

# Immune Focusing and Enhanced Neutralization Induced by HIV-1 gp140 Chemical Cross-Linking

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**Experimental vaccine antigens based upon the HIV-1 envelope glycoproteins (Env) have failed to induce neutralizing antibodies (NAbs) against the majority of circulating viral strains as a result of antibody evasion mechanisms, including amino acid variability and conformational instability. A potential vaccine design strategy is to stabilize Env, thereby focusing antibody responses on constitutively exposed, conserved surfaces, such as the CD4 binding site (CD4bs). Here, we show that a largely trimeric form of soluble Env can be stably cross-linked with glutaraldehyde (GLA) without global modification of antigenicity. Cross-linking largely conserved binding of all potent broadly neutralizing antibodies (bNAbs) tested, including CD4bs-specific VRC01 and HJ16, but reduced binding of several non- or weakly neutralizing antibodies and soluble CD4 (sCD4). Adjuvanted administration of cross-linked or unmodified gp140 to rabbits generated indistinguishable total gp140-specific serum IgG binding titers. However, sera from animals receiving cross-linked gp140 showed significantly increased CD4bs-specific antibody binding compared to animals receiving unmodified gp140. Moreover, peptide mapping of sera from animals receiving cross-linked gp140 revealed increased binding to gp120 C1 and V1V2 regions. Finally, neutralization titers were significantly elevated in sera from animals receiving cross-linked gp140 rather than unmodified gp140. We conclude that cross-linking favors antigen stability, imparts antigenic modifications that selectively refocus antibody specificity and improves induction of NAbs, and might be a useful strategy for future vaccine design.**

A prophylactic vaccine against HIV-1 infection is urgently required to reduce the magnitude of the global pandemic. However, the manifold mechanisms HIV-1 has evolved for evasion of the adaptive immune response, in particular, antigenic variation, make vaccine design a major challenge (1). Transfer of broadly neutralizing antibodies (bNAbs) into macaques completely protects them from infection by immunodeficiency viruses, providing a clear correlate of protection that informs antibody-based vaccine design (2). Moreover, results from the recent RV144 efficacy trial imply that antibodies, not cytotoxic T cells, were responsible for the modest protection (3–5). However, a major hurdle to overcome is the design of HIV-1 vaccine antigens able to elicit neutralizing antibodies by active immunization (6). Most studies to date have used soluble components of HIV-1 Env, either the receptor-binding monomeric gp120 subunit or trimeric gp140 complexes containing gp120 coupled to the membrane-external domain of the transmembrane subunit gp41 (7, 8). These studies have consistently revealed that although NAb responses can be elicited in various animal models and trimeric Env elicits improved NAb responses compared to those of monomeric gp120 (9, 10), virus neutralization remains strain specific and relatively weak. Strain specificity is a result of the bulk of NAb responses being made against variable surfaces on Env that the virus can modify without major fitness cost, a dominant HIV-1 antibody evasion strategy (2). An approach to overcome the dramatic variability of Env is to target conserved regions as defined by the isolation of bNAbs (1, 11–15). One such region is the CD4 binding site (CD4bs) on gp120 that contains a highly conserved site of

vulnerability (16). bNAbs to the CD4bs isolated from HIV-1-infected individuals can neutralize from ~30 to 90% of circulating viral strains (17–21), indicating that under the appropriate circumstances, conserved surfaces within the CD4bs are immunogenic *in vivo*.

A second antibody evasion mechanism relates to Env conformational instability. The Env trimer is held together by relatively labile noncovalent interactions that lead to shedding of gp120 producing nonfunctional gp41 “stumps” that will elicit a mostly nonneutralizing antibody response (18, 22–25). Moreover, probing of gp120, gp140, and cell surface-anchored Env with monoclonal antibodies (MAbs) (26, 27) and cryoelectron tomography of both *in situ* viral Env spikes and soluble gp140 trimers (28, 29) has revealed that Env can fluctuate between different conformational states, potentially modulating B cell recognition of particular conformational NAb epitopes, such as the CD4bs (30).

Therefore, we hypothesized that stabilization of Env tertiary and quaternary structure prevented trimer dissociation and al-

Received 1 May 2013 Accepted 4 July 2013

Published ahead of print 10 July 2013

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JVI.01161-13>.

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doi:10.1128/JVI.01161-13

lowed for a more predictable and robust B cell recognition of otherwise conformationally unstable neutralization epitopes. Aldehyde cross-linking of Env *in situ* on HIV-1-infected (31) or Env-expressing transfected cells (26, 27), or of gp120 and gp140 in solution (26, 27), has revealed selective preservation of MAb epitopes. Of particular interest, binding of CD4bs-specific bNAbs was maintained after mild aldehyde cross-linking (26, 27, 31), implying that components of the CD4bs are stabilized without deleterious modification of associated bNAb epitopes. To further investigate this, we treated predominantly trimeric HIV-1 gp140 derived from a clade C (CN54) HIV-1 isolate with glutaraldehyde (GLA) and characterized resulting effects upon antigenicity *in vitro* and immunogenicity *in vivo*. GLA reacts rapidly with amine groups, particularly lysines, at around neutral pH, and it is more efficient than other aldehydes in generating thermally and chemically stable cross-links (32). We confirm that GLA cross-linking preserves multiple bNAb epitopes, including those within the CD4bs, and demonstrate that immunization with GLA-cross-linked gp140 resulted in higher titers of CD4bs-specific antibodies, increased recognition of the C1 and V1V2 regions of gp120, and more potent neutralization. Thus, gp140 stabilization favorably modifies the responding B cell repertoire and may be a useful strategy in antigen design for eliciting NAb to conserved surfaces on HIV-1.

## MATERIALS AND METHODS

**Proteins and antibodies.** Nontagged soluble CN54 gp140 was derived from HIV-1<sub>97CN54</sub> (GenBank accession number AF286226) (33, 34). The gp140 terminating at ALDSWKN and lacking the 2F5 and 4E10 epitopes was produced in CHO cells, purified on an affinity chromatography column using gp41-specific MAb 5F3 (35), and manufactured to good manufacturing practice specifications by Polymun Scientific, Vienna, Austria (36, 37). sCD4, CD4-IgG2, 17b, F105, 15e, b12, 2G12, PG9, and PGT antibodies were obtained from the IAVI Neutralizing Antibody Consortium. 5F3 was from Polymun Inc. (35), and F105 was from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program. HGP68, HR10, and HJ16 were a kind gift from D. Corti and A. Lanzavecchia. WT and D368R mutant gp120 were produced by transient transfection of HEK293T cells and purified as described previously (38). VRC01, RSC3, and ΔRSC3 were a kind gift from J. R. Mascola. The eOD construct employed here was a variant of the “eOD Base” reported in reference 39 that contained two additional mutations (Huang et al., unpublished data) and was produced in HEK293S cells as described previously (39, 40).

**GLA cross-linking of gp140.** Minor contamination (<2%) of the CN54 gp140 by human 5F3 MAb carryover from the purification procedure was removed using protein A agarose (Pierce), and the buffer was exchanged to phosphate-buffered saline (PBS). Cross-linking was performed by addition of glutaraldehyde (GLA; Agar Scientific) to a final concentration of 7.5 mM for 5 min at room temperature (RT), after which the reaction was stopped by addition of 75 mM Tris buffer (pH 7.4) and then buffer exchanged into PBS. Successful cross-linking was tested by denaturing SDS-PAGE or native blue PAGE analysis on 3 to 8% Tris-glycine or 3 to 12% native page gels (Invitrogen), respectively, according to the manufacturer's instructions. Gels were stained using the Silver-Quest staining kit (Invitrogen), and analysis was performed using the AIDA image analyzer v. 3.27.

**ELISAs.** Enzyme-linked immunosorbent assays (ELISAs) were performed essentially as described previously (37). Briefly, antigens were coated onto ELISA plates (Greiner Bio-One) at 0.5 or 2.0 μg/ml for detection of polyclonal sera or MAbs, respectively. Wells were blocked with 2% bovine serum albumin (BSA) in PBS plus 0.05% Tween, followed by incubation with a dilution series of MAbs or sera for 2 h. Bound antibodies

were labeled with horseradish peroxidase-coupled goat anti-human IgG (Sigma-Aldrich) or goat anti-rabbit IgG (Cayman Chemical Company-Aldrich) and detected using TMB-ultra substrate (Thermo Fisher Scientific), and absorption was read at 450 nm. Curves were fitted to the data using Prism V6 (GraphPad Software), and endpoint titers were defined as the concentration that corresponded to an optical density at 450 nm (OD<sub>450</sub>) of 0.01, which was always greater than 3 standard deviations (SD) above background levels. RSC3 ELISAs were run similarly, but serum dilutions were preincubated with 10 μg/ml ΔRSC3 before addition to RSC3-coated plates. GLA binding indices were calculated by dividing the log<sub>10</sub>-transformed titers against GLA antigen by the respective log<sub>10</sub>-transformed titers against unmodified antigen. Similarly, D368R sensitivity values were calculated using the following formula: binding sensitivity = 100% - log<sub>10</sub> IC<sub>50</sub>(D368R)/log<sub>10</sub> IC<sub>50</sub>(wt) × 100%. Cross-competition ELISAs were performed similarly to direct ELISAs, with the difference that competing MAb was added to the wells at the respective 50% binding concentration directly after the addition of 30-fold-diluted sera. Background-subtracted data were normalized to positive-control wells containing MAb without competing serum using the following formula: percent inhibition = 100% - OD<sub>sample</sub>/OD<sub>positive control</sub> × 100%. Preimmune sera or serum pools were tested in parallel in all ELISAs and showed responses that were undetectable or insignificantly above plate background levels compared to postimmunization samples.

**SPR.** Surface plasmon resonance (SPR) data were collected at 37°C on a Biacore 3000 instrument (GE Healthcare) at a flow rate of 50 μl/min in HBS-EP buffer (GE Healthcare). Rabbit anti-human IgG antibody (Jackson ImmunoResearch) was immobilized on a CM5 sensor chip (GE Healthcare) using standard amine coupling at pH 4.5. In each cycle, approximately 700 RUs of anti-HIV-1 MAbs or irrelevant human control MAbs were captured onto the flow cells, and 2-fold concentration series of WT or GLA-treated gp140, starting at 256 nM, were passed over the captured MAbs for 5 min, followed by a 5-min dissociation time. For kinetic analysis of MAbs with very slow dissociation times, an extended dissociation time of 30 min was used for the highest concentration. Between cycles, the sensor surface was regenerated by two 30-s injections of 10 mM glycine, pH 2.0. Data analysis was performed with the BIA evaluation software (version 4.0.1) by subtracting the data from the control antibody flow cell followed by subtraction of a blank injection cycle (double referencing). The binding index was calculated using the area under the curve (AUC) as a read-out, since this is sensitive to changes in on-rate, off-rate, and maximum binding and circumvents problems with fitting data from a 3:2 binding event. The formula used was AUC(GLA)/AUC(WT) × CL(WT)/CL(GLA), where AUC is the area under the curve and CL is the capture level of the MAb. Kinetic data were obtained by fitting data to a 1:1 Langmuir model.

**CELISA.** A modified version of the cell-based ELISA (CELISA) developed by Haim et al. (65) was used for this study. Briefly, HEK293A cells were grown and transfected with UG37 gp160ΔCT or control DNA by calcium-phosphate precipitation in 15-cm dishes. Twenty-four h after transfection, they were lifted and resuspended into tissue culture-treated, clear-bottom, black-well 96-well plates. The next day, cells were left untreated or were cross-linked with 7.5 mM GLA in wash buffer (25 mM HEPES, 140 mM NaCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, pH 7.4) for 5 min, and the reaction was stopped by addition of an excess of Tris buffer. Cells were blocked and primary antibody was added in blocking buffer (10% fetal calf serum [FCS] in Dulbecco's modified Eagle medium [DMEM]). Bound MAb was labeled with horseradish peroxidase (HRP)-coupled goat anti-human IgG (Sigma) secondary antibody and detected using 35 μl Western Lightning reagents supplemented with 150 mM NaCl on a Spectromax X5. Data analysis was performed as described above for the standard ELISA.

**Flow cytometry.** HEK293T cells were grown and transfected with ZM53 gp160ΔCT or control DNA by calcium-phosphate precipitation in 15-cm dishes. Forty-eight h after transfection, cells were lifted with 10 mM EDTA in PBS and incubated with serial dilutions of sera. Bound antibodies

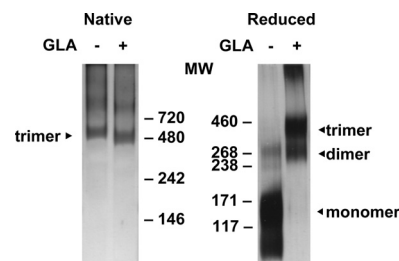
ies were detected using phycoerythrin (PE)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) on a FACSCalibur flow cytometer. Data were analyzed as described for ELISAs using the mean fluorescence intensity (MFI) of cell populations gated in FlowJo (VX.0.6).

**Animals and immunizations.** New Zealand White rabbits ( $n = 8$  per group) were immunized intramuscularly with 20  $\mu\text{g}$  unmodified (WT group) or GLA cross-linked (GLA group) CN54 gp140 per dose by Covance Inc. Immunizations were performed at weeks 0, 6, 12, and 22 using Carbopol 971p (1% wt/vol; 1 ml) for the prime and the first boost and polyethyleneimine (PEI; 25 kDa; branched; 1 mg/ml; 1 ml) for both successive boosts. Serum samples were collected at week 0 and 2 weeks after each immunization.

**Peptide array serum specificity mapping.** Reactivity of sera ( $n = 4$  per group, including 3 randomly chosen animals plus the highest responder from each group) to immobilized 15-mer peptides covering homologous CN54 gp140 were measured by Pepsan peptide binding analysis in an ELISA-based format in which the colored substrate was quantified with a charge-coupled device (CCD) camera and an image processing system. Statistical analysis was performed by averaging the responses to 8 regions for each animal and performing a one-way analysis of variance (ANOVA) with Bonferroni's posttest. Further epitope mapping of heterologous strains was performed essentially as previously described (42). Briefly, a peptide library of overlapping peptides (15-mers overlapping by 12), covering 7 full-length HIV-1 gp160 Env consensus sequences (clades A, B, C, and D, group M, CRF1, and CRF2) and 6 vaccine and laboratory strain gp120 sequences (A244\_1, TH023\_1, MN\_B, 1086\_C, TV1\_C, and ZM651\_C), was printed onto epoxy glass slides (provided by JPT Peptide Technologies GmbH [Germany]). All serum samples were diluted 1:250 and hybridized to the slides using a Tecan HS4000 hybridization workstation, followed by incubation with DyLight 649-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch). Fluorescence intensity was measured using a GenePix 4300 scanner (Molecular Devices) and analyzed with GenePix software. Binding intensity of the postimmunization serum to each peptide was corrected with its own background value, which was defined as the median signal intensity of the prebleed serum for that peptide plus 3 times the standard errors among the 3 subarray replicates present on each slide.

**Neutralization assays.** Neutralizing titers were measured in TZM-bl or A3R5 cells as described previously (43, 44). Briefly, pseudovirus (TZM-bl assay) or replication-competent luciferase reporter viruses (A3R5 assay) were incubated with serial dilutions of sera and added to their respective target cells. Luciferase expression was measured after 2 days (TZM-bl) or 4 days (A3R5), and  $\text{IC}_{50}$ s were determined as the serum concentration that reduced the background-subtracted relative light units by 50% compared to virus-only control wells. For V3 inhibition assays, sera were preincubated with an excess of V3 peptide (TRPNNTRKSIRI GPGQTFYATGDIIGNIRQAH), scrambled control, or PBS only. RSC3 inhibition assays were performed in TZM-bl cells as described above with HIV-1 clade C clone NL-LucR.T2A-MW965.ecto under single-cycle conditions. Serum dilutions were preincubated with 10  $\mu\text{g}/\text{ml}$  circularized CN54 V3 loop (CTRPGNNTRKSIRIGPGQTFYATGDIIGDIRQAH CGC) plus either 20  $\mu\text{g}/\text{ml}$  RSC or  $\Delta\text{RSC3}$ . Statistical analysis of neutralizing titers was performed using a one-way ANOVA of log-transformed data with Bonferroni's posttest. Statistical analysis of overall differences was performed using a Mantel-Cox log-rank test on magnitude-breadth curves. Comparison of RSC3 neutralization curves was performed by an F-test of pooled curves from all animals of each group. Fold inhibition was calculated as  $\text{IC}_{50}$  (no V3 loop)/ $\text{IC}_{50}$  (V3 loop) for the V3 loop and  $\text{IC}_{50}$  (V3 +  $\Delta\text{RSC3}$ )/ $\text{IC}_{50}$  (V3 + RSC3) for the CD4bs.

**Computational modeling of modification sites.** Crystal structures of gp120 or fragments of gp120 in complex with CD4 (Protein Data Bank [PDB] entry 2NXY), 17b (2NXY), F105 (3HI1), b12 (2NY7), VRC01 (3NGB), or PG9 (3U4E) were analyzed and rendered using UCSF Chimera 1.6.2 (45). Lysine residues were identified based on alignments of the sequence of CN54 (GenBank accession number AF286226) or UG37



**FIG 1** PAGE analysis of unmodified and GLA cross-linked CN54 gp140. HIV-1 gp140, untreated or treated with GLA, was migrated by native polyacrylamide gel electrophoresis (PAGE; left) or denaturing SDS-PAGE (right). Monomeric, dimeric, and trimeric bands are indicated according to molecular weight (MW; in thousands) analysis.

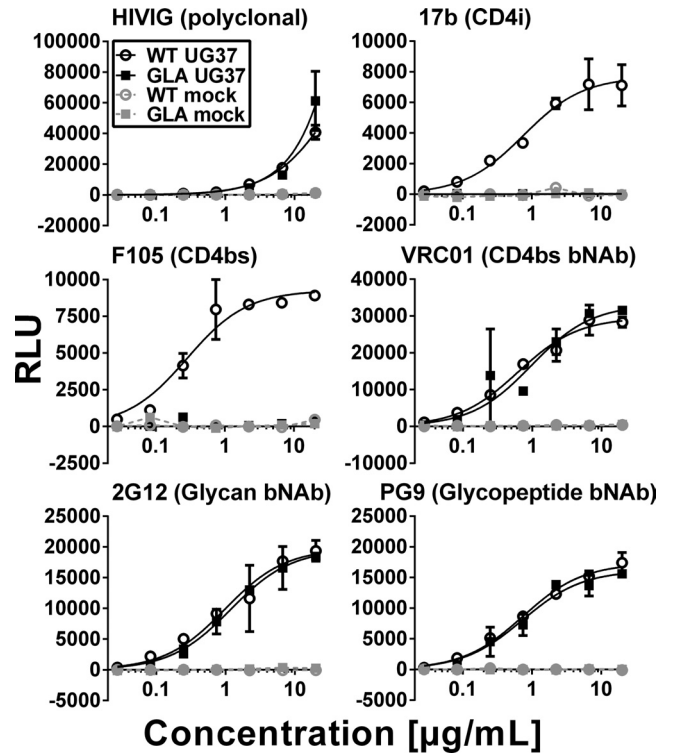
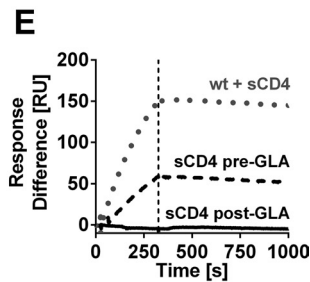
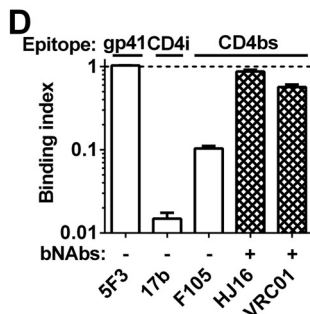
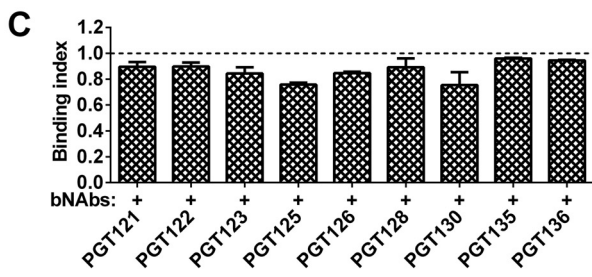
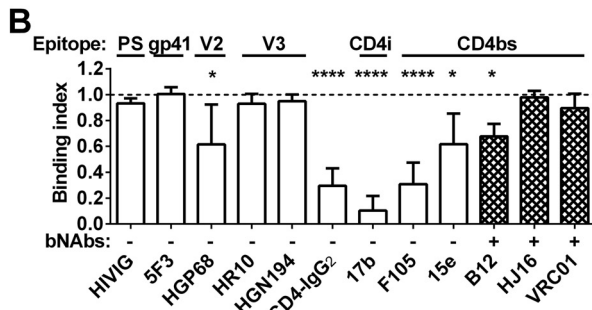
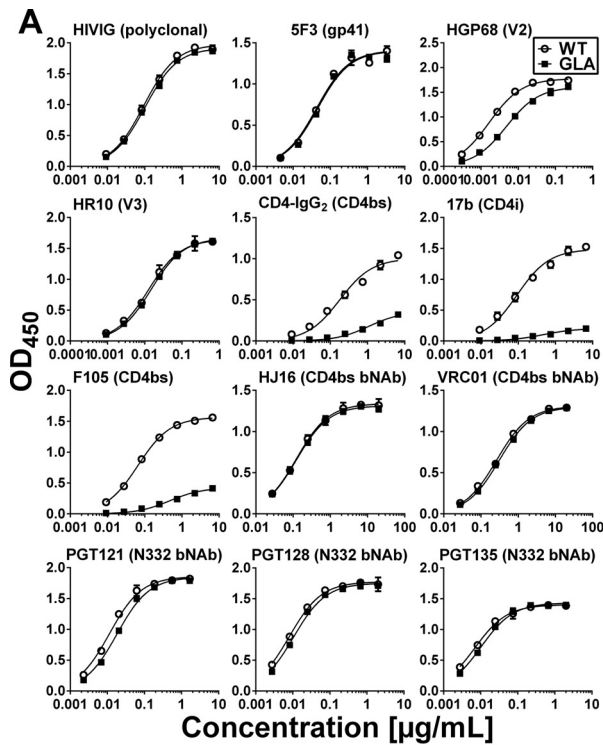
(AY494974) with the respective crystal structure gp120 sequence using the EMBL-EBI EMBOSS Needle online service (46).

## RESULTS

**Stability and antigenicity of GLA cross-linked gp140.** Soluble HIV-1 trimeric gp140 from the clade C CN54 isolate (gp140<sub>CN54</sub>) was cross-linked with 7.5 mM GLA for 5 min, free aldehydes were neutralized, and the resulting glycoprotein was desalted. Control gp140<sub>CN54</sub> received the same treatment without addition of GLA. Unmodified control protein migrated predominantly as a mixture of trimeric  $\sim 450$ -kDa complexes and higher-molecular-mass complexes by blue native PAGE (Fig. 1). GLA treatment yielded a band that ran slightly faster than the untreated material, potentially as a result of modest trimer compaction by cross-linking. Reducing SDS-PAGE disassembled the untreated gp140 into monomeric species, whereas GLA cross-linked material preserved trimers and smaller amounts of dimer and higher order oligomers.

We analyzed the effect of GLA cross-linking on antibody recognition of gp140<sub>CN54</sub> by ELISA. Polyclonal HIVIG binding was not significantly affected by cross-linking, indicating no major overall modification in antigenicity (Fig. 2). Binding of the non-neutralizing gp41-specific MAb 5F3 (35) was likewise unaltered. Binding of MAbs HGP68 (gp120 V2 loop) and HR10 (gp120 V3 loop) (18) was weakly affected ( $P = 0.02$ ) or unaffected by GLA treatment, respectively. Binding of a second V3 loop-specific MAb (HGN194) was similarly unaffected by GLA treatment (Fig. 2B). In contrast, binding of a soluble sCD4-IgG fusion protein (CD4-IgG2) to gp140<sub>CN54</sub> was highly significantly ( $P < 0.0001$ ) reduced after cross-linking, consistent with the inhibition of structural rearrangements in gp120 required for high-affinity CD4 binding (30, 47). In line with this result, the 17b MAb that binds a CD4-induced (CD4i) epitope (48) showed dramatically reduced binding to GLA cross-linked gp140 ( $P < 0.0001$ ). The binding of non- or weakly neutralizing CD4bs MAbs 15e and F105 was substantially reduced ( $P = 0.02$  and  $P < 0.0001$ , respectively). This was expected, because, similar to CD4, these MAbs require conformational rearrangement of gp120 for tight binding (30) and might also be subject to steric inhibition in a rigid trimer due to a predicted restricted angle of approach (49). This may also explain the partial reduction in IgG1b12 binding after GLA treatment (Fig. 2B). In contrast, binding of the more broadly neutralizing CD4bs MAbs VRC01 and HJ16 was unaffected by gp140 cross-linking, confirming a predicted lack of requirement for gp120 conformational change during binding. Several bNAbs (PGT121, PGT122, PGT123, PGT128, PGT135, and 2G12) recognize an epitope cen-

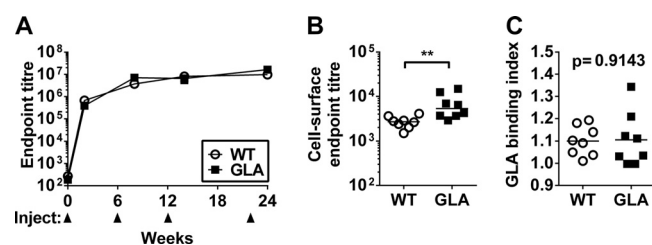




**FIG 3** Antigenicity of GLA cross-linked gp160. HEK293-A cells were transiently transfected with UG37 gp160 $\Delta$ CT (clade A) or mock transfected as indicated. Antibody binding to unmodified (WT) or cross-linked (GLA) gp160 was analyzed by CELISA. Representative titration series of MAbs (epitopes are indicated in parentheses) show maintenance (HIVIG, VRC01, 2G12, and PG9) or selective decreases (17b and F105) of binding. Lines indicate results of the nonlinear fit where possible.

tered on an N-linked glycan at position N332. None of these MAbs showed any significant difference in binding to GLA-treated and untreated gp140 (Fig. 2C). We further investigated the effects of cross-linking by SPR. Using this more sensitive technique, a modest reduction in binding of CD4bs bNAb VRC01 was detected, although the effect was far less pronounced than that with the nonneutralizing CD4bs F105 (Fig. 2D; also see Fig. S1 in the supplemental material). Furthermore, binding of the CD4i antibody 17b was undetectable when sCD4 and 17b were added post-GLA treatment (Fig. 2D and E; also see Fig. S1). However, if an excess of sCD4 was added to gp140 prior to cross-linking, detectable 17b

**FIG 2** Antigenicity of GLA cross-linked gp140. Antibody binding to unmodified (WT) or cross-linked (GLA) gp140 from strain CN54 (clade C) was analyzed by ELISA (A to C) and SPR (D and E). (A) Representative titration series against CN54 gp140 using MAbs covering tested epitopes (indicated in parentheses) show selective decreases of binding of nonneutralizing CD4bs antibody and CD4i antibodies. Curves indicate results of the nonlinear fit. (B and C) The binding index (cross-linked/unmodified) for CN54 gp140 is shown for all antibodies tested, with glycan-dependent antibodies presented in panel C. Values lower than 1.0 indicate loss of binding. Error bars indicate SD from at least five (B) or two (C) independent experiments. (D) The binding index from SPR measurements of CN54 gp140 is shown, with error bars indicating SD of technical repeats. (E) SPR traces are shown for unmodified or cross-linked CN54 gp140 binding to the CD4i Mab 17b. sCD4 was added either before or after cross-linking as indicated. The dotted vertical line indicates the end of the injection. PS, polyclonal serum; \*,  $P < 0.05$ ; \*\*\*\*,  $P < 0.0001$  according to a Kruskal-Wallis test.

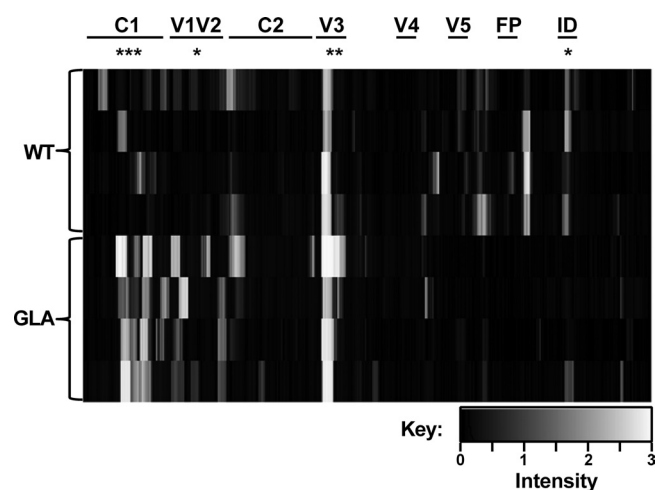


**FIG 4** Immunogenicity of GLA cross-linked CN54 gp140. (A) CN54 gp140-specific total IgG endpoint titers of sera from New Zealand White rabbits ( $n = 8$  per group) immunized with unmodified (WT) or GLA cross-linked (GLA) CN54 gp140 showed no significant differences at any time point (one-way ANOVA of log-transformed data with Bonferroni's posttest). Inject represents time points of immunizations. (B) Binding of week 24 sera to cell surface-expressed heterologous ZM53 Env was analyzed by fluorescence-activated cell sorting (FACS). \*\*,  $P < 0.01$  according to a  $t$  test of log-transformed data. (C) Binding of sera to a GLA cross-linked or unmodified irrelevant protein (HEL) was analyzed with week 24 sera, and results are expressed as an index of binding to GLA-treated/untreated HEL. Student's  $t$  test was performed for statistical analysis.

binding was observed, indicating that cross-linking interferes with structural rearrangements required for the exposure of the CD4i epitope (Fig. 2E; also see Fig. S1).

To determine whether GLA cross-linking modified antigenicity of membrane-associated gp160 in a manner similar to that of soluble gp140, we tested a panel of MAbs for binding using a cell-based ELISA (CELISA) using gp160-expressing HEK293 cells. The same trends were observed, in that HIVIG and VRC01 bound equivalently to the cross-linked and unmodified gp160, whereas the binding of the weak CD4bs NAb F105 and the CD4i antibody 17b were abolished (Fig. 3). Finally, binding of the bNAbs 2G12, which has a glycan epitope, and PG9, whose glycopeptide epitope is dependent upon conservation of the quaternary Env structure, was equivalent in GLA-treated and untreated cells, showing complete preservation of these epitopes (Fig. 3). Lysine residues, which are potential GLA modification sites, were present in several of the analyzed MAb epitopes (see Fig. S2 in the supplemental material). Interestingly, the bNAbs VRC01 and PG9 both directly interacted with two lysine residues on Env, yet neither showed substantially reduced binding to cross-linked protein, suggesting minor or negligible steric effects of the modifications. The epitopes of several other ligands for which crystal structures are available (CD4, 17b, F105, and b12) also contained lysines, the modification of which by GLA may have influenced binding. Thus, in summary, GLA cross-linking selectively modifies gp140 antigenicity, largely preserving all bNAb epitopes tested but reducing binding of several weakly or nonneutralizing MAbs by conformational stabilization, lysine modification within the epitope, or a combination of both.

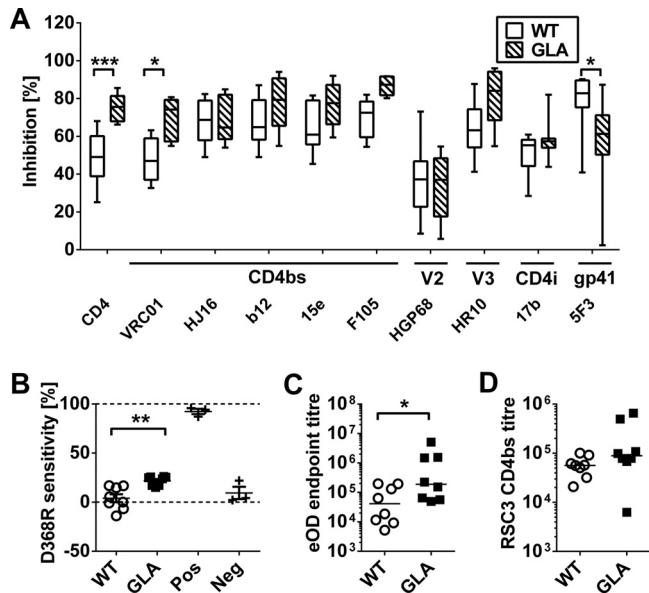
**Immunogenicity of GLA cross-linked gp140.** To investigate how changes in antigenicity translate into immunogenicity *in vivo*, New Zealand White rabbits were immunized with GLA cross-linked or unmodified gp140<sub>CN54</sub>. Immunizations were carried out at weeks 0, 6, 12, and 22 in carbopol (50) and PEI (51) adjuvants, a combination of adjuvants that induces increased antibody titer and avidity compared to either adjuvant alone (unpublished results). Serum samples were collected before the start of the immunization regimen and 2 weeks after each immunization. Indistinguishable binding antibody responses were induced by both antigens at all time points regardless of whether sera were



**FIG 5** Peptide scanning of week 24 serum responses. Binding of sera to 15-mer peptides ( $n = 4$  per group) was tested by peptide array. Binding intensity is plotted in arbitrary units. Regions of gp140 are marked at the top. Statistical analysis for individual regions was performed by one-way ANOVA with Bonferroni's posttest. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

titrated onto untreated (Fig. 4A) or GLA-treated gp140 (data not shown). In contrast, significantly higher ( $P < 0.01$ ) titers were obtained from sera from the GLA group against cell surface-expressed Env (Fig. 4B) than for the WT group, implying better recognition of native Env species. To determine whether antibodies were elicited against the GLA modifications themselves, binding of sera to GLA cross-linked and unmodified irrelevant antigen (hen egg lysozyme [HEL]) was tested. The GLA binding index was calculated as the ratio of the log<sub>10</sub> endpoint titer against GLA cross-linked HEL divided by the log<sub>10</sub> endpoint titer against unmodified HEL (Fig. 4C). There was no difference between animals immunized with unmodified or GLA cross-linked gp140 ( $P = 0.91$ ). Thus, the immunization regimen induced strong binding antibody responses to gp140 and membrane-bound gp160 but did not induce GLA cross-reactive antibodies.

To determine whether GLA cross-linking modified B cell gp140 epitope recognition and antibody production *in vivo*, we first carried out an analysis of linear sequences recognized by sera from animals immunized with WT or GLA-treated antigen. For this, we used a printed overlapping 15-mer peptide Pepsan array spanning the gp140<sub>CN54</sub> sequence. This assay revealed striking differences in the epitopes recognized by sera from GLA or WT gp140-immunized rabbits (Fig. 5; also see Fig. S3 in the supplemental material). Sera from the GLA-gp140 group bound significantly more strongly to peptides from the C1 and V1V2 regions than sera from untreated (WT) gp140-immunized animals ( $P = 0.0005$  and  $P = 0.0293$ , respectively). All animals showed strong responses to the V3 loop, although the responses were higher in the GLA group ( $P = 0.0045$ ). Animals from the WT gp140-immunized group showed strong binding to epitopes in the C terminus of gp120 and gp41, with responses to the immunodominant region being significantly higher than those in the GLA group ( $P = 0.0169$ ). Similar results were obtained when sera were mapped using peptide libraries derived from a panel of gp120s from diverse clades and origins (see Fig. S4). Thus, immunization with cross-linked gp140 refocused antibody responses toward N-ter-



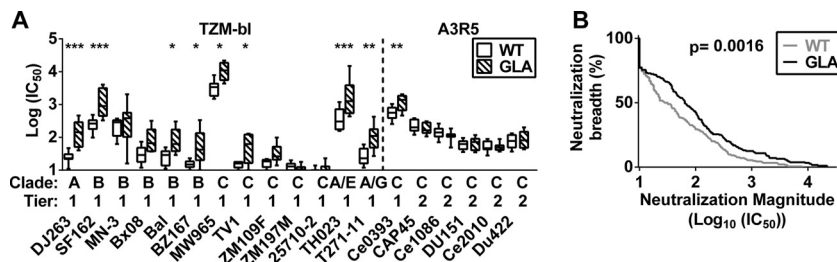
**FIG 6** Conformational epitope analysis of week 24 serum responses. (A) Cross-competition ELISA of sera against a panel of gp140-specific human MAbs. (B) D368R sensitivity ELISA shows amount of CD4bs antibodies. Pos, panel of CD4bs MAbs; Neg, panel of non-CD4bs MAbs. (C) Endpoint titers against an engineered outer domain of ENV. (D) Binding of sera to the CD4 binding site probe RSC3 in the presence of an excess of competing RSC3  $\Delta$ 371I/P363N CD4bs knockout protein. A one-way ANOVA with Bonferroni's posttest (A), Student's *t* test (B), and Student's *t* test of log-transformed data (C and D) were used for statistical analysis. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

minal regions of gp120, including the V1V2 loop, and reduced responses to more C-terminal regions and elements of gp41.

**GLA cross-linked gp140 induces increased CD4bs antibody responses.** Since most bNAb epitopes are conformational in nature and not represented by linear peptide epitopes, we performed epitope mapping by cross-competition using a panel of well-characterized MAbs. Strong competitive responses were induced by WT gp140 against CD4bs, CD4i epitope, V3 loop, and gp41-specific MAbs and weaker responses against a V2 MAb (Fig. 6A). Significant differences in cross-competition were detected with sera from animals immunized with GLA-treated gp140: competition was increased for sCD4 ( $P = 0.0006$ ) and the broadly neutralizing CD4bs MAb VRC01 ( $P = 0.012$ ). Interestingly, cross-competition for the CD4bs MAbs IgG1b12 and HJ16 did not

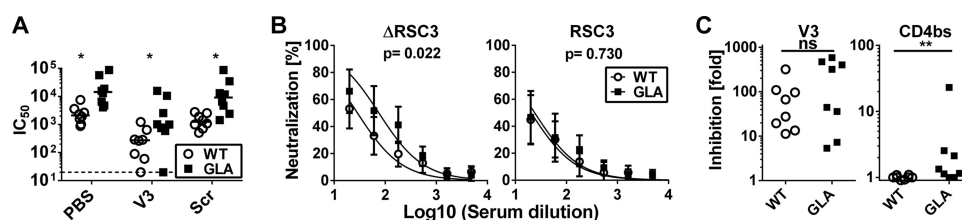
differ significantly between groups, demonstrating selectivity within the CD4bs epitope cluster. Of note, cross-competition was significantly reduced for the 5F3 epitope, suggesting a reduced immunogenicity of this epitope. The increased competition for CD4bs MAbs in sera from GLA-gp140-immunized animals suggested that the modified antigenicity of GLA-treated gp140 was influencing the immunogenicity of this region. To confirm that increased levels of CD4bs antibodies were elicited in animals receiving GLA-treated gp140, we performed three further assays. First, we measured the sensitivity of binding to the D368R mutation introduced into gp120, which abolishes binding of most CD4bs-specific MAbs. Loss of binding to D368R gp120 compared to WT protein was significantly ( $P = 0.0011$ ) stronger in animals receiving GLA cross-linked gp140 than in those receiving unmodified material (Fig. 6B). Second, binding to an engineered gp120 outer domain construct (39, 40), which lacks variable loops and almost exclusively displays elements of the CD4bs, was significantly ( $P = 0.021$ ) increased in the GLA group (Fig. 6C). Three animals in the GLA group showed extremely high titers of binding ( $>10^6$ ) compared to the mean of the non-GLA group (which was  $\sim 4 \times 10^4$ ). Finally, binding to the RSC3 probe (20), which displays the HIV-1 CD4bs in the context of a background engineered to contain non-HIV-1 residues in order to selectively assess HIV-1 CD4bs antibody responses. Binding to this probe in the presence of an excess of RSC3  $\Delta$ 371I/P363N protein (20), which has two mutations in the CD4bs that abolish CD4bs MAb binding and is used to compete out any non-CD4bs binding to the probe, was increased 3-fold in the GLA group, although this was not statistically significant (Fig. 6D). Taken together, these findings strongly support the increased elicitation of CD4bs-specific antibodies in animals immunized with GLA-treated gp140.

**GLA cross-linking induces more potent neutralization responses than unmodified protein.** To assess whether these changes in epitope recognition modified serum neutralization activity, we performed TZM-bl-based neutralization assays (43) using week 24 sera (2 weeks after final boost). All animals showed neutralization of all tier 1 pseudoviruses expressing Envs from 5 different clades (Fig. 7A; also see Table S1 in the supplemental material). Furthermore, sera from the GLA-gp140-immunized group exhibited significantly increased ( $P = 0.0002$ ) neutralization potency against the tier 1 viruses. This increased neutralizing potency could not be detected in the sera at the earlier time point at week 14 (see Table S1). No neutralization of tier 2 viruses could be detected in the TZM-bl assay for either time point (see Table S1); thus, we used the more sensitive A3R5 assay (44) to test a



**FIG 7** Neutralization assays. (A) Neutralization of week 24 sera from animals immunized with unmodified (WT) or GLA cross-linked (GLA) gp140 was tested in the TZM-bl assay against tier 1 viruses (left) and in the more sensitive A3R5 assay against tier 2 viruses (right). (B) Magnitude-breadth curves are shown for pooled data from all animal sera from both assays. A one-way ANOVA of log-transformed data with Bonferroni's posttest (A) and a Mantel-Cox log-rank test (B) were used for statistical analysis. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .





**FIG 8** Characterization of neutralizing responses. (A) Neutralization of HIV-1 strain MW965 by week 24 sera in the presence of V3 peptide (V3), scrambled control (Scr), or vehicle alone (PBS) in TZM-bl cells. (B) Neutralization curves of MW965 by week 24 in the presence of V3 peptide plus either the CD4bs probe RSC3 or the corresponding negative control,  $\Delta$ RSC3. Curves show averages of all animals from the two groups. (C) Loss of neutralization upon addition of inhibitor is shown as the ratio  $IC_{50}$  (no V3 loop)/ $IC_{50}$  (V3 loop) (left) or  $IC_{50}$  (V3 +  $\Delta$ RSC3)/ $IC_{50}$  (V3 + RSC3) (right). A one-way ANOVA of log-transformed data with Bonferroni's posttest (A), a curve-comparison F test (B), and a Mann-Whitney test (C) were used for statistical analysis. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

panel of clade C viruses, including 4 tier 2 viruses (Fig. 6; also see Table S2). All animals showed neutralization against all tier 2 viruses tested (Fig. 7A; also see Table S2). Compared to the TZM-bl assay, differences were smaller in this assay, and only one virus (Ce0393, tier 1, clade C) showed significantly (multiple-comparison adjusted  $P$  of 0.0042) enhanced neutralization by the GLA-gp140 group sera. Pooling all data together to carry out a magnitude-breadth plot (53), we observed a significant ( $P = 0.0016$ ) (Fig. 7B) increase in neutralization in sera from the GLA group compared to the WT group. Thus, immunization with GLA cross-linked gp140 led to the induction of higher titers of neutralizing antibodies than unmodified protein.

Since tier 1 virus serum neutralizing activity in immunized animals is frequently associated with V3 loop recognition, we analyzed the ability of saturating concentrations of a V3 loop peptide to reduce neutralization against a tier 1 clade C virus (MW965). Neutralization titers were reduced by about 10-fold for sera from both WT and GLA-treated gp140-immunized animals, showing a strong component of V3-mediated neutralization (Fig. 8A). However, strong neutralization remained in most sera, suggesting the presence of other species of NAb. Moreover, the significantly greater neutralization activity of the sera from GLA-treated gp140-immunized animals was maintained, implying that the increased neutralizing activity in these sera was mostly non-V3 specific. To test whether the observed increase in CD4bs-specific antibodies was responsible for the increased neutralization in the GLA group, we performed further neutralization assays in which sera were preabsorbed with either RSC3 or  $\Delta$ RSC3. To increase the sensitivity, we performed this assay in the presence of competing V3-peptide in all tests. Curve comparison showed that the GLA group displayed significantly ( $P = 0.022$ ) higher neutralization activity when absorbed with the negative control  $\Delta$ RSC3 (Fig. 8B, left). However, the difference in neutralization disappeared when the CD4bs probe RSC3 was used (Fig. 8B, right). Comparison of the loss of binding between  $\Delta$ RSC3 and RSC3 showed a significant ( $P = 0.0076$ ) difference between the WT and GLA groups (Fig. 8C). The total effect of the CD4bs neutralization was much less pronounced than the effect of the V3 loop (Fig. 8C), which might be partially due to the tier 1 viruses used for this assay, which are known to be very sensitive to V3-mediated neutralization. Taken together, these data show that immunization with GLA cross-linked gp140 leads to the induction of higher titers of neutralizing activity than unmodified protein, which is at least partially due to CD4bs-mediated neutralization.

## DISCUSSION

GLA treatment results in covalent cross-linking of proteins, allowing their conformational stabilization. GLA-modified gp140 largely conserved all bNAb epitopes tested but selectively reduced binding of weakly neutralizing CD4bs MAbs and some other non-neutralizing MAbs. These findings are in line with previous studies that suggested that structural flexibility within CD4bs epitopes is a mechanism by which HIV-1 circumvents the induction and high-affinity binding of CD4bs-directed antibodies, termed "conformational masking" (30). It has further been suggested that weakly neutralizing CD4bs MAbs, such as F105, only recognize one specific gp140 conformation, whereas bNAbs recognize many or all conformations (26, 27). Our data are consistent with this proposal and extend it to include representatives of known bNAb epitope clusters within gp120, including the quaternary epitope-specific bNAbs PG9 and PG16. On the basis of these data, we hypothesize that cross-linking lowers the effective concentration of conformations recognized by nonneutralizing MAbs and redirects immune responses toward features recognized by bNAbs that are conserved between different gp140 conformations. The loss of binding of specific MAbs was unlikely to be due to direct modification of their epitopes by GLA adduction to lysines, since bNAbs VRC01 and PG9 directly interact with two lysine residues each and still retained strong to complete binding. However, we cannot rule out a substantial effect of lysine modification on some of the weakly or nonneutralizing MAbs that lost binding after GLA treatment. A further useful effect of cross-linking the antigen may be that of increasing *in vivo* lifetime after administration, particularly of conformational epitopes such as those contained within the CD4bs that might otherwise be rapidly lost upon protease cleavage or other degradation of the antigen.

Consistent with the antigenicity results, immunization with cross-linked gp140 resulted in substantially different specificities of serum antibody production compared to unmodified gp140, as determined by peptide mapping and binding of sera to the CD4bs. GLA treatment reduced the immunogenicity of C-terminal regions, including the gp41 immunodominant domain, which are considered to elicit nonneutralizing antibodies and are of reduced interest to vaccine design, but the treatment elicited increased antibody responses to the C1 region and the V1V2 loop. Binding of IgG to the V1V2 loop has recently been shown to inversely correlate with the rate of infection in the RV144 trial (3, 5, 54, 55). Although the causality of the relationship between binding V1V2 antibodies and protection from HIV-1 infection is currently un-

clear, induction of this type of antibody response might be a desirable property of an HIV-1 vaccine.

Higher titers of CD4bs-specific antibodies were detected in the GLA-gp140-immunized group. Interestingly, the increased immunogenicity of the CD4bs *in vivo* was achieved despite the reduction in binding of weakly neutralizing CD4bs MAbs *in vitro*. Since elements of the CD4bs, particularly those associated with the gp120 outer domain (16, 56–58), are well conserved and the target of broadly and potently NAb, such as VRC01 (20, 21), rendering this site more immunogenic is considered to be a beneficial property of an HIV-1 vaccine (6, 38). The selectively increased cross-competition of sCD4 and VRC01 but not HJ16, IgG1b12, 15e, and F105 by sera from the GLA group suggest that a very specific subset of CD4bs antibodies have been induced that react predominantly with the outer domain of CD4. This hypothesis is supported by the outer domain binding observed in the GLA group, particularly in some animals that showed 30- to 100-fold increases over the non-GLA group (Fig. 6C). The increase in immunogenicity of the CD4bs in the cross-linked material could be further increased by additional boosting with the homologous material or may be focused using boosting with outer domain constructs, such as that used here as a probe. The reduction in binding of sCD4 induced by GLA treatment might have additional benefits for immunization in humans, since Env binding to cell surface CD4 leads to high titers of CD4i-specific antibodies (59) that are weakly or nonneutralizing (60–62). The neutralizing responses induced by the cross-linked material, although significantly increased in breadth and potency compared to that of the WT material, nevertheless were weaker than desired for vaccine design purposes. Moreover, differences were not observed between sera from animals immunized with GLA-treated and WT gp140 in pseudoviruses carrying tier 2 Envs, suggesting that these Envs have more stringent requirements for CD4bs Nab binding than tier 1 Envs. There are several possible reasons for this limited CD4bs-directed neutralizing response, including insufficient affinity maturation to achieve adequate occupancy at the maximum serum concentration used and an angle of approach to the trimer on the clones of pseudovirus used for neutralization that is sub-optimal for tight binding (49). We propose that these limitations can be overcome by modified immunization protocols that allow greater affinity maturation and boosting with other Env-based constructs to focus antibody responses that have the appropriate angle of approach to the trimer.

We have used a recombinant gp140 that, although predominantly trimeric, contained some dimeric and higher order oligomeric species that were stabilized by cross-linking. Although the higher order species could be detected in both unmodified and cross-linked material in a native page, we cannot rule out that covalent stabilization of the multimers influences immunogenicity. Our GLA-treated material will contain a mixture of Env conformers (27), some of which may have antigenicity and immunogenicity profiles more suitable than others for induction of NAb. Finally, the gp140 used here shows a lack of binding to bNAbs with epitopes dependent upon Env quaternary structure, such as PG9 and PG16. This is typical for soluble Env constructs (63) and suggests a degree of misfolding. Thus, several optimization steps are immediately obvious that might improve the antigenicity of cross-linked gp140, particularly purifying out the trimeric species, potentially leading to better presentation of bNAb epitopes and enhanced immunogenicity and induction of NAbs.

Sera from animals that were immunized with GLA-treated gp140 elicited higher tier 1 virus neutralization titers in the TZM-bl assay than the unmodified gp120-immunized animals. The specificity of this increased activity does not appear to be V3 specific but seems to be at least partially due to CD4bs-mediated neutralization. Robust neutralization of tier 1 viruses after rabbit immunization with Env-based antigens is not in itself novel. However, the fact that higher titers of CD4bs antibodies and neutralization were elicited using an antigen that had undergone a simple modification with a chemical species (aldehyde) used to treat other vaccine antigens with extensive and safe use in humans (64) suggests that this approach deserves further investigation.

## ACKNOWLEDGMENTS

This work was supported by grants from the Bill and Melinda Gates Foundation to the Weiss Neutralizing Antibody Vaccine Design Consortium, the International AIDS Vaccine Initiative (IAVI) Neutralizing Antibody Consortium, and Dormeur Investment Services Ltd. Q.J.S. is a Jenner Vaccine Institute Investigator and a James Martin Senior Fellow.

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