodies have been reported (14–17). It is therefore important to develop strategies that improve the magnitude and breadth of vaccine-elicited NAbs.

As the HIV-1 Env protein is the sole viral antigen on the surface of the virus, it is the target for NAbs. HIV-1 Env is a trimer consisting of three gp120 surface subunits, responsible for interacting with the primary receptor (CD4) and the secondary receptors (CCR5 and/or CXCR4), as well as a trimer of gp41 transmembrane subunits responsible for membrane fusion (18). Previous studies have demonstrated that soluble Env gp140 trimers more closely mimic the antigenic properties of circulating virions and generate more robust neutralizing antibody responses than do Env gp120 monomers (19–24).

Several strategies have been explored with the goal of increasing the magnitude and breadth of vaccine-elicited NAbs, including the development of centralized sequences and multivalent mixtures of Env. Centralized (consensus or ancestral) immunogens are generated *in silico* with the goal of representing the global sequence diversity of Env (8, 25, 26). A previous study comparing trimeric consensus Env to trimeric native Env sequences isolated from acutely and chronically infected individuals showed that

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consensus immunogens were capable of eliciting a higher magnitude of NABs than those elicited by native Envs, but with a limited breadth (23). Other studies utilizing consensus and/or ancestral trimers showed only a modest advantage over native immunogens (27–29). Multivalent vaccination approaches utilize cocktails of HIV-1 Env immunogens with the goal of improving NAB responses. A DNA prime, adenoviral serotype 5 (Ad5) vector boost vaccine expressing multiclade Env inserts elicited a greater breadth of NABs than that of a comparator single Env immunogen (30, 31). Similarly, a multiclade DNA prime, gp120 protein boost vaccine elicited a greater breadth of NABs than that of the comparator single gp120 immunogen in rabbits (32, 33). However, these previous studies did not directly compare the cocktail with each individual component of the vaccine; thus, the potential advantage of an Env immunogen cocktail remains unclear.

In this study, we report the generation of three novel, acute clade C HIV-1 Env trimers. Each of these trimers possessed unique antigenic properties, and when combined in a mixture with our previously described chronic clade C (C97ZA012) HIV-1 Env trimer (34), the cocktail induced a greater magnitude of NAB responses than that of any single trimer component in the mixture.

MATERIALS AND METHODS

Plasmids, cell lines, protein production, and antibodies. Four to 10 full-length gp160 envelope sequences for HIV-1 Env 405C, 459C, and 939C were generated from virus in 15 acutely infected participants (<90 days postinfection) from the South African HVTN503/Phambili vaccine trial (35). The codon-optimized synthetic genes of the derived consensus sequences for the HIV-1 Env gp140 trimers were produced by GeneArt (Life Technologies). All constructs contained a consensus leader signal sequence peptide as well as a C-terminal foldon trimerization tag followed by a His tag, as described previously (34, 36). The codon-optimized synthetic genes for the full-length HIV-1 Env 405C, 459C, and 939C gp120s were cloned from their respective gp140 construct, and a C-terminal His tag was added. HIV-1 Env C97ZA012.1, 92UG037.8, and Mosaic (MosM) gp140 were produced as described previously (34, 37).

All constructs were generated in 293T cells utilizing transient transfections with polyethylenimine. Cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) to confluence and then changed to Freestyle 293 (Invitrogen) expression medium for protein expression. Cell supernatants were harvested 5 days after medium change, centrifuged for clarification, and brought to a final concentration of 10 mM imidazole.

All His-tagged proteins were purified by a HisTrap nickel-nitrilotriacetic acid (Ni-NTA) column (GE Healthcare). Ni-NTA columns were washed with 20 mM imidazole (pH 8.0), and protein was eluted with 300 mM imidazole (pH 8.0). Fractions containing protein were pooled and concentrated. Protein constructs were further purified by utilizing gel filtration chromatography on a Superose 6 column (GE Healthcare) for gp140 trimeric constructs and a Superdex 200 column (GE Healthcare) for gp120 monomeric constructs in running buffer containing 25 mM Tris (pH 7.5) and 150 mM sodium chloride. Purified proteins were concentrated using Centricon YM-50 concentrators (Millipore), flash frozen in liquid nitrogen, and stored at -80°C . To assess protein stability, 5 μg of protein was run on an SDS-PAGE gel (Bio-Rad) either after a single freeze/thaw cycle or after incubation at 4°C for 2 weeks.

Soluble two-domain CD4 was produced as described previously (38). 17b hybridoma was provided by James Robinson (Tulane University, New Orleans, LA) and purified as described previously (22). VRC01 was obtained through the NIH AIDS Reagent Program (39). 3BNC117 and 10-1074 were provided by Michel Nussenzweig (Rockefeller University,

New York, NY). PGT121, PGT126, and PGT145 were provided by Dennis Burton (The Scripps Research Institute, La Jolla, CA).

Surface plasmon resonance binding analysis. Surface plasmon resonance (SPR) experiments were conducted on a Biacore 3000 (GE Healthcare) at 25°C utilizing HBS-EP (10 mM HEPES [pH 7.4], 150 mM NaCl, 3 mM EDTA, 0.005% P20) (GE Healthcare) as the running buffer. Immobilization of CD4 (1,500 response units [RU]) or protein A (Thermo Scientific) to CM5 chips was performed following the standard amine coupling procedure as recommended by the manufacturer (GE Healthcare). Immobilized IgGs were captured at 300 to 550 RU. Binding experiments were conducted with a flow rate of 50 $\mu\text{L}/\text{min}$ with a 2-min association phase and a 5-min dissociation phase. Regeneration was conducted with one injection (3 s) of 35 mM sodium hydroxide and 1.3 M sodium chloride at 100 $\mu\text{L}/\text{min}$ followed by a 3-min equilibration phase in HBS-EP. Identical injections over blank surfaces were subtracted from the binding data for analysis. Binding kinetics were determined using BIAevaluation software (GE Healthcare) and the Langmuir 1:1 binding model. A bivalent binding model was used to fit PGT145 IgG binding. All samples were run in duplicate and yielded similar kinetic results. Single curves of the duplicates are shown in all figures.

Guinea pig vaccinations. Outbred female Hartley guinea pigs (Elm Hill) were used for all vaccination studies and were housed at the Animal Research Facility of Beth Israel Deaconess Medical Center under approved Institutional Animal Care and Use Committee (IACUC) protocols. Guinea pigs ($n = 5$ to 14 animals/group) were immunized with protein trimers intramuscularly in the quadriceps bilaterally at 4-week intervals for a total of 3 injections. Vaccine formulations for each guinea pig consisted of a total of 100 μg of trimer per injection formulated in 15% Emulsigen (vol/vol) oil-in-water emulsion (MVP Laboratories) and 50 μg CpG (Midland Reagent Company) as adjuvants. In multivalent vaccination regimens, the total amount of injected protein was maintained at 100 μg and divided equally among the total number of immunogens in the mixture. Multivalent mixtures included the C97ZA012 and 459C gp140 trimers (2C mixture), C97ZA012, 459C, and 405C gp140 trimers (3C mixture), and C97ZA012, 405C, 459C, and 939C gp140 trimers (4C mixture). Serum samples were obtained from the vena cava of anesthetized animals 4 weeks after each immunization.

Endpoint ELISAs. Serum binding antibodies against gp140 were measured by endpoint enzyme-linked immunosorbent assays (ELISAs) as described previously (34). Briefly, ELISA plates (Thermo Scientific) were coated with individual trimers and incubated overnight. Guinea pig sera were then added in serial dilutions and later detected with a horseradish peroxidase (HRP)-conjugated goat anti-guinea pig secondary antibody (Jackson ImmunoResearch Laboratories). Plates were developed and read using a Spectramax Plus ELISA plate reader (Molecular Devices) and Softmax Pro 4.7.1 software. Endpoint titers were considered positive at the highest dilution that maintained an absorbance >2 -fold above background values.

TZM.bl neutralization assay. Functional neutralizing antibody responses against HIV-1 Env pseudovirions were measured using the TZM.bl neutralization assay, a luciferase-based virus neutralization assay in TZM.bl cells as described previously (40). The ID_{50} was calculated as the serum dilution that resulted in a 50% reduction in relative luminescence units of TZM.bl cells compared to results for virus-only control wells after the subtraction of a cell-only control. Briefly, serial dilutions of sera were incubated with pseudovirions and then overlaid with TZM.bl cells. Murine leukemia virus (MuLV) was included as a negative control in all assays. HIV-1 Env pseudovirions, including tier 1 isolates from clade A (DJ263.8, Q23.17, MS208.A1), clade B (SF162.LS, BaL.26, SS1196.1, 6535.3), and clade C (MW965.26, TV1.21, ZM109F.PB4, ZM197M.PB7), as well as one isolate from tier 2 clade C (Du422), were prepared as described previously (41).

Phylogenetic trees. Maximum-likelihood phylogenetic trees were generated using the PhyML 3.0 program (42), with the web interface at the

FIG 1 Acute clade C HIV-1 Env gp140 trimer expression, stability, and homogeneity. (A) Expression levels of novel, acute gp140 envelope protein sequences. Supernatant collected from 293T cells transiently transfected with HIV-1 Env gp140 sequences was assessed for protein expression by Western blotting. (B) Coomassie-stained SDS-PAGE gel of pooled peaks of acute, clade C trimers after a single freeze/thaw cycle or incubation at 4°C for 2 weeks. Trimers are as follows for both SDS-PAGE gels: lanes 1, C97ZA012; lanes 2, 405C; lanes 3, 459C; lanes 4, 939C gp140. (C) Gel filtration chromatography traces of 459C, 405C, and 939C gp140 trimers as run on a Superose 6 column. Molecular mass standards for traces include thyroglobin (670 kDa), ferritin (440 kDa), and γ -globin (158 kDa).

Los Alamos HIV database (<http://www.hiv.lanl.gov/content/sequence/PHYML/interface.html>), using default parameter values.

Statistical analysis of neutralization data. Neutralization data were analyzed using R (43) and GraphPad Prism version 6.00 (GraphPad Software, San Diego, CA) software. Postvaccination raw neutralization data were compared utilizing Mann-Whitney U analysis by pairwise analysis to the 4C-vaccinated animals.

Neutralization thresholds. In order to correct for the high background (see Fig. 7), three distinct thresholds were tested. They are defined as follows, where “pre” is prevaccination sera, “post” is postvaccination sera, and the lowest background below the cutoffs is set to 10.

For cutoff 1, response = post, if (post – pre) is >10, and 10 otherwise.

For cutoff 2, response = post, if (post – pre $\times$ 3) is >10, and 10 otherwise.

For cutoff 3, response = (post – pre), if (post – pre) is >10 and if (post – pre) is >(MuLV post – MuLV pre).

All three cutoffs gave statistically consistent outcomes within the scope of the tests performed in our studies. Surprisingly, the general phenomenon of guinea pig assay background levels was also apparent in the MuLV negative control; the background was higher in the MuLV negative control postvaccination relative to prevaccination ($P = 2.71 \times 10^{-9}$, paired Wilcoxon test). To account for this, we considered a vaccine response to be positive when the “post – pre” difference was both greater than 10 and greater than the postvaccination increase in MuLV control responses, a generalized increase in background stimulated by the vaccine. As a result, we considered the response to be equal to (post – pre) if (post – pre) was >10 and if (post – pre) was >(MuLV post – MuLV pre), or 10 otherwise. Cutoff 3 was ultimately chosen and used for the generation of figures, as we believe it provides the most accurate measure of vaccine effects and the best method for removing background values in a pseudovirion-specific manner as detected by MuLV background values.

Generalized linear model analysis. Generalized linear models (GLMs) are a generalization of linear regression, which allows the fitting of response variables with non-normal error distribution models. GLM analysis was performed with R, using the glmer4 package. We fit an inverse Gaussian GLM that included both random effects (animal, pseudovirions) and fixed effects (vaccine/trimer, tier, clade).

Equations for vaccine and tier interactions are as follows.

$$g0 = \text{glmer}[\log_{10}(\text{Response})] \sim \text{clade} + \text{Vaccine} * \text{Tier} + (1|\text{Animal}) + (1|\text{Env})$$

$$g1 = \text{glmer}[\log_{10}(\text{Response})] \sim \text{clade} + \text{Vaccine} + \text{Tier} + (1|\text{Animal}) + (1|\text{Env})$$

$$g2 = \text{glmer}[\log_{10}(\text{Response})] \sim \text{Vaccine} * \text{Tier} + (1|\text{Animal}) + (1|\text{Env})$$

$$\text{anova}(g0, g1), \text{anova}(g0, g2)$$

1|Animal is the notation for treating an animal as a random effect. Vaccine*Tier is the notation for an interaction between the vaccine and the tier of the test Env. We started with a complex model and used analysis of variance (ANOVA) to see if we could simplify the model.

Clade effect was not significant. The interaction between vaccine and tier, however, was found to be significant ($P = 0.000274$ for cutoff 3), indicating that the effect of vaccine was different on tiers 1A and 1B. In order to estimate the effect, we fit the model on tiers 1A and 1B separately.

Equations for effect of vaccine are as follows.

$$g0 = \text{glmer}[\log_{10}(\text{Response})] \sim \text{Vaccine} + (1|\text{Animal}) + (1|\text{Env})$$

$$g1 = \text{glmer}[\log_{10}(\text{Response})] \sim 1 + (1|\text{Animal}) + (1|\text{Env})$$

$$\text{anova}(g0, g1)$$

For both tier 1A and tier 1B, the vaccine effect was significant ($P = 2.151 \times 10^{-6}$ and $P = 0.0100$, respectively), indicating that the vaccine was significantly predictive of the magnitude of the neutralizing responses in the vaccinated animals for both tiers. Even though the specific vaccine effects were different across the two tiers, in both cases we found that the

FIG 2 Maximum-likelihood trees and sequence alignments of clade C gp140 sequences. (A) Phylogenetic tree comparing each of the four clade C vaccine envelope (Env) sequences to 489 clade C sequences sampled from the year 2004. Colors indicate the country of origin for each sequence according to the key provided (ZA, South Africa; MW, Malawi; ZM, Zambia; TZ, Tanzania; CN, China; CY, Cyprus; BR, Brazil; GB, Great Britain; X, all other countries sampled; ES, Spain; IN, India; Ccon, consensus C; FR, France; US, United States; ZW, Zimbabwe). (B) Phylogenetic tree comparing each of the four clade C vaccine Env sequences to 506 clade C sequences from South Africa starting from the year 2000 (00). Years of origin are shown in shades of gray according to the key provided. For panels A and B, vaccine Env strains are highlighted in red, the consensus clade C Env sequence is shown in cyan, and the HXB2 sequence (outgroup) is indicated in dark blue. The scale bar indicates phylogenetic distance, with the bar length corresponding to 0.01 genetic change, or nucleotide substitution, per site. (C) Alignment of CD4 binding site contact residues for clade C immunogens, (D) alignment of PG9 contact residues for clade C immunogens, (E) alignment of V3 loop and C-terminal glycan contact residues for clade C immunogens. For panels C, D, and E, sequence alignments were compared to a consensus C sequence and are aligned using HXB2 numbering, with ranking of sequence centrality denoted with red numbers, 1 being most central and 4 being least central. pos, positions.

mixtures, on average, elicited higher magnitude responses than those of the single-strain vaccines, and of the single-strain vaccines, 459C was the best (see Table 2). The complete R code used for these analyses is available from the authors upon request.

Geometric mean analysis. Geometric means were calculated over test pseudovirions for each animal (producing one point per vaccine per animal) and analyzed by Kruskal-Wallis and 1-sided Mann-Whitney U tests to compare the 4C mixture and the 459C trimer with all other vaccines. All

tests were performed with the complete pseudovirion panel and then repeated with the tier 1B panel only (the other tiers had too few data points for this kind of analysis).

RESULTS

Generation of novel, acute clade C Env trimers. Fifteen acute HIV-1 clade C envelope sequences from South Africa (35) were

FIG 3 Presentation of CD4 and CD4i epitopes by acute, clade C trimers. (A) Soluble two-domain CD4 was irreversibly coupled to a CM5 chip, and 459C, 405C, or 939C gp140 was flowed over the chip at concentrations of 62.5 to 1,000 nM. (B to D) Protein A was irreversibly coupled to a CM5 chip, and (B) 17b IgG was captured. HIV-1 Env 459C, 405C, or 939C gp140 was flowed over the bound IgG at a concentration of 1,000 nM in the presence or absence of CD4 bound to the immunogen. 17b binding alone is indicated in red, and CD4 coupled to trimer binding to 17b IgG is shown in blue. VRC01 IgG (C) and 3BNC117 IgG (D) were captured, and HIV-1 Env 459C, 405C, and 939C gp140 trimers were flowed over the bound IgG at concentrations of 62.5 to 1,000 nM. Sensorgrams are presented in black and kinetic fits in green. RU, response units.

cloned into a pCMV expression vector and transiently transfected in human endothelial 293T kidney cells utilizing polyethylenimine. Expression levels of Env gp140 were compared by Western blotting utilizing the supernatant from transfected cells (Fig. 1A), and expression data were verified by quantitative binding ELISAs (data not shown). Western blot analysis showed that eight of the 15 Env gp140s expressed at a level similar to or greater than that of our previously characterized C97ZA012 gp140 (22, 34): 405C, 459C, 939C, 823cD6, 756C, 823C, 349C, and 706C gp140. The

remaining Env gp140s, 426C, 590C, 072C, 327C, 431C, 885C, and 140C, exhibited low expression levels. The eight sequences with the highest expression levels were then screened for expression from large-scale purifications.

From this large-scale screen, three trimers (459C, 405C, and 939C) expressed at higher levels than the other trimers and were therefore selected for further study. Negligible degradation was seen both after a freeze/thaw cycle and after incubation at 4°C for 2 weeks (Fig. 1B). Additionally, each of these trimers represented

FIG 4 Presentation of V3 and glycan-dependent epitopes by acute, clade C trimers. For all experiments, protein A was irreversibly coupled to a CM5 chip and IgGs were captured. HIV-1 Env 459C, 405C, and 939C gp140 trimers were flowed over bound PGT126 IgG (A), PGT121 IgG (B), and 10-1074 IgG (C) at concentrations of 62.5 to 1,000 nM. Sensorgrams are presented in black and kinetic fits in green. RU, response units.

a homogenous population as measured by gel filtration chromatography (Fig. 1C).

Phylogenetic characterization of novel, acute clade C immunogens. To compare these Env sequences to more recent clade C sequences circulating worldwide, we generated a maximum likelihood phylogenetic tree that included the three novel, acute clade C and the C97ZA012 (22, 34) Env sequences, as well as 489 clade C sequences from different countries from 2004 (Fig. 2A). To assess where the novel, acute clade C sequences stood in terms of their relatedness to other South African strains, a second tree compared the four sequences to 506 South African clade C sequences from the years 2000 to 2009 (Fig. 2B). Both of these analyses determined that Env 459C gp140 was the most central of the four sequences, whereas Env 405C gp140 was somewhat of an outlier.

Sequence analyses of specific epitope regions critical to known bNAbs were also conducted. Env 459C and Env 939C gp140 were closer to the consensus sequence for the CD4 binding site epitope (b12 [44] and VRC01 [15]) than were C97ZA012 or 405C gp140 (Fig. 2C). In contrast, Env 405C gp140 was the most central of all of these sequences for PG9/PG16/PGT145-like glycan-dependent variable loop 1 and 2 (V1/V2) binding antibodies (14, 45–47) (Fig. 2D). The Env 939C trimer lacked the amino acid sequence motif (NXS/T) for N-linked glycosylation at amino acid position 332 (N332; HXB2 reference numbering), which is important for the

glycan-dependent variable loop 3 (V3) binding, PGT family of antibodies (17, 48–50) (Fig. 2E). These phylogenetic and sequence analyses suggest that each trimer had unique phylogenetic and antigenic characteristics.

Antigenic properties of novel, acute clade C immunogens. We next analyzed the antigenic properties of the novel, acute clade C trimers by surface plasmon resonance (SPR). All of the clade C trimers presented the CD4 binding site (CD4bs), bound well to CD4 (Fig. 3A), and showed a substantial increase in the binding of 17b IgG (51) in the presence of CD4, as expected (Fig. 3B). All trimers also bound to the CD4bs antibodies VRC01 (15) and 3BNC117 (52), but the magnitude of binding differed among the different isolates (Fig. 3C and D). In particular, the Env 405C trimer bound VRC01 and 3BNC117 at about a 5-fold-lower magnitude than Env 459C and 939C trimers, suggesting that 459C and 939C may present the CD4bs epitope more optimally than 405C, which is consistent with the sequence analysis showing that Env 405C may have mutations in important CD4bs contact residues (Fig. 2C). Similar to Env 459C and 939C gp140s, C97ZA012 gp140 also bound soluble CD4 (sCD4) and VRC01 (22).

The Env 405C and 459C trimers bound the V3/glycan-dependent antibodies PGT121 and PGT126 at a higher magnitude than did the Env 939C trimer (Fig. 4A and B). This is consistent with the sequence analysis showing that Env 939C gp140 lacks the

FIG 5 Presentation of V1/V2, glycan-dependent, quaternary-preferring epitopes by acute, clade C trimers and monomers. For all experiments, protein A was irreversibly coupled to a CM5 chip and IgGs were captured. 459C, 405C, and 939C trimers and monomers were flowed over bound PGT145 IgG at concentrations of 62.5 to 1,000 nM. Sensorgrams are presented in black and kinetic fits in green. RU, response units.

amino acid sequence motif necessary for the addition of the N332 N-linked glycan (NXS/T), which is important for the V3/glycan-dependent antibodies (17, 48, 50) (Fig. 2E). Additionally, while Env 405C and 459C gp140s both bound 10-1074, 939C exhibited essentially no binding to this antibody (Fig. 4C), which is expected as N332 is critical for 10-1074 binding (49). Similar to Env 405C

and 459C gp140s, C97ZA012 gp140 also bound V3/glycan-dependent antibodies (22).

The quaternary structure of the acute, clade C gp140 trimers was assessed utilizing PGT145 IgG, which preferentially binds to intact trimers and targets variable loops 1 and 2 (V1/V2) and N-linked glycans in this region (24, 45). PGT145 bound all the Env

FIG 6 Binding antibody titers from guinea pigs vaccinated with clade C trimers. (A) Vaccination scheme for all vaccinated guinea pigs. Animals were vaccinated at weeks 0, 4, and 8 and bled at weeks 0, 4, 8, and 12. (B) Binding antibody titers from guinea pig sera against gp140 antigens after vaccination with clade C trimeric immunogen. Sera were tested in endpoint ELISAs against a panel of trimeric antigens in guinea pigs vaccinated with HIV-1 Env C97ZA012 ($n = 14$ animals), 459C ($n = 10$), 405C ($n = 5$), and 939C ($n = 5$) gp140 trimeric protein immunogens. 2C mixture ($n = 5$), C97ZA012+459C gp140; 3C mixture ($n = 5$), C97ZA012+459C+405C gp140; 4C mixture ($n = 10$), C97ZA012+405C+459C+939C gp140. Colors correspond to coating proteins as listed. The horizontal dotted line indicates background, and error bars indicate standard deviations.

FIG 7 Magnitude of neutralizing antibody titers after vaccination with single clade C or multivalent vaccination regimens. Guinea pig sera obtained prevaccination (pre) and 4 weeks after the third vaccination (post) were tested against a multiclade panel of tier 1 clade C, clade B, and clade A neutralization-sensitive isolates in the TZM.bl neutralization assay. Horizontal bars indicate median titers, and the dotted black line indicates the limit of detection for the assay. The x-axis immunogen names refer to the vaccination regimen. C97 is HIV-1 Env C97ZA012 gp140, 2C includes HIV-1 Env C97ZA012+459C gp140, 3C includes HIV-1 Env C97ZA012+459C+405C gp140, and 4C includes HIV-1 Env C97ZA012+459C+405C+939C gp140 trimeric immunogens.

gp140 trimers but exhibited essentially no binding to the sequence-matched Env gp120 monomers (Fig. 5). PGT145 bound the clade C trimers at a magnitude comparable to that of the other bNAbs tested, but it exhibited a faster off-rate. These data suggest that the PGT145 epitope is present at least to some extent on all of the gp140 trimers but not on the gp120 monomers.

Immunogenicity of novel, acute clade C trimers. To assess the immunogenicity of our novel, acute clade C trimers, we immunized guinea pigs with trimers three times at monthly intervals, and animals were bled 4 weeks after each vaccination (Fig. 6A). Four groups of guinea pigs were vaccinated with the single Env trimers, including C97ZA012, 459C, 405C, or 939C ($n = 5$ to 14/group). In addition, guinea pigs were vaccinated with multivalent trimer cocktails, including mixtures of two (2C; C97ZA012+459C), three (3C; C97ZA012+459C+405C), or all four clade C trimers (4C; C97ZA012+459C+405C+939C) ($n = 5$ to 10/group). Binding antibody responses were assessed by utilizing a panel of Envs as coating proteins from clade C (C97ZA012, 459C, 405C, and 939C), clade A (92UG037), clade B

(PVO.4), and a mosaic (MosM) sequence. All guinea pigs developed similar magnitudes of binding antibody titers by ELISA (Fig. 6B). Animals showed low levels of binding antibodies after the first vaccination and higher levels after the second vaccination, at which point the titers of binding antibodies largely plateaued. These data show that the single immunogens and cocktails developed high titer binding antibodies with similar kinetics and breadth.

To determine the neutralization capacity of antibodies elicited by each of the novel trimers, a multiclade panel of tier 1A, 1B, and 2 pseudovirions was utilized in the TZM.bl neutralization assay (Fig. 7) (40). To evaluate the differences in the magnitude of NABs elicited by each single trimer, Kruskal-Wallis unpaired tests and Mann-Whitney U tests comparing the geometric means over vaccines by animal after background subtraction were utilized (Fig. 8A; Table 1). Env 459C elicited higher-magnitude NAB responses than those of all other single trimers when tested against all pseudovirions ($P = 3.5 \times 10^{-6}$ to 3.8×10^{-2}) as well as against tier 1B pseudovirions alone ($P = 3.5 \times 10^{-6}$ to 2.8×10^{-2}). To further

FIG 8 Comparison of titers of neutralizing antibodies elicited by vaccination regimens by clade C trimers as measured by the TZM.bl neutralization assay. (A) Geometric means over test pseudovirions for each animal. Each point represents a geometric mean response per animal for all pseudovirions, compared with the entire pseudovirion panel (top) and tier 1B pseudovirions only (bottom). Boxes show median and interquartile ranges, and vaccines are indicated with colors according to the key. (B) Geometric means of background subtracted neutralizing antibody ID₅₀ titers by vaccination group at week 12 stratified by test pseudovirion (1 point per vaccination group per test pseudovirion). Each vaccination group is indicated in a color according to the key and connected by a single line.

TABLE 1 Comparison of magnitudes of geometric means of neutralizing titers across test pseudovirions and by animal^c

Test	Comparison	<i>P</i> value
Kruskal-Wallis	All pseudovirions	3.58E-07
Mann-Whitney U	4C ≅ 3C	0.42957
	4C > 2C	0.01998 ^a
	4C > 459C	0.0007 ^{a,b}
	4C > 405C	0.0045 ^{a,b}
	4C > 939C	0.00033 ^{a,b}
	4C > C97ZA012	5.10E-07 ^{a,b}
Kruskal-Wallis	Tier 1B pseudovirions	1.40E-06
Mann-Whitney U	4C ≅ 3C	0.5704
	4C > 2C	0.050 ^a
	4C > 459C	0.0007 ^{a,b}
	4C > 405C	0.012 ^a
	4C > 939C	0.0003 ^{a,b}
	4C > C97ZA012	5.10E-07 ^{a,b}
Kruskal-Wallis	All pseudovirions	0.0022
Mann-Whitney U	459C > 405C	0.038 ^a
	459C > 939C	0.00033 ^{a,b}
	459C > C97Z	3.57E-06 ^{a,b}
Kruskal-Wallis	Tier 1B pseudovirions	0.0073
Mann-Whitney U	459C > 405C	0.028 ^a
	459C > 939C	0.0013 ^{a,b}
	459C > C97Z	3.57E-06 ^{a,b}

^a Significant by pairwise comparisons ($P < 0.05$).

^b Significant after Bonferroni correction.

^c 4C is compared to all vaccination regimens and 459C to the single-strain vaccines.

support this finding, we fit an inverse Gaussian generalized linear model analysis after background subtraction, using the vaccine as a fixed effect and the animal and Env as random effects. We also explored interactions with the additional fixed effects of clade and tier. By this analysis, animals vaccinated with Env 459C gp140 similarly elicited higher-magnitude NAb responses than those of all other single gp140s against tier 1B pseudovirions ($P = 1.5 \times 10^{-4}$ to 2.7×10^{-3}) (Fig. 8B; Table 2). Furthermore, 459C trended toward higher NAb responses against tier 1A pseudovirions than those of any other single trimer ($P = 1.0 \times 10^{-9}$ to 7.6×10^{-2}). These data show that 459C was more potent at eliciting NAb responses than any other single immunogen tested.

We next assessed the potential benefits of the cocktail of trimers. We observed trends toward higher NAb magnitudes as each additional component was added to the mixture, suggesting that the unique antigenic properties of each trimer may contribute to the improved NAb responses (Fig. 7). To evaluate the differences in NAb responses elicited by the quadrivalent mixture of clade C trimers (4C) compared with each individual immunogen in the mixture, we performed Kruskal-Wallis unpaired tests and Mann-Whitney U tests as described above (Fig. 8A; Table 1). The 4C mixture proved superior to each individual component of the mixture against all pseudovirions ($P = 5.1 \times 10^{-7}$ to 4.5×10^{-3}) as well as against tier 1B pseudovirions ($P = 5.1 \times 10^{-7}$ to 1.2×10^{-2}). Similarly, the inverse Gaussian generalized linear model analysis described above showed that in a comparison of all vaccination groups against tier 1A pseudovirions, animals vaccinated with the 4C mixture elicited a greater magnitude of NAb responses than any single trimer within the mixture (Fig. 8B; Table 2) ($P = 1.2 \times 10^{-13}$ to 1.7×10^{-2}). Furthermore, when testing NAb responses elicited against only

TABLE 2 Comparison of magnitude of neutralizing titers by generalized linear model analysis

Vaccine	Estimate ^a	SE	<i>t</i> value	Pr(> <i>z</i>)
Comparison across Tier 1A pseudovirions to 4C mixture				
3C	1.672	0.227	−0.982	3.260e−01
2C	1.775	0.226	−1.101	2.707e−01
459C	2.725	0.183	−2.375	1.755e−02 ^b
405C	6.030	0.213	−3.664	2.485e−04 ^{b,c}
939C	36.98	0.198	−7.903	2.722e−15 ^{b,c}
C97ZA012	16.16	0.163	−7.411	1.250e−13 ^{b,c}
Comparison across Tier 1B pseudovirions to 4C mixture				
3C	0.681	0.138	1.201	2.297e−01
2C	1.723	0.123	−1.919	5.500e−02
459C	1.228	0.105	−0.842	3.992e−01
405C	2.724	0.118	−3.699	2.168e−04 ^{b,c}
939C	2.903	0.117	−3.968	7.258e−05 ^{b,c}
C97ZA012	2.704	0.093	−4.640	3.501e−06 ^{b,c}
Comparison across Tier 1A pseudovirions to 459C				
4C	0.385	0.181	2.290	2.200e−02 ^b
3C	0.635	0.218	0.903	3.665e−01
2C	0.686	0.217	0.754	4.506e−01
405C	2.299	0.204	−1.773	7.623e−02
939C	14.23	0.189	−6.098	1.075e−09 ^{b,c}
C97ZA012	6.082	0.153	−5.141	2.729e−07 ^{b,c}
Comparison across Tier 1B pseudovirions to 459C				
4C	0.814	0.106	0.843	3.991e−01
3C	0.554	0.137	1.866	6.204e−02
2C	1.403	0.121	−1.213	2.250e−01
405C	2.219	0.116	−2.991	2.780e−03 ^{b,c}
939C	2.364	0.115	−3.259	1.119e−03 ^{b,c}
C97ZA012	2.202	0.091	−3.783	1.549e−04 ^{b,c}

^a The “Estimate” column shows how much larger the comparison vaccine (4C or 459C) is, on average, than the vaccine in the left column.

^b Significant by pairwise comparisons ($P < 0.05$).

^c Significant after Bonferroni correction.

tier 1B pseudovirions, the 4C mixture was statistically superior to 405C, 939C, and C97ZA012 ($P = 3.5 \times 10^{-6}$ to 2.1×10^{-4}) and trended toward being superior to 459C. Moreover, the 4C mixture trended toward eliciting a greater magnitude of NAb than the 2C or 3C mixture by both statistical models (Tables 1 and 2). Taken together, these data show that the 4C mixture was superior to each single trimer within the mixture. However, the breadth of NAb was not significantly improved by the 4C mixture, and tier 2 NAb activity was marginal. These neutralization data suggest that multivalent mixtures of trimeric HIV-1 Env immunogens represent a feasible strategy for increasing the magnitude of NAb responses.

DISCUSSION

In this study, we report the generation and characterization of three novel, acute clade C HIV-1 Env gp140 trimers. All trimers proved relatively stable and homogenous, and phylogenetic and epitope analysis suggested that Env 459C gp140 was the most central sequence in terms of clade C diversity. Antigenicity studies similarly demonstrated that Env 459C gp140 bound to a larger number of bNAbs than did the other trimers, and it elicited the most potent NAb responses compared with all other single immunogens that were tested. While all single and multivalent combinations of Env immunogens raised similar titers of binding antibodies, the cocktail containing all four clade C trimers elicited a greater magnitude of

NAb than any individual component and any other vaccination regimen tested. These data suggest an immunological advantage to utilizing a vaccine cocktail of antigenically diverse Envs.

Developing bNAbs remains an elusive goal of the HIV-1 vaccine field, and several strategies have been explored to increase the magnitude and breadth of NAb. One strategy includes the use of centralized (consensus or ancestral) immunogens (27–29, 53). In a study of consensus versus natural sequence Envs, the global consensus immunogen ConS elicited more potent NAb than those elicited by the natural Envs that were tested (23), suggesting that the use of central sequences warrants further investigation. A second strategy involves the development of cocktails of Env immunogens. Some cocktails failed to elicit NAb (54–57), while others have reported modest increases in the breadth of NAb compared to results with a single, wild-type control (30–33, 58, 59). Studies utilizing DNA vaccines or virus-like particles elicited only negligible levels of NAb (32, 33, 54–57, 60). It has also been shown that DNA prime followed by a soluble Env gp120 boost elicited a greater breadth of NAb than gp120 alone (32, 33, 60). Most prior studies utilized cocktails of Envs from different clades. In the present study, we show that a cocktail of clade C Envs substantially increased the overall magnitude of NAb compared with results for any individual component, but the cocktail did not substantially increase the breadth of the NAb responses. Future

epitope mapping studies are warranted to yield further insight into these observations, but it is likely that fundamentally different Env immunogens and vaccination strategies will be required for the generation of broad, heterologous, tier 2 NAbs.

The Env sequence analysis accurately predicted the observed antigenic properties of the trimers. For example, the Env 939C gp140 sequence lacks the potential N-linked glycosylation motif at position 332 (HXB2 reference numbering) (Fig. 2E), which impacts the ability of 939C gp140 to bind the V3/glycan-dependent antibodies PGT126, PGT121, and 10-1074 (Fig. 4A to C). Additionally, 405C, which was the least central sequence for the CD4bs bNAbs (Fig. 2C), bound these antibodies at a lower magnitude than that of trimers containing sequences more central to this epitope (Fig. 3C and D). In particular, the 405C gp140 contains two potential N-linked glycosylation sites in V5, which may be related to certain VRC01 resistance mutations, and deglycosylation of 405C gp140 restored VRC01 binding (data not shown). Sequence analyses utilizing epitopes of known bNAbs may prove useful for screening large numbers of Env isolates in the future prior to the production of immunogens, allowing for the generation of immunogens containing epitopes or antigenic properties of interest. Selecting immunogens with unique antigenic properties might also be beneficial in developing strategies to drive the development of NAb to a greater diversity of epitopes. Furthermore, assessing phylogeny may be beneficial, as 459C was the most central sequence, and in our study, this immunogen elicited the greatest magnitude of NAb compared to results with the other single immunogens.

In summary, our data demonstrate that a cocktail of soluble HIV-1 clade C Env trimers represents a feasible strategy for increasing the magnitude of NAb in guinea pigs. These findings suggest that the development of Env cocktails to improve NAb responses warrants further investigation.

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