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# Anti-V3/Glycan and Anti-MPER Neutralizing Antibodies, but Not Anti-V2/Glycan Site Antibodies, Are Strongly Associated with Greater Anti-HIV-1 Neutralization Breadth and Potency

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**ABSTRACT**

**The membrane-proximal external region (MPER), the V2/glycan site (initially defined by PG9 and PG16 antibodies), and the V3/glycans (initially defined by PGT121–128 antibodies) are targets of broadly neutralizing antibodies and potential targets for anti-HIV-1 antibody-based vaccines. Recent evidence shows that antibodies with moderate neutralization breadth are frequently attainable, with 50% of sera from chronically infected individuals neutralizing ≥50% of a large, diverse set of viruses. Nonetheless, there is little systematic information addressing which specificities are preferentially targeted among such commonly found, moderately broadly neutralizing sera. We explored associations between neutralization breadth and potency and the presence of neutralizing antibodies targeting the MPER, V2/glycan site, and V3/glycans in sera from 177 antiretroviral-naive HIV-1-infected (>1 year) individuals. Recognition of both MPER and V3/glycans was associated with increased breadth and potency. MPER-recognizing sera neutralized 4.62 more panel viruses than MPER-negative sera (95% prediction interval [95% PI], 4.41 to 5.20), and V3/glycan-recognizing sera neutralized 3.24 more panel viruses than V3/glycan-negative sera (95% PI, 3.15 to 3.52). In contrast, V2/glycan site-recognizing sera neutralized only 0.38 more panel viruses (95% PI, 0.20 to 0.45) than V2/glycan site-negative sera and no association between V2/glycan site recognition and breadth or potency was observed. Despite autoreactivity of many neutralizing antibodies recognizing MPER and V3/glycans, antibodies to these sites are major contributors to neutralization breadth and potency in this cohort. It may therefore be appropriate to focus on developing immunogens based upon the MPER and V3/glycans.**

**IMPORTANCE**

**Previous candidate HIV vaccines have failed either to induce wide-coverage neutralizing antibodies or to substantially protect vaccinees. Therefore, current efforts focus on novel approaches never before successfully used in vaccine design, including modeling epitopes. Candidate immunogen models identified by broadly neutralizing antibodies include the membrane-proximal external region (MPER), V3/glycans, and the V2/glycan site. Autoreactivity and polyreactivity of anti-MPER and anti-V3/glycan antibodies are thought to pose both direct and indirect barriers to achieving neutralization breadth. We found that antibodies to the MPER and the V3/glycans contribute substantially to neutralization breadth and potency. In contrast, antibodies to the V2/glycan site were not associated with neutralization breadth/potency. This suggests that the autoreactivity effect is not critical and that the MPER and the V3/glycans should remain high-priority vaccine candidates. The V2/glycan site result is surprising because broadly neutralizing antibodies to this site have been repeatedly observed. Vaccine design priorities should shift toward the MPER and V3/glycans.**

A relatively small number of epitopes that are targets of broadly neutralizing antibodies (Abs) have been identified on the HIV-1 envelope glycoproteins, gp120 and gp41 (1–5). Prominent among them, the membrane-proximal external region (MPER), the V2/glycan site, and the V3/glycans are models for candidate vaccine antigens (1–3). Sophisticated efforts have been made to attach these targets to protein scaffolds in order to create vaccine immunogens to elicit neutralizing antibodies (6), highlighting their importance in vaccine development.

The membrane-proximal external region (MPER) is the target of three broadly neutralizing monoclonal antibodies (MAbs) (7, 8). The MPER appears to be a relatively simple, linear antigen (9) but harbors substantial complexity (10–14). Another set of potent and broadly neutralizing antibodies, PGT121–128 and PGT130–131, bind primarily to glycans at either position 301 or position 332 in the V3 loop ("V3/glycans") (15). The V2/glycan site is a quaternary epitope (16) that is thought to be stabilized by the

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presence of the N160 glycan, without forming a direct part of the epitope (17). Antibodies recognizing MPER and the V3/glycans have been reported to be self-reactive (2, 18–20). It has long been suspected that self-reactivity checkpoints may limit the ability of many individuals to produce broadly neutralizing responses to such targets (2, 19, 20).

Little is known about the likelihood that any particular neutralizing anti-HIV antibody will become broadly neutralizing, even though the route of somatic hypermutation to arrive at rare broadly neutralizing antibodies is being elucidated (21, 22). Recent evidence shows that antibodies with this moderate neutralization breadth are frequently attainable (perhaps even in response to a vaccine [23]), more so than the very well-studied and highly broadly neutralizing antibodies found in sera from the top 1% to 2% "elite neutralizers" (24). A total of 50% of sera from chronically infected individuals achieve moderate neutralization breadth, neutralizing ≥50% of a large, diverse set of viruses (23).

There is little systematic information about which specificities are preferentially targeted among moderately broadly neutralizing sera. In this study, we observed that neutralization breadth and potency were significantly positively associated with the presence of MPER-specific neutralization and V3/glycan-specific neutralization but not with anti-V2/glycan site-specific neutralization. These data suggest that many individuals are capable of developing antibody responses of moderate to high neutralization breadth recognizing the MPER and V3/glycans. This may suggest that it would be easier to elicit such antibodies in response to a vaccine.

## MATERIALS AND METHODS

**Samples.** Blood samples were collected in December 2009 to July 2011 from donors who were >18 years old and HIV-1 infected (>1 year) and were not exposed to antiretroviral therapy (ART), except for ART given for prevention of mother-to-child transmission (>3 months prior). Study participants were recruited from among (i) caregivers of patients at the pediatric HIV clinic at Groote Schuur Hospital and (ii) attendees of the HIV wellness clinic at the Khayelitsha Site B clinic. Both clinics are in Cape Town, South Africa. Written informed consent was received from study participants. This study was approved by the Human Research Ethics Committee, Faculty of Health Sciences of the University of Cape Town. Data were included from another project approved by the Human Research Ethics Committee, Faculty of Health Sciences of the University of Cape Town, and the National Ethics Committee of the Republic of Cameroon.

**Pseudovirus constructs.** The envelope constructs for COT6.15, Du151.2, Du156.12, and murine leukemia virus (MLV) envelope were kind gifts from Lynn Morris and Penny Moore, National Institute for Communicable Diseases (NICD), Johannesburg, South Africa. The SG3 HIV-1 genome with an inactivated envelope gene (SG3-Δenv) and envelope constructs (unless specified otherwise) were received via the NIH AIDS Research Reagent Reference Program. The 7312A HIV-2 genomic construct and chimeric versions of it with MPER sequences swapped in from Yu2 (C1 [25]) and consensus C MPER (C1C [26]) were kind gifts from George Shaw, University of Pennsylvania, USA. We generated the chimera displaying 253-11 MPER sequence from C1 by site-directed mutagenesis. CAP45.2.00.G3 N160A and K169E and Du156.12 N160K, K169E, and N332A were kind gifts from Lynn Morris and Penny Moore. QH343.A10.N160A, I169E, and N301A/N332A, Du156.12 N301A/N332A, and CAP45.2 N301A were made from QH343.21M.ENV.A10, Du156.12 N332A, or CAP45.2.00.G3 by site-directed mutagenesis. All constructs made by site-directed mutagenesis were confirmed by sequencing both strands of the open reading frame of the envelope gene.

**Neutralization assay.** Neutralization was tested using a standard pseudovirus-based neutralization assay (27). Titers (50% infective doses [$ID_{50}$]) were calculated using curve fit functions in Prism (GraphPad, La Jolla, CA, USA), except that for the purposes of determining neutralization breadth or potency, many $ID_{50}$ values were predicted (see below). MLV was used as a negative control; MLV neutralization was low (<20% neutralization), except for two sera with 20% to 30% at a 1/100 dilution.

**Pseudovirus panel and assessment of neutralization breadth and potency of sera.** A pseudovirus panel ($n = 24$) representing the global HIV-1 pandemic was assembled to evaluate the neutralization breadth of sera. The panel was selected based upon neutralization resistance (28–30; R. A. Jacob, unpublished data), subtype, and geographic diversity. Panel viruses are listed and described (see Fig. 2D). All tier (neutralization resistance) designations are according to Seaman and colleagues (28). A neutralization score for each serum was determined by calculating a geometric mean $ID_{50}$ of all 24 viruses as neutralized by that serum. A neutralization sensitivity score for each virus was determined by calculating a geometric mean $ID_{50}$ titer of all 177 sera neutralizing that virus. Fold difference in sensitivity of subtypes of panel viruses was determined by calculating the ratio of geometric means of all measurements for viruses of each subtype. The 95% confidence interval (95% CI) of the fold difference for each subtype comparison was calculated from a log linear mixed-regression model.

**Detection of anti-MPER, anti-V2/glycan site, and anti-V3/glycan antibodies.** Chimeric 7312A HIV-2 viruses engrafted with a consensus subtype C MPER (C1C [26]) or a Yu2 MPER (C1 [26, 31]) or the MPER sequence of a CRF02_AG virus, 253-11, were used to detect anti-MPER antibodies. Samples were scored positive for anti-MPER antibodies if they neutralized at least one of three chimeric viruses at an $ID_{50}$ of >1,000. Anti-MPER-positive sera did not detectably neutralize the 7312A control (data not shown). Dominant anti-V2/glycan antibodies were detected using pseudoviruses with individual mutations at positions 160 and 169. The N160A/K and K/I169E single-amino-acid substitutions abrogate PG9 and PG16 MAb neutralization and have been used to identify anti-V2/glycan antibodies from blood samples (32–35; T. Moyo, unpublished data). Dominant anti-V3/glycan antibodies were detected using pseudoviruses with mutations at position N301 and/or N332. Either N301 or N332 is necessary for the full neutralization activity of anti-V3/glycan MAbs PGT120–131 (15), and N332 is important for neutralization by anti-glycan MAb 2G12 (36). We used three parent (wild-type) viruses: CAP45.2.00.G3 (37), with virus mutants N160A, K169E, and N301A (N at position 332 is not glycosylated in CAP45); Du156.12 (38), with virus mutants N160K, K169E, and N301A/N332A; and QH343.21M.ENV.A10 (29), with virus mutants N160A, I169E, and N301A/N332A. Neutralization mapping was scored positive if a ≥3-fold drop (32) in neutralization $ID_{50}$ was observed with ≥1 mapping mutant(s) for the site concerned compared to the corresponding wild-type virus.

**Comparison of neutralization breadth and potency between groups of sera.** Neutralization breadth and potency were compared for anti-V3/glycan, anti-MPER, and anti-V2/glycan site neutralizing sera by means of the ratios of the geometric mean $ID_{50}$ titer (potency) and differences (breadth) for the numbers of viruses neutralized at an $ID_{50}$ of >100, i.e., based on the measured $ID_{50}$ values ($n = 312$) and predicted $ID_{50}$ values ($n = 3{,}936$). We calculated the aggregate neutralization breadth and potency for the sera mapped to three different sites and summarized them by means of their ratios and differences compared to sera that did not map to each site or to other groups. Additive linear mixed models were used to model the association of log $ID_{50}$ and percent neutralization. Bootstrap estimation (39) (1,000 replicates) was used to estimate the confidence intervals of these ratios and differences and to estimate the prediction error associated with the $ID_{50}$ estimation and model fit.

**Depletion of anti-MPER antibodies.** An 11-virus panel was assembled to test sera for their capacity to neutralize HIV-1 viruses by recognition of the MPER. The panel consisted of COT6.15 (subtype C), Du151.2 (C), CAP45.2.00.G3 (C), Du422.1 (C), 001428-2.47 (C), TRO.11 (B), REJO4541.67 (B), RHPA4259.7 (B), 928-28 (CRF02_AG), 269-12 (CRF02_AG), and 253-11 (CRF02_AG). Antibodies were depleted in two

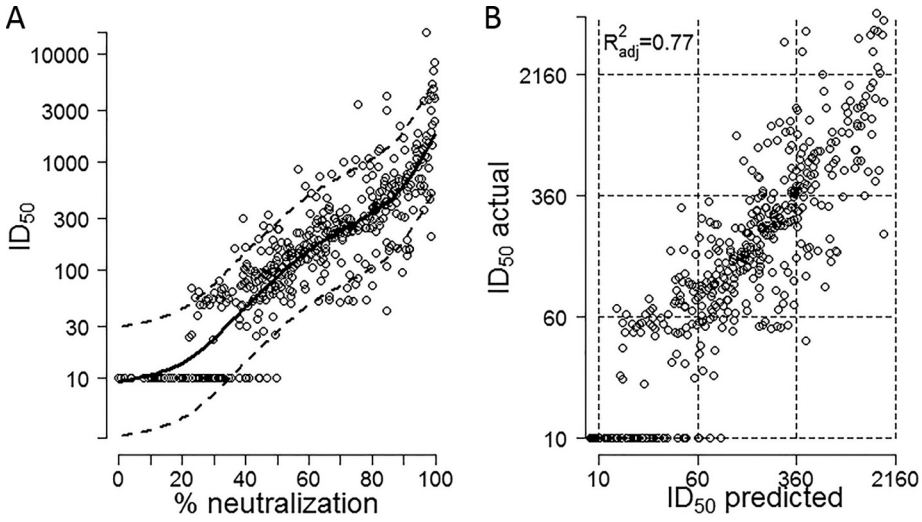


**FIG 1** Statistical prediction model of $ID_{50}$ values from percent neutralization. (A) Prediction function of $ID_{50}$ determined by percent neutralization at a 1/100 dilution. The dashed lines correspond to ±2 times the residual standard error. This reflects the conditional normal distribution related to the underlying linear model. (B) Testing of the prediction model was performed using a set of 474 virus/serum combinations with measured 1/100 dilution screening values and $ID_{50}$ values measured by titration. The data were split into 10 different subsets of approximately the same size; 9 of the subsets were pooled to estimate the model which was used to predict values for the 10th subset. This procedure was repeated 10 times so that a predicted value was obtained for each percent neutralization value. The predicted and measured $ID_{50}$ values are shown for each of the 474 virus/serum combinations.

rounds of depletion (34, 40) as previously described using a biotinylated MPER peptide (MPR.03 [31, 34, 40, 41]; KKKNEQELLELDKWASLWN WFDITNWLWYIRKKK-biotin-NH2; Peptide Synthetics, Hampshire, United Kingdom). Control depletions were performed as described above using streptavidin-magnetic Dynabeads (Invitrogen, Darmstadt, Germany) and a biotinylated control peptide with a scrambled sequence (K KKNEKSNNDWERLWLEWLYIWLQDWAFTLIKKK-biotin-NH2). A threshold of a ≥2-fold drop (34, 40, 42) in $ID_{50}$ compared to control peptide depletion was accepted as positive for MPER-mediated neutralization, i.e., as indicating that more than half of the neutralizing activity was directed against MPER. Six serum samples from the cohort and one CRF02_AG-infected plasma sample (30, 43) were tested against at least 7 of the viruses in the 11-virus panel.

## RESULTS

**Study participants.** The median age of the study participants was 33 years (interquartile range [IQR], 28 to 37 years), with 17 (10%) males and 160 (90%) females, reflecting the general gender imbalance in adults seeking care at our recruiting facilities. The median $CD4^+$ T cell count value was 407 (IQR, 286 to 533). The median known duration of infection was 3.0 years (IQR, 1.7 to 5.9 years). The known duration of infection was determined by the duration of time since the diagnosis of HIV infection or by the earliest $CD4^+$ T cell count documented in clinical records, if possible, or from the study participant's verbal report.

**Use of $ID_{50}$ prediction and its validation.** We directly measured ($n = 312$) or predicted ($n = 3,936$) the neutralization effect of each of the 177 sera on 24 panel viruses and devised a model to estimate the additional error that was generated by the prediction model (prediction error). To generate the prediction model, the effect of the percentage of neutralization at a 1/100 dilution was modeled both linearly and nonlinearly (spline-based model [44]). We chose the nonlinear model (Fig. 1A) because of its better predictive ability (cross-validation error [cv] [39] = 5.42 versus cv = 5.73 for the linear model). The values for the serum/virus pairs used to generate the model were from this study (290/474) or a previous study (36) (72/474) or other unpublished values (112/474). The model was validated by 10-fold cross-validation, i.e., the data were split into 10 different subsets of the approximately same size; 9 of the subsets were pooled to estimate the model used to predict values for the 10th subset (39). This was repeated 9 times until all data points appeared once in the comparison of predicted versus measured $ID_{50}$ values (Fig. 1B). The fit was very good (adjusted $R^2 = 0.7664$), and the estimated slope was 0.99 (95% CI, 0.94 to 1.04), close to the expected slope of 1.

**Measurement of potency/breadth of neutralization and relationship to $CD4^+$ T cell count.** A total of 18% (32/177) of the sera neutralized at least three-fourths of the virus panel (Fig. 2A) and were categorized as broad. A total of 16% (29/177) of the sera had geometric mean $ID_{50}$ titers of >220 (Fig. 2B), our cutoff for highly potent sera. These frequencies appear similar to previously observed frequencies (24, 31, 32, 34, 45–47), although differences in criteria for neutralization breadth/potency, in the panel viruses used, and in cohort characteristics make precise comparisons difficult.

Neutralization breadth and potency correlated well (Spearman's correlation coefficient, $\rho = 0.97$, $P < 0.0001$; data not shown), and each was negatively associated with the $CD4^+$ T cell count (Fig. 2C). The $CD4^+$ T cell count dropped by an average of 8.1 (95% CI, 2.1 to 14.0) for each increase of 1 virus neutralized (adjusted $R^2 = 0.037$, $P = 0.009$) and by an average of 39.6 (95% CI, 12.4 to 66.7) for each 2-fold increase in geometric mean $ID_{50}$ (adjusted $R^2 = 0.044$, $P = 0.005$).

**Neutralization sensitivity of panel viruses.** We ranked viruses by neutralization sensitivity using the geometric mean of the $ID_{50}$ values for all 177 serum samples neutralizing each virus (Fig. 2D). Within-subtype neutralization, i.e., better neutralization of viruses matched to the sera by subtype (8, 28, 48–51), was clearly observed: Four subtype C pseudoviruses were in the most sensitive quartile and none were in the least sensitive quartile when neu-

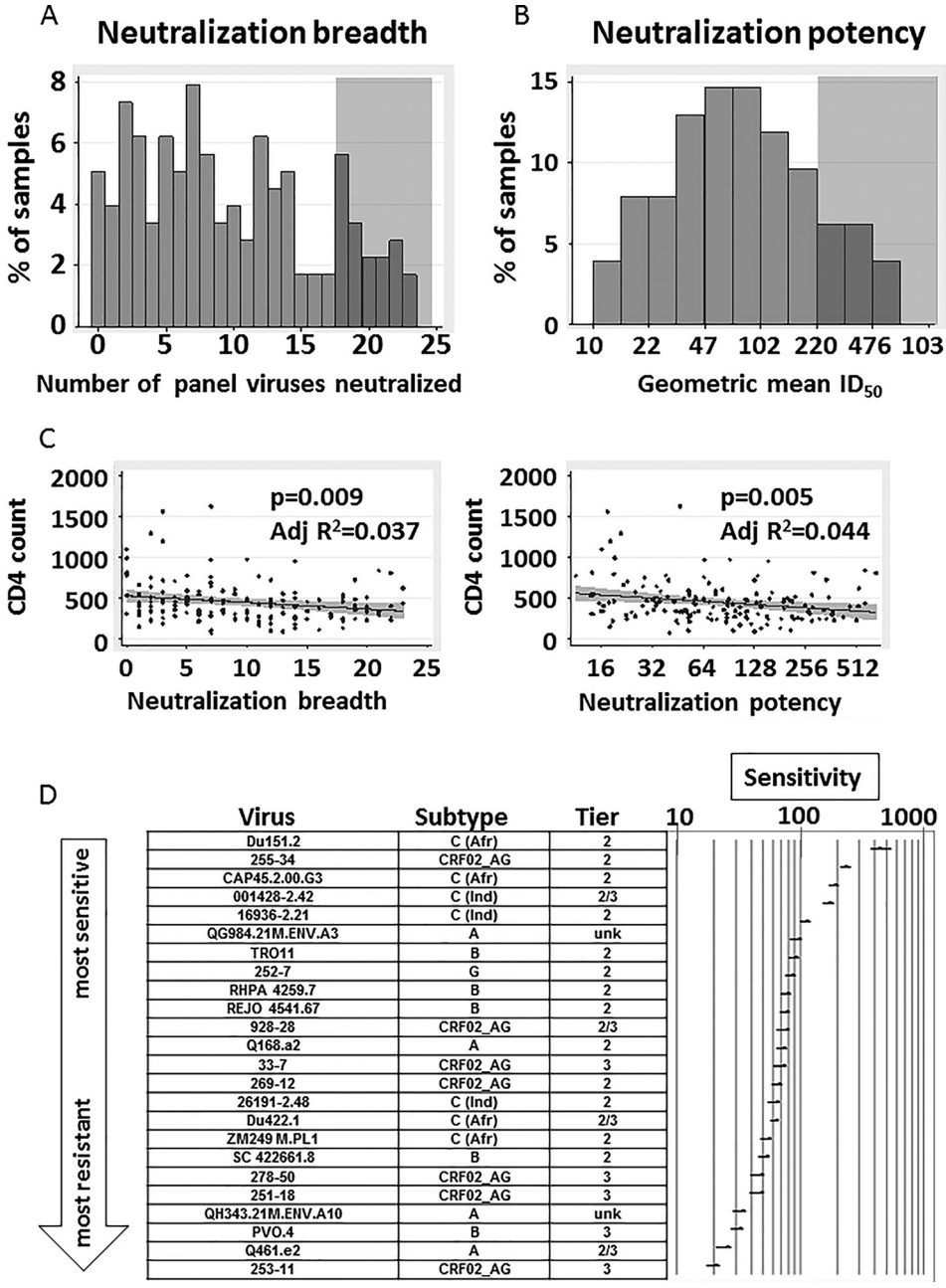


**FIG 2** Neutralization breadth and potency of cohort sera and association with $CD4^+$ T cell count. (A) The distribution of neutralization breadth of the 177 cohort sera is shown by displaying the number of viruses neutralized by each serum. Gray shading indicates at what level samples were scored positive for high neutralization breadth (>3/4 of panel viruses neutralized). (B) The distribution of neutralization potency of the 177 cohort sera is shown by displaying the geometric mean $ID_{50}$ of each serum neutralizing the 24 panel viruses. Gray shading indicates at what level samples were scored positive for high neutralization potency (geometric mean $ID_{50}$, >220). (C) Comparison of neutralization breadth and potency to the $CD4^+$ T cell count measured in the same sample. Potency is shown on a $\log_2$ scale. Line fits, *P* values, and adjusted (Adj) $R^2$ values were calculated from a linear regression model. Gray shading represents the 95% CI of the linear regression line. (D) Relative sensitivity ranking of viruses with respect to the 177 cohort sera. Viruses were ranked by the geometric mean $ID_{50}$ values for all 177 sera neutralizing that virus; 95% prediction intervals (95% PI) from the marginal prediction of a log linear mixed model are depicted. C (Afr), subtype C and derived from an African donor; C (Ind), subtype C and derived from an Indian donor; unk, unknown. Tier designations are from Seaman et al. (28); Tier 2/3, found to be between tiers 2 and tier 3.

tralized by the South Africa sera (~98% subtype C [52]) (Fig. 2D). Subtype C panel viruses were 2.51 times (95% CI, 2.23 to 2.82) more sensitive than subtype A panel viruses, 1.94 times (95% CI, 1.74 to 2.16) more sensitive than subtype B panel viruses, and 1.94 times (95% CI, 1.75 to 2.14) more sensitive than CRF02_AG panel viruses (data not shown). This hierarchy (C→B~AG→A) is substantially different from that of an almost identical virus panel when neutralized by plasma from CRF02_AG-infected donors; in that case, CRF02_AG viruses were the most sensitive subtype of the viruses (30).

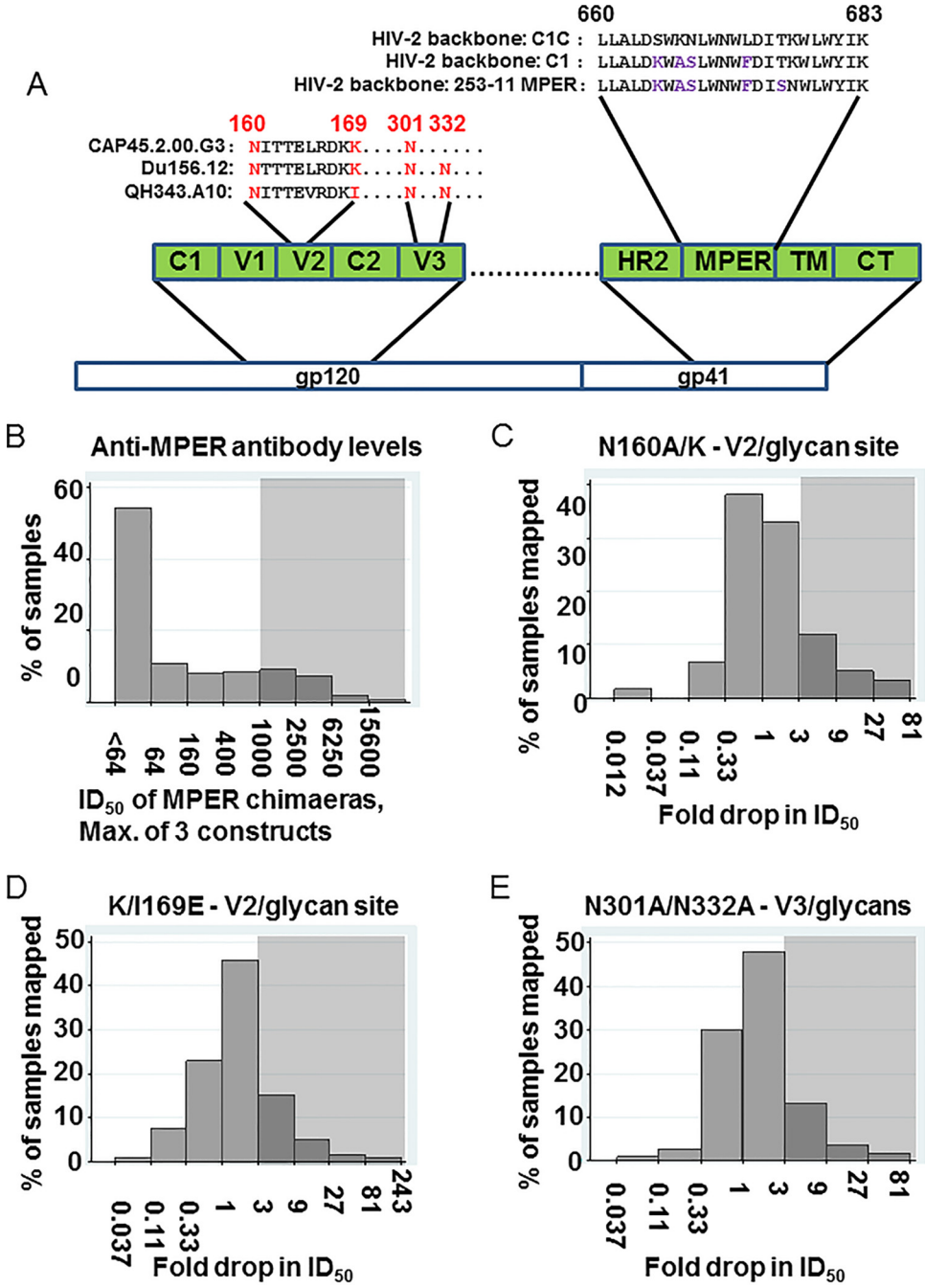


**FIG 3** Mapping of anti-MPER, anti-V2/glycan site, and anti-V3/glycan antibodies in the cohort sera. (A) A depiction of the location of the MPER and the MPER sequences inserted into the HIV-2/HIV-1 MPER chimeric viruses and of the location of the mutations used for mapping the V2/glycan site and V3/glycan epitopes. C, constant region; V, variable loop; HR, heptad repeat; TM, transmembrane domain; CT, cytoplasmic tail. (B) The distribution of anti-MPER $ID_{50}$ (log scale) is shown, using the highest of the three $ID_{50}$ values obtained against the three HIV-2/HIV-1 MPER chimeric viruses. Gray shading indicates at what level samples were scored positive for anti-MPER antibodies ($ID_{50} > 1{,}000$). (C, D, and E) The distribution of drops in neutralization due to the introduction of the N160A/K (C), K/I169E (D), or N301A/N332A (E) mutation compared to the unmutated parent virus. Gray shading indicates at what level samples were scored positive for the indicated mapping mutant (≥3-fold drop compared to unmutated parent virus). If mapping of more than one virus was measured, the maximum fold drop is shown, except when the maximum was less than 3 and the minimum was less than 1; minimum fold drop is shown in order to display presumed masking of neutralization epitopes by glycans. Values below 1 indicate an increase in neutralization of the mutant virus compared to the parent.

**Anti-MPER antibodies within the cohort.** A total of 19% (33/177) of the cohort exhibited significant neutralization activity ($ID_{50} > 1{,}000$) against one or more of the three chimeric construct viruses used to detect anti-MPER neutralizing activity (Fig. 3A and B and Table 1). Previous data demonstrate that high neutralization ($ID_{50} > 1{,}000$) of HIV-2/HIV-1 MPER construct viruses is associated with neutralization of HIV-1 isolates via recognition of MPER (32, 40), while sera with an $ID_{50}$ of <400 do not neutralize

**TABLE 1** Comparison of the likelihood of an antibody being broadly or potently neutralizing depending upon target recognition of neutralizing antibodies

| Epitope mapping category[a] | No. of samples with indicated neutralization potency and mapping category | | Relative risk (95% CI) | *P* value ($\chi^2$)[d] | No. of samples with indicated neutralization breadth and mapping category | | Relative risk (95% CI) | *P* value ($\chi^2$)[d] |
|---|---|---|---|---|---|---|---|---|
| | Less potent[b] | Potently neutralizing[c] | | | Less broad[e] | Broadly neutralizing[f] | | |
| Anti-MPER neg | 124 | 20 | 1.00 (reference) | | 122 | 22 | 1.00 (reference) | |
| Anti-MPER pos | 24 | 9 | 1.96 (0.99–3.91) | 0.061 | 23 | 10 | 1.98 (1.04–3.78) | **0.043** |
| Anti-V2/glycan site neg | 63 | 21 | 1.00 (reference) | | 62 | 22 | 1.00 (reference) | |
| Anti-V2/glycan site pos | 29 | 5 | 0.59 (0.24–1.43) | 0.222 | 27 | 7 | 0.79 (0.37–1.67) | 0.522 |
| Anti-V3/glycan neg | 75 | 17 | 1.00 (reference) | | 73 | 19 | 1.00 (reference) | |
| Anti-V3/glycan pos | 12 | 9 | 2.32 (1.21–4.46) | **0.017** | 12 | 9 | 2.08 (1.10–3.92) | **0.033** |

[a] neg, negative; pos, positive.
[b] Geometric mean $ID_{50}$, <220.
[c] Geometric mean $ID_{50}$, >220.
[d] Bold values indicate a *P* value of <0.05.
[e] <18/24 panel viruses neutralized.
[f] ≥18/24 panel viruses neutralized.

HIV-1 isolates via recognition of MPER (32, 53). In addition, we tested seven samples with neutralization against HIV-2/HIV-1 MPER chimeric viruses ranging from 1,781 to 21,000. Six samples were from among the sera we mapped in this study, and the seventh was a CRF02_AG-infected plasma sample from Cameroon (30, 43). All were tested in MPER peptide depletion assays (34, 40) for dominant (>50%) neutralizing activity in the serum directed against MPER. All seven samples showed dominant neutralizing activity against at least one virus tested. On average, samples neutralized 45% of viruses tested with dominant anti-MPER antibodies, confirming the usefulness of the HIV-2/HIV-1 MPER chimeric viruses for this purpose (Fig. 4).

**Antibodies against the V2/glycan site and V3/glycans within the cohort.** We assessed sera for the presence of dominant neutralizing antibodies recognizing the V2/glycan site or the V3/glycans in any of the pseudoviruses used for mapping, by measuring the drop in neutralization of the mutants with the respective target ablated (mutants depicted in Fig. 3A): N160A/K (Fig. 3C) and K/I169E (Fig. 3D) mutants in V2 and N301A/N332A mutants (Fig. 3E) in V3 (15, 32–36). Because we could assess recognition of V2/glycan and V3/glycan sites only in sera that neutralized Du156.12, CAP45.2, or QH343.A10 parent viruses, we were able to map these epitopes in only a subset of the 177 sera. Mutants were compared to parent viruses CAP45.2.00.G3 (97/177; 95 mapped for V2/glycan site, 90 measured for V3/glycans), Du156.12 (80/171; 80 mapped for V2/glycan site, 77 mapped for V3/glycans), and QH343.21M.ENV.A10 (13/177; 12 measured for V2/glycan site and V3/glycans). In all, 118/177 (66.7%) sera were mapped for the V2/glycan site recognition, and 113/177 (63.8%) were mapped for V3/glycan recognition on ≥1 pseudovirus(es).

Of the tested sera, 29% (34/118) exhibited diminished (≥3-fold drop) neutralization against ≥1 V2 mutant(s) (Table 1). Ten samples (Fig. 3C, values of <0.33) neutralized one N160 mutant substantially better than the corresponding wild type, suggesting that antibody-targeted epitopes shielded by the glycan added at position 160 (54) may be relatively common.

A total of 19% (21/113) of the sera exhibited diminished (≥3-fold drop) neutralization against ≥1 V3 mutant(s) (Table 1). Sera that exhibited increased neutralization for the N301A/N332A mutants appeared less common than those that exhibited increased neutralization for the N160 mutants (Fig. 3D, 2/113 values < 0.33).

**Association of anti-MPER antibodies and anti-V3/glycan with neutralization breadth.** We evaluated associations between the presence of neutralizing anti-MPER antibodies and the presence of neutralizing anti-V3/glycan antibodies with neutralization potency and breadth. We used Wilcoxon rank sum analysis to detect differences in distributions among breadths and potencies. In addition, we calculated differences in neutralization breadths and ratios of potencies between groups. We included an estimate of the error arising from our $ID_{50}$ prediction method that was generated using bootstrapping (95% prediction interval [95%PI]).

Anti-MPER-positive sera were more broadly (Wilcoxon $z = -3.864$, $P < 0.0001$) (Fig. 5A) and potently ($z = -3.916$, $P < 0.001$) (Fig. 5B) neutralizing than anti-MPER-negative sera. We also compared the values representing the magnitude of the difference in breadth or fold increase in potency. Anti-MPER-positive sera neutralized 4.62 (95% PI, 4.41 to 5.20) (Fig. 6A) more panel viruses and were 1.95-fold more potent (95% PI, 1.91 to 2.06) (Fig. 6B) than anti-MPER-negative sera. Anti-MPER-positive sera were 1.98 times more likely to be highly broadly neutralizing (Table 1) (95% CI, 1.04 to 3.78, $P = 0.043$), with a trend toward being more likely to be highly potent (1.96-fold) (95% CI, 0.99 to 3.91, $P = 0.061$) than anti-MPER-negative sera.

Anti-V3/glycan-positive sera were more broadly ($z = -2.470$, $P = 0.0135$) (Fig. 5C) and potently ($z = -2.901$, $P = 0.037$) (Fig. 5D) neutralizing than anti-V3/glycan-negative sera, neutralizing 3.24 (95% PI, 3.15 to 3.52) (Fig. 6A) more panel viruses. They were also moderately more potent (1.68-fold; 95% PI, 1.66 to 1.76) (Fig. 6B) than anti-V3/glycan-negative sera. Anti-V3/glycan-positive sera were 2.08 times more likely to be highly broadly neutralizing (Table 1) (95% CI, 1.10 to 3.92, $P = 0.033$) and 2.32 times more likely to be highly potent (95% CI, 1.21 to 4.46, $P = 0.017$) than anti-V3/glycan-negative sera.

| serum ID number: | | BNAB0060 | BNAB0063 | BNAB0075 | BNAB0088 | BNAB0149 | BNAB0197 | BS50 | | |
|---|---|---|---|---|---|---|---|---|---|---|
| C1C $ID_{50}$: | | 1781 | 21028 | 7664 | 7968 | 4232 | 1974 | (C1) 1855 | | |
| C1C (consensus C) HIV-2/HIV-1 MPER chimera | | 19 | ND | 23 | ND | ND | 43 | ND | test for completeness of depletion | Fold depletion of neutralization activity by MPER peptide-coated beads, compared to depletion with neg control peptide |
| C1 (YU2) HIV-2/HIV-1 MPER chimera | | ND | 33 | 257 | 20 | 219 | ND | 19 | | |
| 253-11 HIV-2/HIV-1 MPER chimera | | 107 | 22 | 168 | 371 | 71 | 152 | 30 | | |
| TRO.11 (B) | Tier 2 | VR | 32.2 | 4.0 | 27.3 | 3.4 | 5.3 | 1.8 | Test HIV-1 viruses | |
| REJO 4541.67 (B) | Tier 2 | 2.8 | 9.7 | VR | 26.4 | 2.8 | VR | 1.7 | | |
| RHPA 4259.7 (B) | Tier 2 | VR | VR | ND | VR | VR | ND | 1.2 | | |
| COT6.15 (C) | not assigned | 7.6 | 12.1 | 8.4 | 9.2 | 2.1 | 3.0 | 3.0 | | |
| Du151.2 (C) | Tier 2 | 3.4 | VR | 2.6 | ND | ND | 59.5 | 1.4 | | |
| Du422.1 (C) | Tier 2/3 | VR | ND | VR | VR | VR | VR | ND | | |
| CAP45.2.00.G3 (C) | Tier 2 | VR | VR | VR | ND | 3.8 | VR | VR | | |
| 001428-2.42 (C) | Tier 2/3 | VR | VR | VR | 1.5 | 1.0 | ND | VR | | |
| 928-28 (CRF02_AG) | Tier 2/3 | 6.5 | 27.0 | 6.5 | 56.4 | ND | 3.5 | 1.3 | | |
| 269-12 (CRF02_AG) | Tier 2 | VR | 17.0 | 15.4 | ND | ND | 4.6 | ND | | |
| 253-11 (CRF02_AG) | Tier 3 | VR | VR | VR | ND | VR | ND | 1.3 | | |
| SF162.L.S (neg control for depletion) | | ND | 1.0 | 1.4 | 1.5 | 1.6 | 1.1 | 1.8 | Neg con for depletion | |
| Num HIV-1 isolates tested | | 11 | 10 | 10 | 7 | 8 | 8 | 9 | | |
| Num HIV-1 isolates w/depl > 2-fold | | 4 | 5 | 5 | 4 | 4 | 5 | 1 | average | |
| % of tested isolates neutralized via recognition of MPER | | 36% | 50% | 50% | 57% | 50% | 63% | 11% | 45% | |

**FIG 4** Verification of anti-MPER neutralizing antibodies in samples recognizing HIV-2/HIV-1 MPER chimeric viruses. Data represent comparison of $ID_{50}$-recognizing HIV-2/HIV-1 MPER chimeric target viruses (top; C1C $ID_{50}$) to tests for dominant anti-MPER neutralizing antibodies measured by bead depletion with anti-MPER coated beads. Fold depletion of neutralizing activity compared to control bead depleted sera is displayed. Depletion of activity against HIV-2/HIV-1 chimeric viruses is displayed to indicate the level of depletion of anti-MPER activity. Tests for depletion of activity against 7 to 11 HIV-1 pseudoviruses are shown, with a >2-fold drop in activity accepted as positive. SF162.L.S was used as a negative control (neg con) for depletion because it is usually recognized by anti-V3 loop neutralizing antibodies (65). The subtype and tier (overall neutralization resistance [28]) of each HIV-1 test virus are indicated. BS50 is a subtype CRF02_AG-infected plasma sample from Cameroon. Neutralization of the Yu2 MPER-swapped chimeric construct (C1) is shown for that plasma sample instead of neutralization of the C1C construct, which contains a consensus C MPER sequence. VR, the virus is resistant to neutralization by the corresponding sample; ND, not determined; num, number; depl, depletion. Color coding: red, >10-fold drop; yellow: 2-to-10-fold drop; gray, <2-fold drop or virus resistant.

**No association observed between the presence of anti-V2/glycan site antibodies and neutralization breadth or potency.** Anti-V2/glycan site-positive sera were not more broadly (Wilcoxon $z = -0.476$, $P = 0.64$) (Fig. 5E) and not more potently ($z = -0.208$, $P = 0.84$) (Fig. 5F) neutralizing than anti-V2/glycan-negative sera. They neutralized only 0.38 (95% prediction interval [95% PI], 0.20 to 0.45) (Fig. 6A) more panel viruses and were not more potent (0.98-fold; 95% PI, 0.97 to 1.00) (Fig. 6B) than anti-V2/glycan-negative sera.

The trend for potency and breadth of neutralization was anti-V3/glycan-positive sera > anti-MPER-positive sera > anti-V2/glycan site-positive sera (Fig. 6). Of these comparisons, only the distributions of neutralization breadth for anti-V3/glycans versus anti-V2/glycan site were significantly different (Wilcoxon $z = -2.362$, $P = 0.0182$; data not shown), with a strong trend in the same direction for potency ($z = -1.941$, $P = 0.0522$; data not shown). We looked for associations between positivity for antibodies to one site and positivity for antibodies to each other site. We were unable to detect any associations (data not shown; all $P$ values > 0.05).

## DISCUSSION

In this study, we found neutralization breadth to be strongly positively associated with the presence of neutralizing anti-MPER antibodies and neutralizing anti-V3/glycan antibodies but not with the presence of anti-V2/glycan site-directed antibodies. This is a surprising finding because anti-V2/glycan site antibodies have been observed frequently in broadly neutralizing sera (32, 34, 53) and because anti-MPER and anti-V3/glycan antibodies are often autoreactive (18–20). Self-reactivity has long been proposed to limit responsiveness to targets of broadly neutralizing anti-HIV antibodies (2, 19, 20). It has long been known that many anti-MPER antibodies are self-reactive (20). Recently, of 3 tested anti-V3/glycan MAbs, PGT125 and PGT128 were shown to be polyreactive (18). In contrast, only one of five tested anti-V2/glycan site MAbs was polyreactive (18) and a recent review indicated that, among all broadly neutralizing antibodies responding to this site, only CH103 is polyreactive (2). Our findings suggest that autoreactivity may not be as large a barrier to generating moderately broadly neutralizing and potent anti-HIV-1 neutralizing antibody responses as commonly thought.

Despite these apparent self-reactivity barriers, we show that a substantial proportion of chronically HIV-infected individuals are able to produce at least moderately broadly neutralizing antibody responses to both MPER and the V3/glycans, while such moderately to highly neutralizing antibodies are not enriched among the V2/glycan site-recognizing sera. It has been suggested that moderately broadly neutralizing responses occur frequently and may therefore be more attainable from a vaccine than highly broadly neutralizing responses (23). For example, clusters of related, moderately broadly neutralizing antibodies occur in natural infection and can give high neutralization breadth and potency in

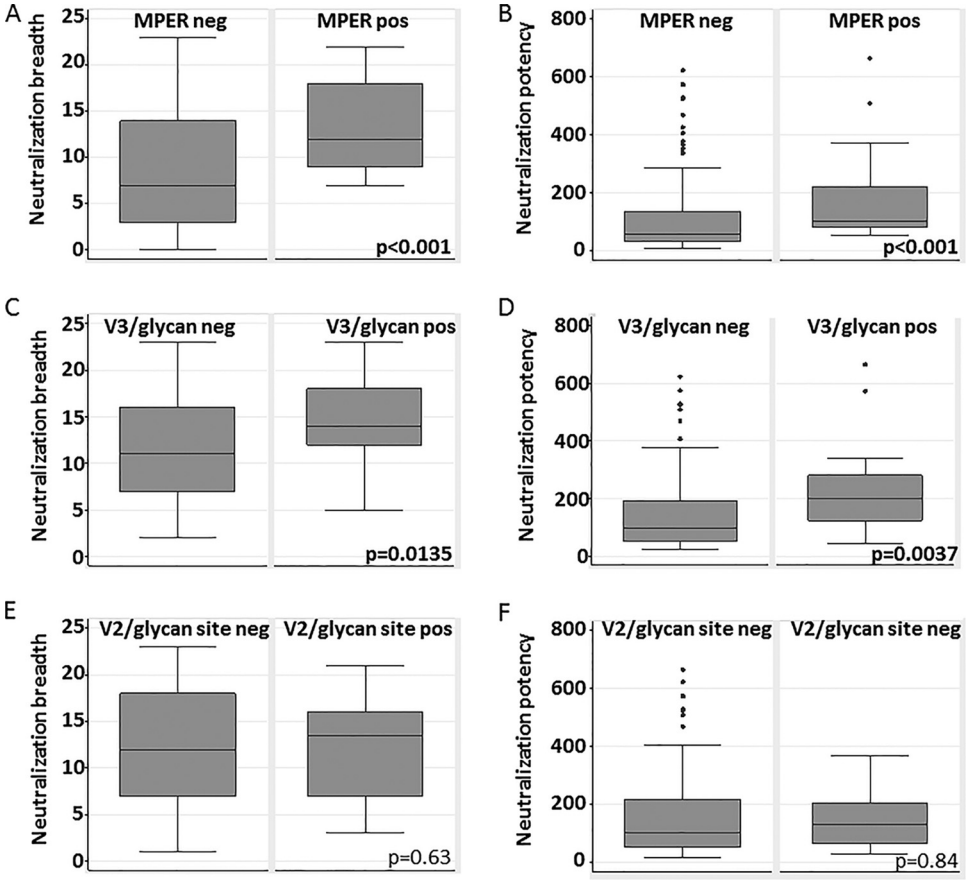


**FIG 5** Differences in neutralization breadth and potency between groups of sera recognizing particular targets. Data represent the results of comparison of the distributions of neutralization breadth scores (A, C, and E) and neutralization potency scores (B, D, and F) based upon detection of functional anti-MPER antibodies (A and B), dominant anti-V3/glycan antibodies (C and D), or dominant anti-V2/glycan site antibodies (E and F). *P* values were calculated from Wilcoxon rank sum tests. neg, negative; pos, positive.

aggregate (55). The (presumably more attainable) precursors of broadly neutralizing antibody PGT121 are also, at least sometimes, moderately neutralizing (56).

In this study, 19% (33/177) of the cohort had high titers ($ID_{50} > 1{,}000$) of anti-MPER antibodies (Fig. 3B), which is similar to the proportions of previously studied North American HIV-1 subtype B cohorts (with an $ID_{50}$ cutoff value of >1,000), 12% (42) and 19% (7), but lower than the prevalence in a sample from one European cohort (57). MPER antibody prevalence in this cohort was higher than that in a blood bank cohort from South Africa (4%) (58), presumably because the blood bank cohort included individuals infected for shorter time periods than our study participants, thus containing lower levels of neutralizing antibodies (32, 35, 47). Our results indicate that prevalence of anti-MPER does not differ substantially by subtype and is often reasonably high.

We found a negative association between neutralization breadth and contemporaneous $CD4^+$ T cell count (Fig. 2C), similarly to a previous study (47). Others found no association with contemporaneous $CD4^+$ T cell count but did find higher neutralization with a greater $CD4^+$ T cell decline (32) and/or with a lower $CD4^+$ T cell count earlier in infection (32, 46). The relationship between $CD4^+$ T cell count and neutralization potency and breadth that we observed in adults appears different from that in children, in whom neutralization potency was lower with depletion of $CD4^+$ T cells (59).

A limitation of our study is that we did not evaluate other targets of broadly neutralizing monoclonal antibodies: the CD4 binding site (CD4bs [60]), the site recognized by MAbs 3BC176 and 3BC315 (61), and the newly identified hinge site at the interface between gp120 and gp41 (5). The hinge region is currently too poorly characterized to allow mutational mapping, and mapping approaches for the 3BC176/315 site are not established. The CD4bs site is not amenable to mapping on the scale necessary for this analysis because the mapping process (62) requires large amounts of serum and recombinant proteins.

Sera with anti-V2/glycan site antibodies (i) are neither more potently neutralizing nor substantially more broadly neutralizing than sera without detectable anti-V2/glycan antibodies and (ii) are less broadly neutralizing than sera with anti-V3/glycan neutralizing sera. There are a series of examples of highly broadly and potently neutralizing V2/glycan site-recognizing MAbs such as PG9 and PG16 (16) and V2/glycan site-recognizing sera (32–34, 53), demonstrating that broadly neutralizing antibodies targeting the V2/glycan site are possible. Higher variability of the V2/glycan site (33) did not prevent the production of very broadly neutralizing and potent antibodies such as PG9 and PG16. It is clear that broadly neutralizing and potent antibodies generally arise after a

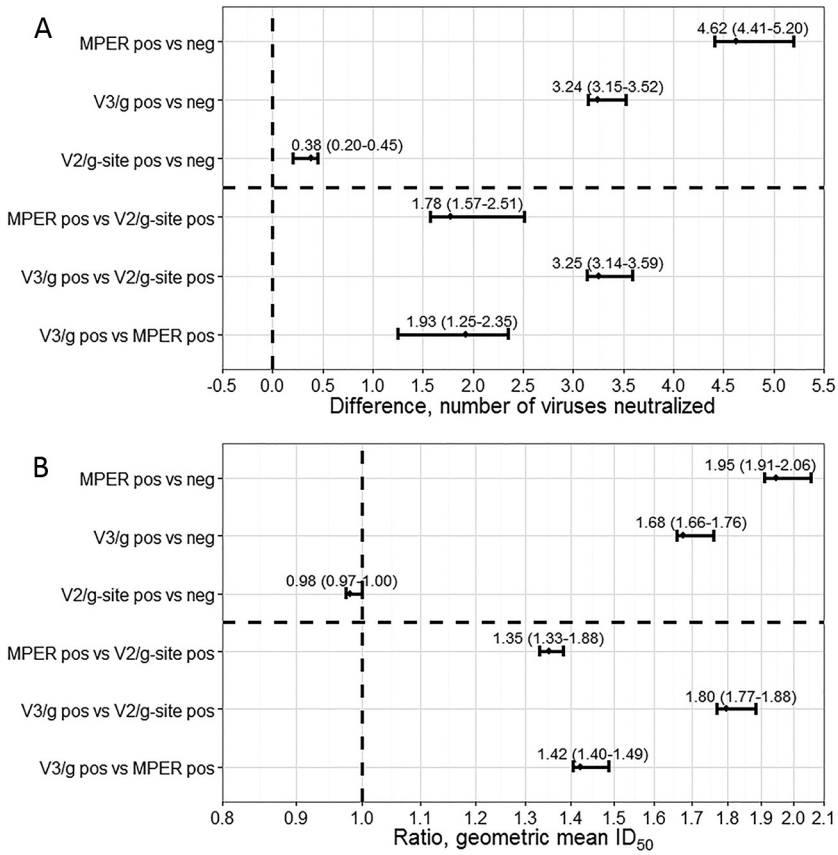


**FIG 6** Changes in neutralization breadth and potency based upon recognition of MPER, the V2/glycan site, or the V3/glycans. We explored changes in neutralization breadth (A) and potency (B) based upon target mapping of the neutralizing antibodies in each serum. The groups that were compared are indicated on the *y* axis. The differences in neutralization breadth between the indicated groups are indicated by the differences in the number of viruses neutralized (no difference = 0), and the difference in neutralization potency is indicated by the ratio of aggregate geometric mean $ID_{50}$ values to those seen with the panel viruses (no difference = 1). Values of 95% prediction intervals (PI) are shown and indicate the bootstrap-based estimate of the error associated with the $ID_{50}$ prediction algorithm in this data set. g, glycan; g-site, glycan site.

complex process of somatic hypermutation-mediated evolution of the antibodies (1–3, 63). Reasons for the failure of most serum anti-V2/glycan site antibodies to be broadly neutralizing and potent may include complexity in this evolution process as well as technical difficulties affecting the ability of an immune response to produce a highly broadly neutralizing and potent anti-V2/glycan site antibody response. For example, some part of the structure of these antibodies, perhaps the extended anionic loops (64), may be difficult to fashion effectively by somatic hypermutation of germ line antibodies. If so, our data suggest that a fast progression to high neutralization breadth is not frequently found among the anti-V2/glycan antibodies such as those produced by donor CAP256 (~5 months [22]). Importantly, such difficulties may also extend to immune responses to an HIV vaccine. Thus, antibodies of moderate neutralization breadth against the MPER or V3/glycans may be easier to induce with a vaccine than those against the V2/glycan site, and these sites might be more amenable models than the V2/glycan site for vaccine design.

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