# Title: Antibody responses elicited by immunization with BG505 trimer-immune complexes

Running title: Immune complex immunization

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## ABSTRACT

Immune complex (IC) vaccines have been successfully used to increase immune responses against various pathogens, including HIV-1. Additionally, IC vaccines can induce qualitatively different antibody responses with distinct antigenic specificities compared to the same antigens used alone. Here we measured the HIV-1-specific antibody response in female New Zealand White rabbits after immunization with ICs made from BG505 SOSIP.664 trimers (BG505 trimers) and three different rabbit monoclonal antibodies (mAbs) with varying neutralization profiles. Two of the mAbs were specific for a hole in the glycan shield of the BG505 trimer while the third, which bound less avidly, was specific for determinants at the gp41/gp120 interface. We found that immunizing with one of the glycan hole-specific ICs resulted in lower levels of trimer-binding antibodies compared to vaccination with the uncomplexed trimer and that ICs made using either of the glycan hole-specific mAbs resulted in lower rates of anti-trimer antibody decay. We conclude that ICs based on mAbs that bound to the immunodominant glycan hole epitope likely diverted antibody responses, to some extent, away from this site and to other regions of the trimer. However, this outcome was not accompanied by a widening of the breadth or an increase in the potency of neutralizing antibody responses compared with uncomplexed trimers.

## IMPORTANCE

Immunodominant epitopes may suppress immune responses to more desirable determinants, such as those that elicit potentially protective neutralizing antibody

responses. To overcome this problem, we attempted to mask immunodominant glycan holes by immunizing rabbits with immune complexes (ICs) consisting of the BG505 SOSIP.664 gp140 trimer and monoclonal antibodies that target the glycan holes. We found that IC vaccination likely diverted antibody responses, to some extent, away from glycan holes and toward other regions of the trimer. IC vaccination resulted in a slower decay of HIV-1-specific antibodies than did immunization with uncomplexed trimer. We did not observe a widening of the breadth or an increase in the potency of neutralizing antibody responses compared to uncomplexed trimers. Our results suggest that selective epitope dampening of BG505 trimers by ICs is rather ineffective. However, IC vaccination may represent a novel means of increasing the duration of vaccine-induced antibody responses.

## INTRODUCTION

A major goal of HIV-1 vaccine design is to elicit neutralizing antibody (NAb) responses with activity against a broad array of virus strains. This task has proven to be difficult and will likely require immunogens that expose or mimic vulnerable sites on the native HIV-1 trimer (40). Toward that end, the BG505 SOSIP.664 envelope glycoprotein (Env) trimer has been used in multiple animal immunization studies (4, 9, 20-23, 30, 39, 44, 48). The BG505 Env glycoprotein lacks N-glycosylation sites at positions 241 and 289 (23, 30). The resulting holes in the glycan shield expose immunodominant targets that elicit NAbs specific to the sequence-matched, i.e., autologous, BG505.T332N virus, which lacks the same N-glycosylation sites (22, 23, 30). Antibodies against sites of vulnerability associated with neutralization breadth, such as the CD4 binding site

(CD4bs), V1/V2 loop region, V3/Asn332 glycan patch, gp120/gp41-interface, or the membrane proximal external region have not yet been elicited consistently by immunizing with the BG505 SOSIP trimer or other recombinant Env proteins (52, 53).

It has been proposed that suppressing immunodominant non-NAb or narrow-specificity NAb epitopes may help drive the emergence of neutralization breadth (2, 6, 8, 55). One way to decrease the immunogenicity of immunodominant regions is via epitope masking (1, 11, 15, 34, 42, 43, 45). For example, adding N-glycosylation sites to the V3 region or the 241/289-glycan hole epitope of the BG505 trimer suppresses the immunogenicity of its non-NAb epitopes and, in some cases, diverts the Nab responses to neo-epitopes (15, 37, 38). Antibodies, by forming immune complexes (ICs) with antigens, can also be used to mask immunogenic epitopes (49, 54, 56). Antibody binding can also change antigen stability and thereby affect processing pathways (7, 50) and T-cell epitope presentation (19). ICs have also been shown to induce qualitatively different antibody responses with distinct antigenic specificities from those elicited by antigens alone and can enhance immune responses against various viral pathogens including HIV-1 (5, 19, 26-28, 49). Guided by these observations, we immunized rabbits with ICs formed between the BG505 SOSIP.664 trimer and rabbit mAbs that targeted either a glycan hole at positions 241 and 289 or an epitope located at the gp120-gp41 interface around residue 611 (3).

## MATERIALS and METHODS

### Reagents

BG505 SOSIP.664 trimers were expressed in CHO cells and purified as described previously [4]. Rabbit mAbs 11A, 11B, and 12A were transiently expressed, affinity purified and checked for purity and integrity as previously described [6]. Human mAbs PGT145 and F105, as well as the HIV-1 MN Env (15-mer) V3 peptides CTRPNYNKRKRIHIG, RKRIHIGPGRAFYTT, and HIGPGRAFYTTKNII were obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH.

### Rabbit Immunization

Immunizations and blood sampling were carried out under subcontract by Pacific Immunology (Ramona, CA). Prior to immunization, the BG505 trimers (30 µg/rabbit) were incubated with or without one of the rabbit mAbs (32 µg/rabbit) for 30 min at room temperature at a molar ratio of 1:3 and then formulated in 75 Units of ISCOMATRIX™ adjuvant. The immunization mixture was injected intramuscularly into female New Zealand White (NZW) rabbits (5 rabbits per group). The use of rabbit antibodies for immune complex formulation avoids anti-immune globulin responses after rabbit immunization. The animals were immunized at weeks 0, 4, and 20 and bled at weeks 0, 6, 8, 12, 16, 22, and 26 as previously described (10, 47).

### Ethic statement

The NZW rabbits were housed, immunized and bled at Pacific Immunology, in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and in adherence to the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996.

**ELISA for anti-trimer antibodies**

96-well ELISA plates were coated overnight at 4°C with the gp120 C5-epitope-specific antibody D7324 (500 ng/well). Wells were then washed and blocked in 5% blocking solution (5% non-fat dry milk in DPBS, containing 0.05% Tween) for 1 hour at 37°C (39). After washing and blocking, plates were further incubated with D7324-epitope tagged BG505 trimers (50 ng/well) for 2 hours at 37°C. Duplicates of serially diluted (1:5 in 1% blocking buffer) rabbit serum samples (starting at a dilution of 1:20) or of mAbs (starting at 5 µg/ml) were added and incubated for 1 hour at 37°C. Unbound antibodies were washed away and trimer-specific antibodies were detected with a goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) diluted in 1% blocking solution. After 1 hour, plates were washed, developed with 3,3', 5,5'-tetramethylbenzidine (TMB) solution, and subsequently read at 450 nm with a Synergy 2 plate reader (BioTek). Half maximum effective concentrations ($EC_{50}$) were determined and calculated using GraphPad Prism 7.0 software after applying a nonlinear regression curve fit on the antibody binding curves.

**Immune complex conformation assay**

ELISA plates were coated with 2 µg/mL (100 ng/well) of a goat anti rabbit Fc-specific antibody and incubated overnight at 4°C. Plates were then washed and blocked with 5% blocking solution. In the meantime 1 µg/mL of mAbs 11A, 11B, and 12A were mixed with 1 µg/mL of BG505 trimers and incubated for 45 min at 37°C. After blocking, ELISA plates were washed and incubated with the mAb/BG505 trimer mixture for 1 hour at 37°C. IC formation was tested with a serial dilution (1:3) of human mAbs PGT145 and F105. Diluted antibodies were incubated for 1 hour at 37°C. Bound antibodies were detected by a HRP labeled goat anti-human Fab-specific antibody. Plates were developed as described above.

**Peptide ELISA**

HIV-1 MN V3 envelope peptides (15-mers) were coated at 500 ng per well (10 µg/mL) and incubated over night at 4°C. After washing, wells were blocked in 5% blocking solution for 1 hour at 37°C. Replicates of diluted rabbit sera at week 0 (preimmune serum control) as well as week 22 (1:100 dilutions in 1% blocking buffer) were added and incubated for 1 hour at 37°C. Unbound antibody was removed by washing and bound antibodies were detected by a goat anti rabbit IgG HRP conjugate. After 1 hour at 37°C, plates were washed and detected as described above. Fold increase in signal intensity of week 22 serum compared to week 0 serum from the same animal was calculated.

**Neutralization assays**

Neutralization activity of the rabbit sera at week 22 was measured with Env-pseudotyped viruses in TZM-bl cells as described previously (31). In brief, heat-inactivated test samples were serially diluted 1:3 in duplicate and incubated with a pre-titered dose of the respective pseudotyped HIV-1 virions for 1 hour at 37°C. Freshly trypsinized TZM-bl cells (10,000 cells per well) were then added to each well and incubated for 48 hours at 37°C in the presence of DEAE-dextran (15 µg/mL). Luminescence was measured using the Britelite luminescence reporter gene assay system (PerkinElmer Life Sciences). A virus control (cells and virus) and a background control (cells only) were used to calculate neutralization titers ($ID_{50}$), defined as the dilution at which relative luminescence units (RLU) were reduced by 50% compared to that in the virus control wells after subtraction of background control RLUs. All stocks of HIV-1 Env-pseudotyped viruses for neutralization assays were prepared by transfection in 293T-cells and were titrated in TZM-bl cells, as previously described (31).

**Epitope mapping assays**

Epitope mapping of selected rabbit serum samples was performed at Duke University Medical Center and Weill Cornell Medical College (WCMC). Mapping at Duke was conducted using the following BG505 virus mutants provided by WCMC: N133A, N142A, N280D, S241N, P291T, and S241N + P291T. Further mapping at Duke was carried out with the CH119.10 virus variants: N88A, N160A, V295N, N332A, N611A, and N625A as well as the 25710-2.43 virus mutants: N88A, N160K, N332A, and N625A. Human monoclonal antibodies VRC01, CH01-31, VRC34.01, PG16, and PGT128 were used as controls. All mutants were prepared and titrated as previously

described (31). Differences in serum mapping were considered real when the calculated $ID_{50}$'s of the parental strain and the respective mutant were at least 3-fold different. For values that were below our limit of detection ($ID_{50}$ <30), we used one-half the cut-off ($ID_{50}$ =15) for calculations. Mapping at WCMC was performed using the BG505 virus mutants 133aN + 136aA, N241 KI + N289 KI, N356 KI, and N465 KI. Relative $ID_{50}$ ($RID_{50}$) and relative extent of neutralization (REN) were calculated at WCMC as described elsewhere (22). In brief, for $RID_{50}$, the $ID_{50}$ against the mutant is divided by the $ID_{50}$ against the parental strain; for REN, the effect on neutralization is expressed as the extent of inhibition of mutant strains divided by that of the parental strain at a 1/50 dilution of serum.

**Statistics**

Kruskal-Wallis tests with Dunn's multiple comparison tests were used to analyze differences in continuous variables between groups. Correlations were analyzed using Spearman's rho. Statistical analyses were conducted using GraphPad Prism 8.0.

## RESULTS

IC immunization modifies titers of anti-trimer binding antibodies

Four groups of rabbits were immunized with uncomplexed BG505 SOSIP.664 trimers (group D) or ICs formed between the trimer and three different rabbit mAbs (**Table I**). The ICs consisted of the trimer bound to mAb 11A (group A), mAb 11B (group B), or mAb 12A (group C) (30). MAbs 11A and 11B bind to similar glycan hole-epitopes that are both in the vicinity of residue S241 and have very similar binding affinities (4.6 x $10^{-10}$ M and 4.5 x $10^{-10}$ M, respectively; **Table I**) (30). 11A and 11B neutralize the parental BG505.T332N virus with $IC_{50}$ titers of 0.17 and 0.11 µg/mL, respectively (30). MAb 12A binds to the gp41/gp120 interface close to the epitope for the PGT151 bNAb and has a lower binding affinity for the BG505 trimer (7.9 x $10^{-8}$), compared to mAbs 11A or 11B (**Table I**) (3). MAb 12A very weakly neutralizes the parental virus ($IC_{50}$ titer of 100 µg/mL), but very potently neutralizes the same virus from which the N611 glycan is removed ($IC_{50}$ titer of 1.06 µg/mL) (30).

The BG505 trimer batch used for the immunization was confirmed to have an appropriate antigenic conformation by demonstrating its binding to mAbs 11A, 11B, and 12A (**Figure 1A)** as well as the trimer-specific mAb PGT145 but not to the gp120 monomer-specific mAb F105 (**Figure 1B**). We found no allosteric changes induced upon IC formation based on our antibody controls PGT145 and F105 (**Figure 2**).

Serum samples from all immunization groups at week 0, 22, and 26 were assessed for anti-trimer binding, and the midpoint titers (i.e. $EC_{50}$ titers) were calculated after applying a nonlinear regression fit to the antibody binding curves. Using serum samples obtained two weeks after the last immunization (i.e., week 22), we observed a

223 significant difference in median anti-trimer binding antibody titers between the individual
224 groups ($p$ = 0.0056). In pair-wise analyses, the difference was due to a lower median
225 binding titer in group A (mAb 11A ICs) compared with the trimer-only immunogen group
226 D ($p$ = 0.002; **Figure 3A**). By week 26 (six weeks after the last immunization), anti-
227 trimer titers had declined in all four groups (**Figure 3B**). However, compared to group D,
228 the rate of BG505-specific antibody midpoint titer decline was significantly slower for
229 groups A ($p$ = 0.042; Kruskal-Wallis with Dunn's multiple comparison test) and B ($p$ =
230 0.006) but not for group C ($p$ = 1.0). There was a trend toward a slower rate of decline
231 for group B compared to group C ($p$ = 0.090). Group A titers declined more slowly than
232 group C as well, but the difference was not statistically significant after adjusting for
233 multiple comparisons ($p$ = 0.39).
234
235 Neutralizing antibody responses are generally lower with IC immunization
236 To determine if IC vaccination had an impact on virus neutralization, sera were
237 tested against a panel of tier-1 (n = 5) and tier-2 (n = 19) HIV-1 isolates at Duke (**Figure**
238 **4**). Autologous NAb responses to the autologous BG505.T332N virus were significantly
239 lower in group A than in group D animals ($p$ = 0.01; **Figure 5A**). Correspondingly, Nab
240 titers against the parental strain BG505 were also significantly lower in group A
241 compared to group D ($p$ = 0.049); **Figure 5A**). As previously reported, there was a
242 strong correlation ($r$ = 0.83; $p$ < 0.0001) between BG505.T332N NAb responses
243 measured as $ID_{50}$ and BG505.T332N anti-trimer binding antibody titers measured as
244 $EC_{50}$ (**Figure 5B**) (14, 35).

We also looked at NAb titers against tier-1 strains that were neutralized by sera from at least 4 out of 5 of the animals in each group ($ID_{50} > 20$). Consistent with the antibody binding data, group A animals responded with lower NAb titers against each of these strains compared to the control group D, although the differences were not always statistically significant: *p*-values = 0.4 for MN_3; 0.049 for SF162_LS; and 0.21 for MW965_26 (**Figure 5C**). In the case of MN_3, the NAb responses for both group A and group B were significantly lower than for group C ($p = 0.01$ and 0.03, respectively). For MW965_26, the responses in group A were significantly lower than in group C ($p = 0.008$) (**Figure 5C**).

Overall, neutralization breadth and potency against heterologous tier-2 strains (n=17) in our virus panel were limited and did not differ between the groups except for NAb titers in group B, which were significantly higher than in group C, ($p = 0.039$) (**Figure 5D)**.

Epitope mapping reveals various neutralizing antibody determinants

The most frequently targeted autologous NAb epitope in BG505 SOSIP-trimer immunized rabbits is a hole in the glycan shield created by the absence of the N241 and N289 glycans (22, 30, 47). To assess whether the same or a different epitope(s) was targeted in the IC-immunized rabbits, we used the same method, based on BG505.T332N mutant viruses, to analyze all the sera from groups B, C, and D (**Figure 6A**). The group A sera were not tested since the NAb titers against the wild-type BG505.T332N virus were too low to be mapped with any precision (22). According to the Duke mapping data, the virus mutants with the N241-glycan and/or P291T

substitution knocked in were predominantly resistant to neutralization by three of the five group B (1B, 3B, and 5B) and group C sera (2C, 3C, and 5C) as well as by four of the five group D sera (2D, 3D, 4D, and 5D) (**Figure 6A**). In contrast, the NAbs in sera 2B, 4B, 1C, 4C and 1D did not target the N241/N289-glycan hole. This analysis suggests that, in most cases, immunization with ICs containing glycan-hole specific mAb 11B or gp120-gp41 interface-specific mAb 12A did not divert the NAb response away from the glycan hole that is predominantly targeted in the trimer-only group D.

We conducted further mapping studies (WCMC) to characterize the NAb responses of samples 4B, 4C, 5C, and 1D against a previously described immunodominant C3/465 epitope (22). Based on partial resistance of the N142A.6 mutant, the response in serum 4B targeted the V1 loop, a rare but not unprecedented response to BG505 SOSIP trimers (**Figure 6A**) (22). The use of the 133aN + 136aA virus mutants at WCMC confirmed that the 4B serum neutralizing activity was directed against a V1 epitope (**Figure 6B**) (22). For serum 5C, the NAbs were directed against the C3/465 epitope. Of note is that the neutralization potency of serum from animal 5C and the CD4bs-specific mAb VRC01 were highly affected by the N280D mutation (**Figure 6A**). NAbs in sera 4C and 1D recognized the C3/465 epitope, albeit, to a lesser extent than serum 5C (**Figure 6B**).

Serum from animal 2B, which had modest activity against the heterologous tier-2 virus, HIV-$1_{CH119.10}$ (**Figure 4**) and which did not target the N241/N289-glycan hole on the BG505.T332N virus (**Figure 6A**), was further evaluated at Duke using a panel of HIV-$1_{CH119.10}$ variants that can be differentiated by binding to bNAbs VRC34.01 (gp120/gp41 interface), PG16 (V2 glycan), and PGT128 (V3 glycan) (**Figure 7A**).

Serum 1D, which neutralized the heterologous tier 2 virus, HIV-$1_{a25710\_2\_43}$ (**Figure 4**), was studied in an analogous way via mutants of that virus (**Figure 7B**). None of the HIV-$1_{CH119.10}$ mutants was significantly resistant to serum 2B, implying that the NAbs present did not target the gp120/gp41 interface, the V2 glycan or the V3 glycan epitopes; overall the Nab activity present in this serum could not be mapped to a known epitope. In contrast, reduction of neutralization in serum 1D by the N160K mutation was consistent with targeting of gp120/gp41 (VCR34.01-like). Targeting of V2 glycan (PG16-like activity) is also suggested by the lack of a >3-fold reduction in activity with the N160K mutation; however, the <3 fold-reduction in the 1D serum associated with the N88A mutation makes V2 glycan targeting unclear.

Vaccination with ICs made with glycan-hole specific antibodies results in lower serum anti-V3 antibody responses.

HIV-1 tier 1 viruses are highly sensitive to anti-V3 Abs (18). To test whether or not V3-specific Ab was made to the V3 crown, we analyzed the immune sera of all groups against a set of HIV-$1_{MN}$ V3 peptides. Sera in groups A and B revealed lower binding signals (>2-fold) compared to sera in groups A and B (Figure 8A). The HIV-$1_{MN}$ V3-specific binding signals correlated with the HIV-$1_{MN\_3}$ neutralization titers ($r$ = 0.45; $p$ = 0.049; Figure 8B) suggesting the presence of neutralizing antibodies against the V3 crown.

## DISCUSSION

In this study, we assessed the immunogenicity in rabbits of ICs composed of BG505 SOSIP.664 trimers and rabbit mAbs directed against N241/N289 glycan hole or gp120-gp41-interface epitopes. The ICs formed using the 11A mAb (glycan hole epitope) induced lower titers of anti-trimer binding antibodies, compared with ICs made with the other mAbs (including 11B to a similar epitope) or with the uncomplexed BG505 SOSIP trimer. Furthermore, immunizing with ICs based on either of the two glycan hole mAbs resulted in 2-3 fold lower rates of binding-antibody decay compared to the uncomplexed control group.

There was no increase in the potency or breadth of the NAb responses induced by ICs compared with the uncomplexed trimer. However, the formation of ICs using mAbs to the glycan hole epitope may have diverted the antibody responses, in some cases, away from that immunodominant autologous NAb epitope and to other regions of the BG505 virus.

Previous immunization studies with HIV-1 Env-based IC vaccines generally resulted in increased Env-specific antibody binding titers and tier-1 NAbs titers compared to uncomplexed vaccines (18, 19, 26, 27). However, the increase of Env-specific antibody responses was mostly attributed to allosteric effects between gp120 and CD4bs-specific antibodies that stabilized the V3 loop for better recognition and also rendered the gp120 protein more resistant to proteolytic degradation (18). Here, we observed the opposite, with lower median BG505.T332N trimer-specific antibody titers as well as lower autologous NAb titers against HIV-$1_{BG505.T332N}$ in all three IC groups compared to the uncomplexed trimer group at week 22. We assume that direct masking or shielding of immunodominant determinants by antibody may account for the lower

335 overall anti-trimer binding antibody responses we observed. This is in accordance with a
336 recent study finding that immunization with ICs consisting of HIV-$1_{JR-FL}$ gp120 and the
337 C2-specific mAb 1006-30D (which binds to epitopes that overlap mAb 11A and 11B
338 epitopes) resulted in overall lower antibody binding titers against gp120, the V1/V2 loop,
339 and the V3 loop, as well as lower NAb titers against the tier-1 isolate HIV-$1_{SF162}$,
340 compared to the uncomplexed gp120 control (18, 33).

341 Immunization with ICs containing mAb 11A resulted in notably less antibody
342 binding and neutralization potency compared to the uncomplexed trimer. This outcome
343 was not seen with ICs based on mAb 11B, despite binding to a similar glycan-hole
344 epitope (30). The difference in neutralizing activity may relate to the fact that both mAbs
345 vary slightly in their epitope binding properties. MAb 11B binds closer to the Env apex
346 than does mAb 11A, which binds closer to the viral membrane. In addition, mAb 11A,
347 but not 11B, binds to the BG505 SOSIP.664 trimer with a S241N mutation, suggesting
348 more binding flexibility. In addition, compared to mAb 11B, MAb 11A has a 1.8-fold
349 faster $k_{on}$ rate ($8.5 \times 10^4$ M/s vs. $4.6 \times 10^4$ M/s) and a 1.8-fold faster $k_{off}$ rate ($3.8 \times 10^{-5}$
350 1/s vs. $2.1 \times 10^{-5}$ 1/s; Table 1). These different epitope binding properties might affect
351 binding of other nAbs through steric hindrance. Indeed, competition assays with mAbs
352 11A and 11B against a panel of human gp120-gp41 interface mAbs revealed
353 interference by mAb 11A, whereas mAb 11B had no effect (30). Finally, all of these
354 differences could affect the antigenicity, stability, and half-life of ICs and thus the
355 production of nAbs after rabbit immunization.

356 One of the primary goals of this study was to divert the immune response away
357 from an immunodominant glycan-hole at positions 241 and 289 by forming ICs with the

glycan-hole specific mAbs 11A and 11B. Although it has been demonstrated previously that blocking undesired epitopes on BG505 trimers lowered tier-1 NAb responses, using a different strategy (37), we found only two animals in group B, compared with one each in groups C and D, that had NAb responses against epitopes other than the glycan hole. Unfortunately, group A sera could not be evaluated thoroughly for glycan-hole-specific NAb responses, as NAb titers against the autologous HIV-1 isolate BG505.T332N were too low. Thus, blocking of glycan-hole reactivity by mAb 11B cannot be considered very effective. Nevertheless, mAb 11B might be considered an inferior blocker of the glycan hole than mAb 11A, based on the fact that mAb 11B cannot bind the BG505 trimer in the presence of a glycan at N241 (30). The fact that the gp120-gp41 interface-specific mAb 12A was ineffective at blocking immunodominant glycan hole-specific epitopes is possibly due to its notably lower affinity for BG505 trimers and the minimal effect that the absence of an N241 glycan has on 12A binding to BG505 trimers (30).

Most of the NAb responses were mapped to epitopes in the glycan hole. However, sera from some animals were of particular interest. In the case of animal 2B, we were not able to map the neutralizing activity to any known epitope. This serum had low-level cross-neutralizing activity, with $ID_{50}$ titers >20 against 10 of 17 heterologous tier 2 HIV-1 strains. Animal 5C revealed potential activity directed against the CD4bs, since the BG505.T332N virus mutant N280D was markedly resistant to neutralization. However, the NAb activity in serum 5C was also likely directed against the C3/465 epitope cluster, given the 4-fold reduction in activity with the N241 KI + N289 KI mutant and an even stronger reduction with the N465-KI mutant. We believe that, overall, the polyclonal neutralizing activity of serum 5C was mainly associated with the C3/465

epitope cluster with the possible indirect effects of the N282D mutation. We observed that deleting the N-glycosylation site from position N133 rendered the BG505.T332N virus 5 fold more sensitive to NAbs in the sera of animals 2B and 4B. It has been shown recently that the removal of specific N-glycosylation sites can have significant effects on viral infectivity and antibody-mediated neutralization (51). For example, a mutation in V1 at position N133 (N133Q) increased the sensitivity of the virus (HIV-$1_{CRF07\_BC\ Env,\ FE}$) to a V3-specific antibody (3869) by 5 fold (51).

Finally, we observed a 2-3 fold slower rate of antibody decay in rabbits immunized with ICs. Additional sampling at longer time intervals will be necessary to confirm this finding in future studies. To our knowledge, a decrease in antibody decay has not been previously ascribed to the use of IC immunogens. Although we have gone no further to investigate the mechanisms of this delay in antibody decay, it is plausibly associated with the recognition of ICs by Fcγ receptors expressed on the surface of antigen presenting cells; the ICs may thus be processed differently or at a different rate (depot-effect) than the uncomplexed antigen (12, 17, 29, 36, 41). For example, Fcγ receptor-mediated antigen processing could impact B cell activation and differentiation (13, 46) or germinal center memory B cells (24, 32, 57) and secondary antibody responses (16). In addition, the formation of immune complexes in the presence of complement factors could lead to a more efficient deposition of antigen on follicular dendritic cells (25).

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635 **age-associated deficiency in germinal center response by immunization with**
636 **immune complexes. Clinical immunology 124:131-137.**
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640 **FIGURE LEGENDS**

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642

643 **Figure 1. Binding of mAbs 11A, 11B, and 12A to BG505 SOSIP.664 trimers**. **(A)**
644 MAb binding to trimers immobilized with D7324 antibody was measured by ELISA. **(B)**
645 BG505 SOSIP.664 trimer binds to the quaternary, configuration-dependent human mAb
646 PGT145 but not to the human mAb F105, which is directed against a non-NAb epitope
647 associated with the CD4bs. MAbs were tested in duplicate. Curve fitting was performed
648 with GraphPad Prism 8.0 software.

649

650 **Figure 2. Trimer-mAb immune complex formation does not impact mAb PGT145**
651 **or mAb F105 binding.** Antigenicity of immune complexes captured by polyclonal anti-
652 rabbit antibody was determined by binding with the conformation-dependent mAb
653 PGT145 or the conformation-independent mAb F105. MAbs were tested in duplicate.
654 Curve fitting was performed with GraphPad Prism 8.0 software.

655

656 **Figure 3. IC immunization may decrease anti-trimer binding antibody responses**
657 **and the rate of antibody decay.** (**A**) Antibody binding to the BG505 SOSIP.664 trimer
658 of individual sera from animals in each group was measured by ELISA at week 22. (**B**)
659 Binding responses at weeks 22 and 26 are plotted. Thick lines represent the median
660 rate of decay. Median decay rates are as follows: -0.05 (Group A), -0.04 (Group B), -0.1
661 (Group C), and -0.15 (Group D). *P*-values were calculated using Kruskal-Wallis with
662 Dunn's multiple comparison tests.

663 **Figure 4. IC immunization does not alter the breadth of NAb responses.** Data
664 represent serum $ID_{50}$ titers measured from week-22 sera using Env-pseudotyped
665 viruses and TZM-bl target cells. Boxes are color coded according to the magnitude of
666 neutralization: $ID_{50}$ <40 (white); $ID_{50}$ 40-100 (yellow); $ID_{50}$ 100-1000 (orange); $ID_{50}$
667 >1000 (red). SVA-MLV was used as a negative control virus, and mAb CH01 served as
668 a positive control antibody. All serum samples were assayed in duplicate.

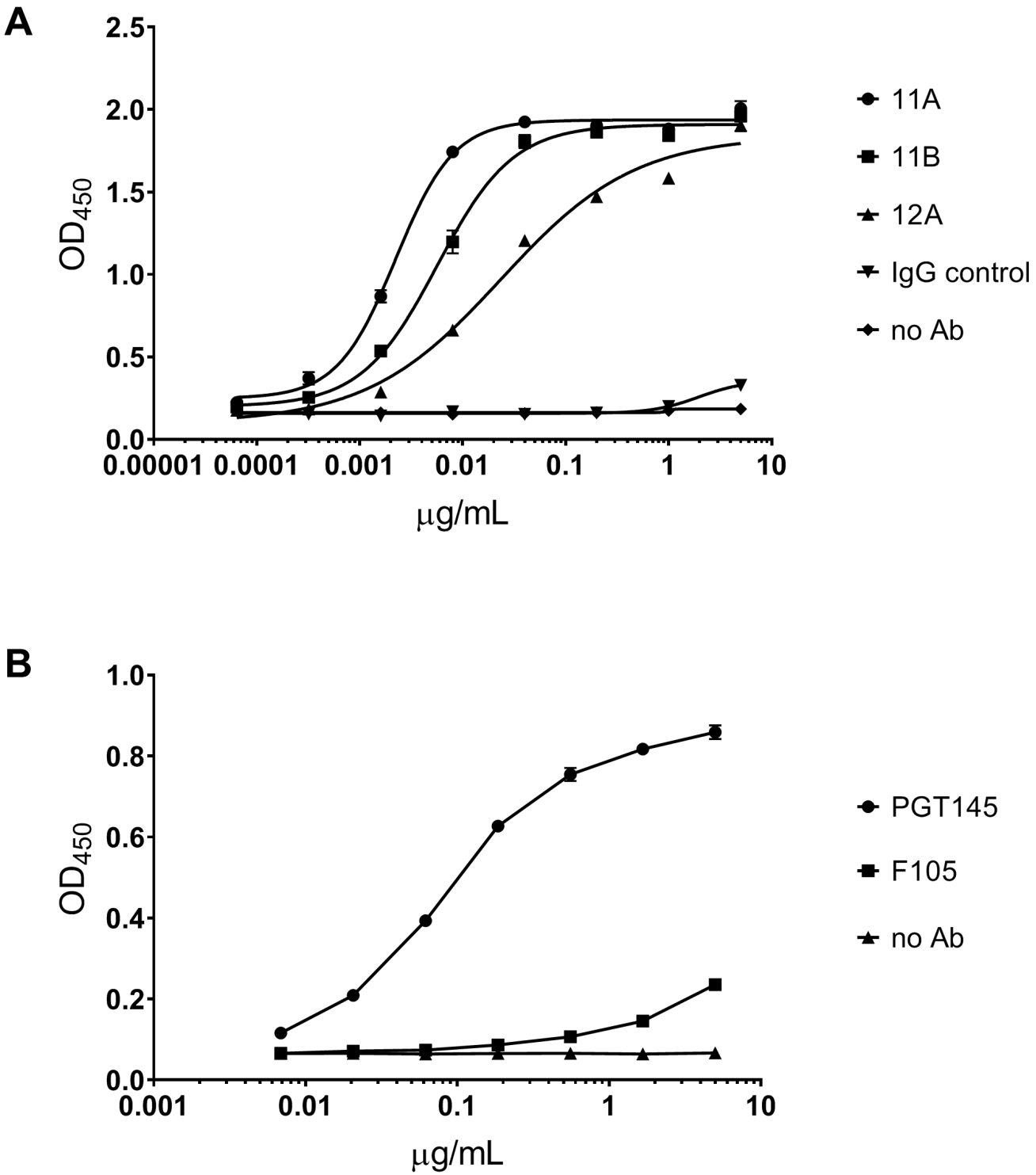
669 **Figure 5. IC immunization affects some NAb responses**. (**A**) Autologous
670 neutralization potency against BG505_T332N and BG505 is reduced in sera from group
671 A animals. (**B**) Neutralizing antibody responses correlate with ELISA binding titers.
672 Binding ($EC_{50}$) and neutralization results ($ID_{50}$) were analyzed by Spearman correlation.
673 (**C**) For certain tier-1 HIV-1 strains, neutralizing activity is decreased by IC
674 immunization. Results are shown for the three HIV-1 strains (indicated above each
675 graph) that were neutralized at an $ID_{50}$ titer > 20 by four out of five animals. (**D**)
676 Neutralizing activity against tier-2 strains is limited and does not differ between
677 vaccination groups except for group B versus group C (see also **Figure 4**). For data
678 analysis, $ID_{50}$ titers <20 and >43740 were considered to equal 20 and 43740,
679 respectively. *P*-values were determined by the Kruskal-Wallis test followed by Dunn's
680 multiple comparisons test.

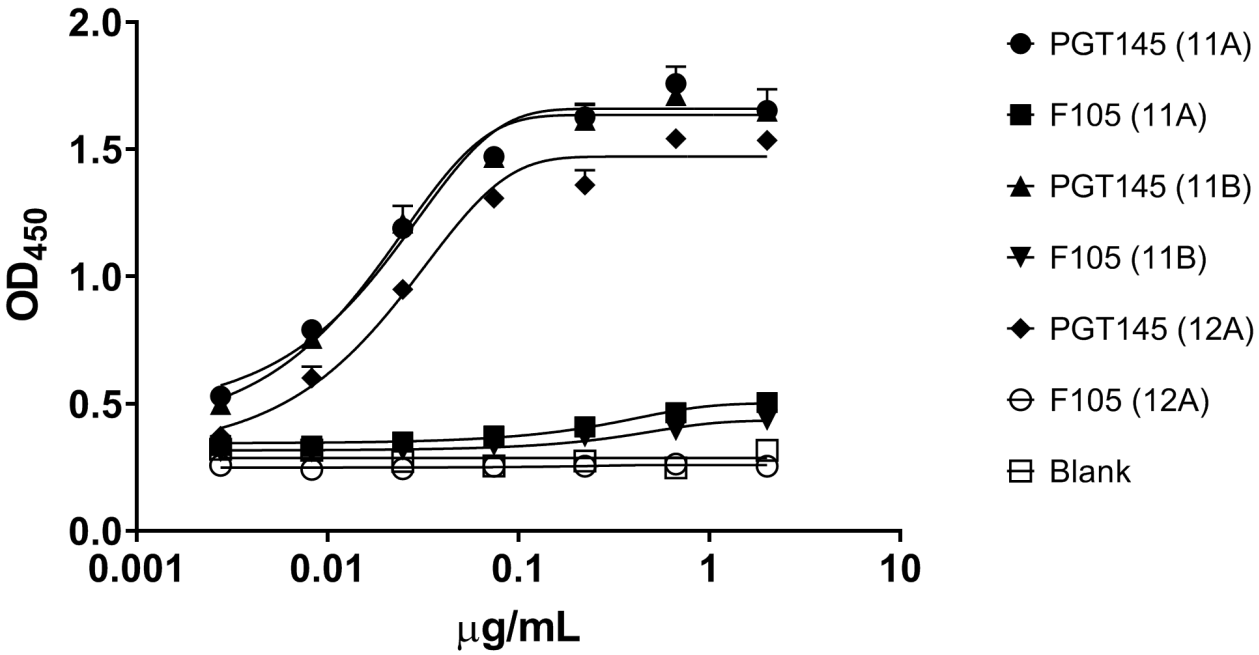
681 **Figure 6. Antibody responses to IC immunogens and uncomplexed trimers are**
682 **predominantly directed against epitopes in the glycan hole.** (**A**) BG505.T332N virus
683 variants were used to map neutralizing determinants in all sera except those in group A,
684 for which the NAb titers were too low. Determinants involved in neutralizing responses
685 are color coded and are identified by an at least 3-fold reduction in NAb titer against

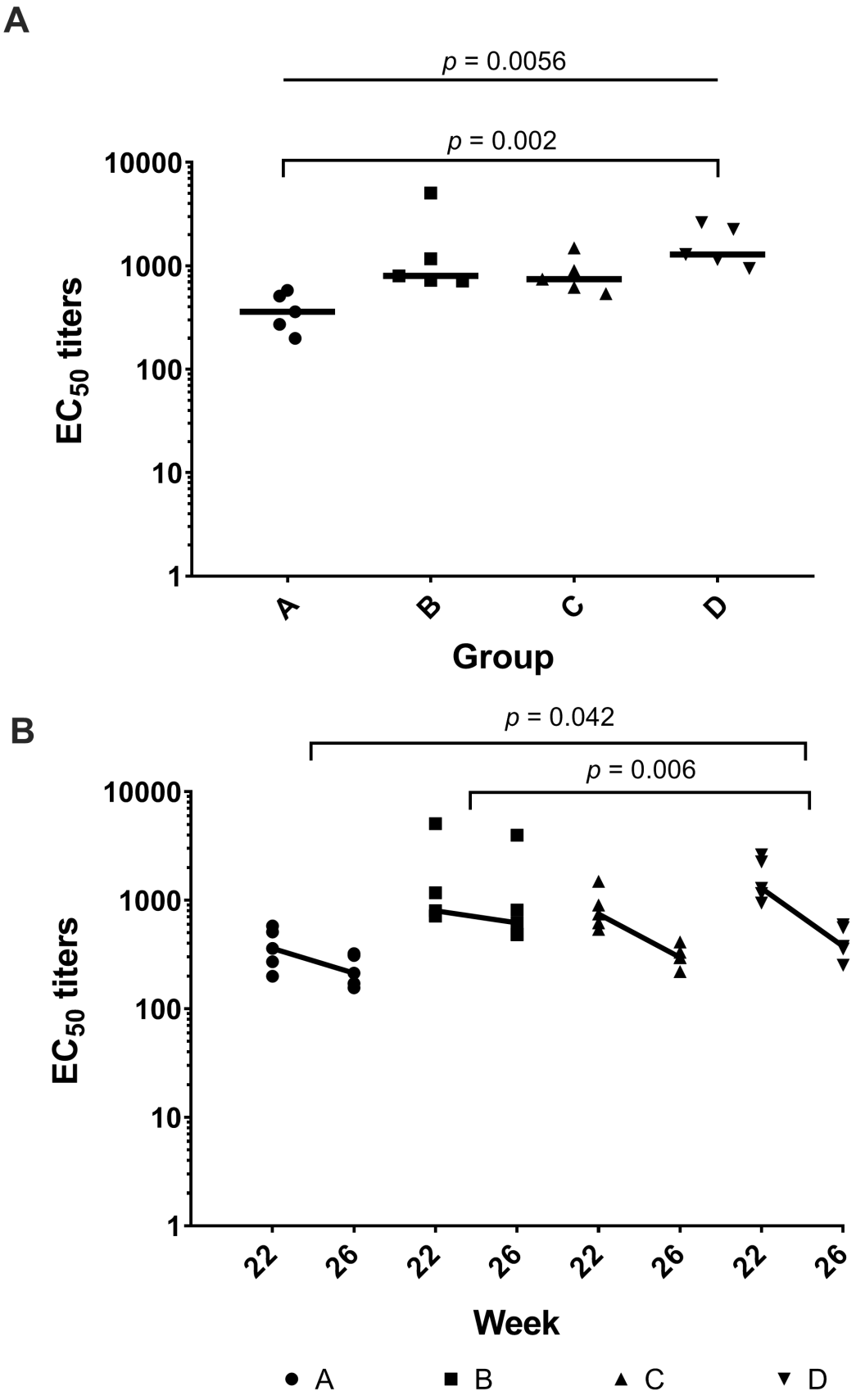
variants containing mutations in relevant epitopes. For values that were below the detection limit ($ID_{50}$ <30), one-half of the cut-off ($ID_{50}$ =15) was used for calculations. Mapping was conducted at Duke. (**B**) Further mapping was performed at WCMC using BG505 virus mutants with sequence changes affecting V1 (133aN+136aA), CD4bs (N356 KI), and a newly identified glycan epitope (N465 KI). $RID_{50}$ (relative $ID_{50}$) refers to the $ID_{50}$ against mutant)/$ID_{50}$ against the parental BG505 strain. REN (relative extent of neutralization) is the ratio of the extent of neutralization of the mutant compared with the parental strain using IgG corresponding to a 1/50 dilution of serum.

**Figure 7. Mapping of neutralizing determinants in sera 2B and 1D.** (**A**) Antibody mapping of serum from animal 2B at week 0 and week 22 was performed with variants derived from HIV-$1_{CH119.10}$. (**B**) Serum from animal 1D was tested against a panel of variants derived from HIV-$1_{25710\text{-}2.43}$. The human mAbs VRC34.01, PG16, and PGT128 were used as controls. $ID_{50}$ values are reported for serum samples and $IC_{50}$ values for the control mAbs. Serum samples were tested in duplicate.

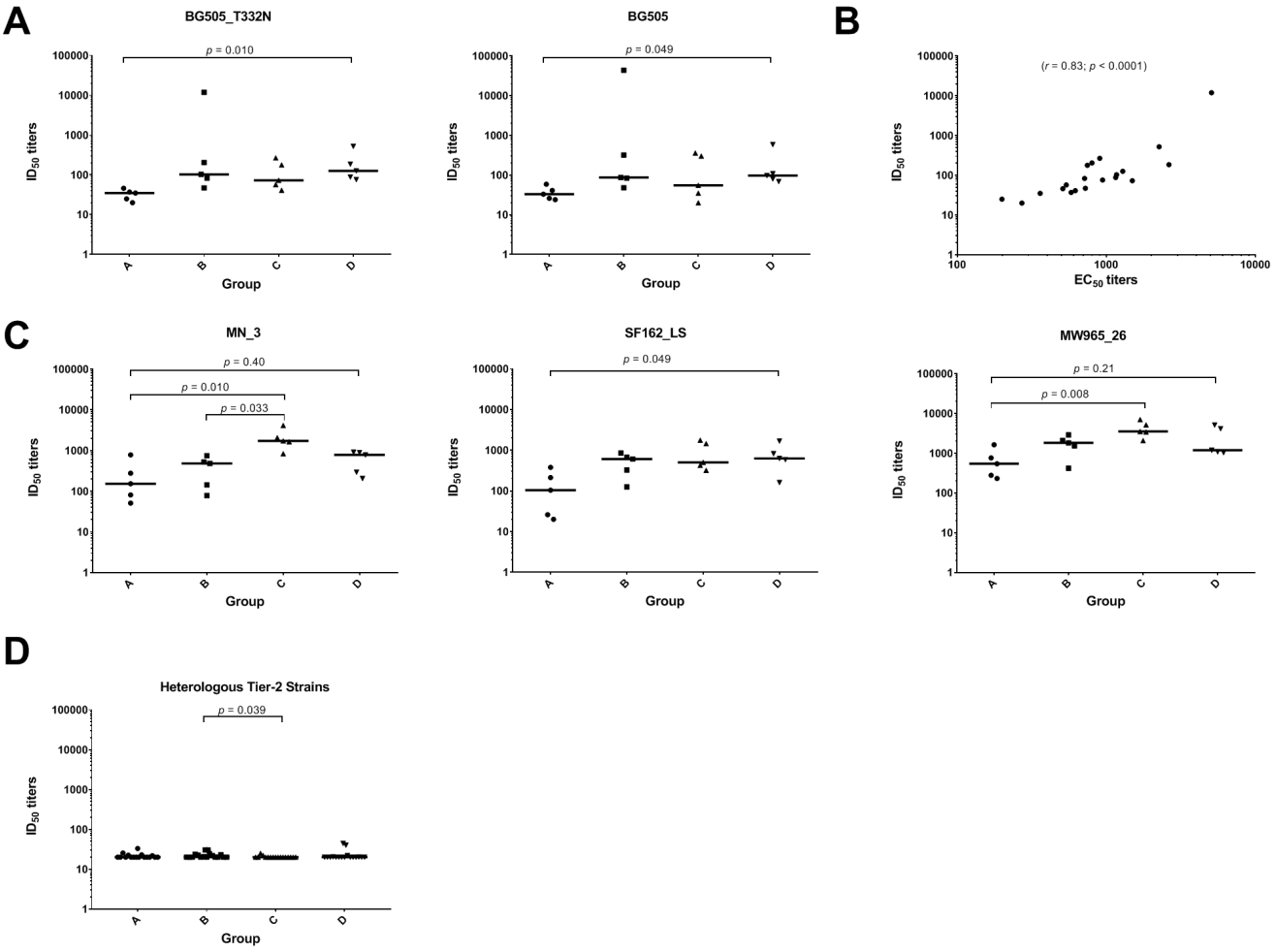
**Figure 8. IC immunization resulted in variable binding to HIV-1 MN V3 peptides, which correlated with neutralizing activity against HIV-$1_{MN\_3}$.** (A) Sera of all groups were tested in by ELISA for binding against three overlapping V3 peptides. All samples were tested in duplicate. (B) HIV-$1_{MN\_3}$ neutralization correlates with HIV-1 MN V3 peptide binding as analyzed by Spearman correlation.







| | | | Group A Animal ID | | | | | Group B Animal ID | | | | | Group C Animal ID | | | | | Group D Animal ID | | | | | Control |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Virus | Clade | Tier | 1A | 2A | 3A | 4A | 5A | 1B | 2B | 3B | 4B | 5B | 1C | 2C | 3C | 4C | 5C | 1D | 2D | 3D | 4D | 5D | CH01 |
| MN_3 | B | 1A | 276 | 782 | 51 | 153 | 81 | 747 | 78 | 481 | 143 | 524 | 1640 | 2066 | 4135 | 1721 | 835 | 292 | 205 | 907 | 887 | 780 | 0.41 |
| SF162_LS | B | 1A | 212 | 378 | <20 | 104 | 26 | 605 | 125 | 854 | 671 | 326 | 1767 | 319 | 1448 | 425 | 502 | 160 | 586 | 822 | 625 | 1672 | 0.19 |
| MW965_26 | C | 1A | 763 | 278 | 232 | 543 | 1634 | 2087 | 419 | 1818 | 2886 | 1536 | 7021 | 3414 | 3517 | 2080 | 5151 | 1079 | 1184 | 1029 | 5103 | 4125 | 1.22 |
| Q23_17 | A | 1B | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 39 | 34 | <20 | <20 | <20 | <0.01 |
| BaL.26 | B | 1B | <20 | <20 | <20 | <20 | <20 | 36 | <20 | 32 | 34 | <20 | 79 | <20 | 73 | 27 | 29 | <20 | 29 | 36 | 37 | 50 | 0.06 |
| BG505 | A | 2 | 33 | 26 | 24 | 41 | 59 | 87 | 319 | 84 | >43740 | 48 | 35 | <20 | 55 | 300 | 359 | 585 | 69 | 97 | 108 | 80 | 0.02 |
| BG505_T332N | A | 2 | 35 | <20 | 25 | 37 | 46 | 103 | 204 | 83 | 11933 | 47 | 41 | 57 | 73 | 267 | 178 | 522 | 88 | 126 | 185 | 76 | <0.01 |
| Q259_d217 | A | 2 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 12.9 |
| Q769_d22 | A | 2 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 0.07 |
| Q842_d12 | A | 2 | <20 | <20 | <20 | <20 | <20 | <20 | 21 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 22 | <20 | <20 | <20 | <20 | <0.01 |
| a398_F1_F6_20 | A | 2 | 33 | 31 | 28 | 45 | 31 | 35 | 61 | 29 | <20 | 21 | 38 | <20 | 27 | <20 | 24 | 145 | 68 | <20 | <20 | 26 | 0.09 |
| a246_F3_C10_2 | AC | 2 | <20 | <20 | 25 | 25 | <20 | 25 | 39 | 22 | <20 | <20 | <20 | <20 | <20 | 20 | <20 | <20 | <20 | <20 | <20 | <20 | 0.08 |
| CNE8 | AE | 2 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 0.2 |
| CNE55 | AE | 2 | <20 | <20 | <20 | <20 | <20 | <20 | 37 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 0.07 |
| YU2 | B | 2 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 0.08 |
| SC422661_8 | B | 2 | <20 | <20 | <20 | <20 | <20 | <20 | 21 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 0.37 |
| JRFL | B | 2 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <0.01 |
| X2278_C2_B6 | B | 2 | <20 | <20 | <20 | <20 | <20 | <20 | 41 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 21 | 0.08 |
| BJOX002000_03_2 | BC | 2 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 1.82 |
| CH119_10 | BC | 2 | 25 | 26 | 27 | 26 | 24 | 26 | 60 | 26 | 22 | 28 | 24 | <20 | 22 | 20 | 25 | 22 | <20 | 26 | 22 | <20 | 1.34 |
| Ce1176_A3 | C | 2 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 0.06 |
| Ce703010217_B6 | C | 2 | 23 | 23 | 21 | 25 | 23 | <20 | 31 | 20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 0.05 |
| a25710_2_43 | C | 2 | 22 | <20 | 27 | 24 | <20 | 26 | 36 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 20 | 223 | 39 | <20 | 20 | 50 | 0.42 |
| X1632_S2_B10 | G | 2 | <20 | <20 | <20 | <20 | <20 | <20 | 23 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 0.07 |
| SVA-MLV | | | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 21 | <20 | <20 | >25 |



**A**

| | $ID_{50}$ | | | | | | | | | | | | | | | $IC_{50}$ (µg/ml) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Group B | | | | | Group C | | | | | Group D | | | | | | |
| | 1B | 2B | 3B | 4B | 5B | 1C | 2C | 3C | 4C | 5C | 1D | 2D | 3D | 4D | 5D | VRC01 | CH01-31 |
| BG505.T332N | 103 | 204 | 83 | 11933 | 47 | 41 | 57 | 73 | 267 | 178 | 522 | 88 | 126 | 185 | 76 | nd | nd |
| N133A.3 | 87 | 1060 | 51 | 56267 | 61 | 50 | 54 | 78 | 275 | 76 | 677 | 84 | 125 | 70 | 129 | 0.04 | 0.01 |
| N142A.6 | 86 | 85 | 78 | 568 | 66 | 47 | 46 | 58 | 130 | 86 | 601 | 81 | 92 | 111 | 113 | 0.07 | 0.03 |
| N280D | 64 | 253 | 38 | 12262 | 50 | 60 | 77 | 60 | 237 | <30 | 419 | 64 | 86 | 34 | 72 | >16.67 | 0.24 |
| S241N | <30 | 183 | 34 | 8427 | <30 | 38 | <30 | 37 | 177 | 74 | 367 | 41 | 66 | 50 | 40 | 0.06 | 0.03 |
| P291T | <30 | 209 | <30 | 17232 | 31 | 34 | <30 | <30 | 90 | 59 | 342 | 30 | 35 | <30 | <30 | 0.09 | 0.04 |
| S241N/P291T | <30 | 170 | <30 | 9383 | <30 | <30 | <30 | <30 | 93 | 42 | 285 | <30 | <30 | <30 | <30 | 0.07 | 0.03 |

| glycan hole | V1 glycan | CD4bs | not mapped |
|---|---|---|---|

**B**

| | BG505 Parental | 133aN + 136aA | N241 KI + N289 KI | N356 KI | N465 KI |
|---|---|---|---|---|---|
| Sample | $ID_{50}$ | $RID_{50}$ | $RID_{50}$ | $RID_{50}$ | $RID_{50}$ |
| 4B | 3074 | <0.01 | 0.85 | 1.23 | 0.58 |
| | EN | REN | REN | REN | REN |
| 4B | 100 | 0.38 | 1.00 | 1.00 | 1.00 |
| 4C | 65 | 0.88 | 0.84 | 0.91 | 0.59 |
| 5C | 67 | 0.88 | 0.79 | -0.09 | -0.29 |
| 1D | 81 | 1.07 | 1.03 | 0.81 | 0.27 |

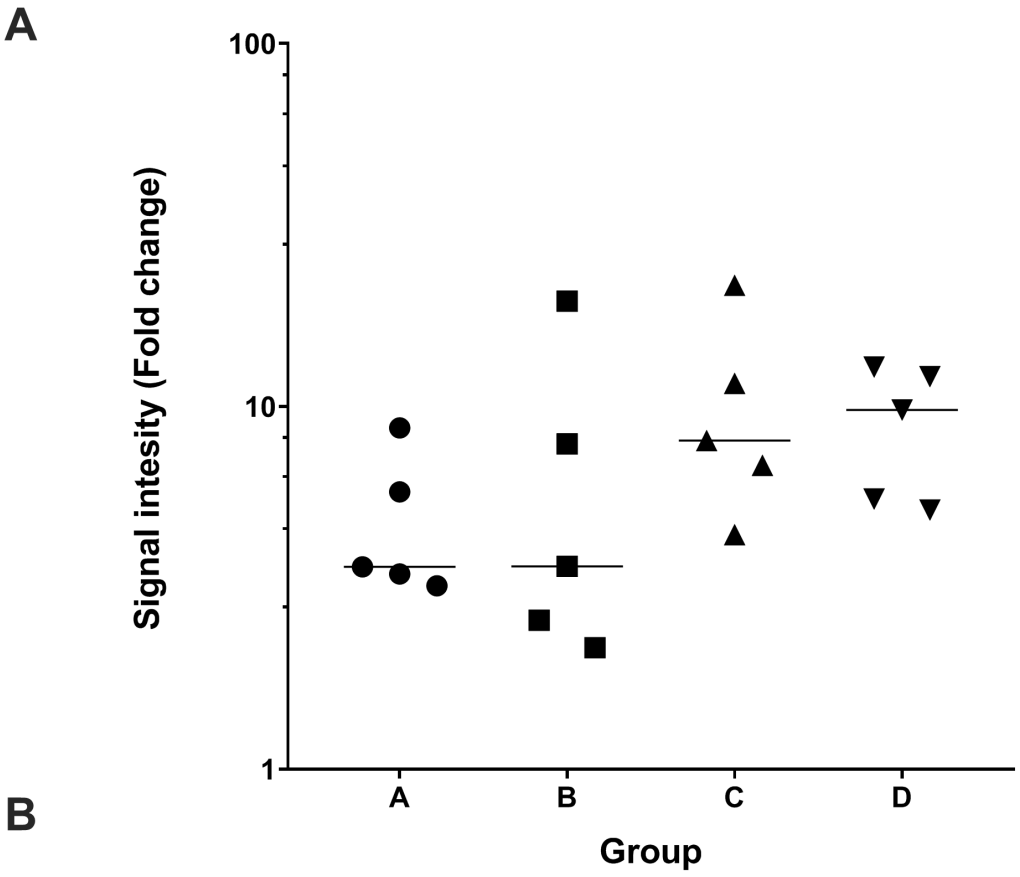
| $RID_{50}$ | Mutant/Parental | $RID_{50}$ <0.25 | $RID_{50}$ ≥0.25, ≤0.5 | $RID_{50}$ >0.5, ≤2.0 |
|---|---|---|---|---|
| REN | Mutant/Parental | REN <0.25 | REN ≥0.25, ≤0.75 | REN >0.75, ≤0.75 |

**A**

| | ID50 (ug/ml) in TZM-bl cells[1] | | | | | | |
|---|---|---|---|---|---|---|---|
| | CH119.10 | CH119.10.N88A | CH119.10.N160A | CH119.10.V295N | CH119.10.N332A | CH119.10.N611A | CH119.10.N625A |
| | CRF07_BC | CRF07_BC | CRF07_BC | CRF07_BC | CRF07_BC | CRF07_BC | CRF07_BC |
| | Tier 1A | Tier 2 | Tier 2 | Tier 2 | Tier 2 | Tier 2 | Tier 2 |
| 2B (week 0) | <30 | <30 | <30 | 31 | <30 | <30 | <30 |
| 2B (week 22) | 84 | 64 | 66 | 81 | 81 | 75 | 63 |
| VRC34.01 (gp120-gp41) | 0.07 | >25 | 0.06 | 0.06 | 0.07 | <0.01 | 0.07 |
| PG16 (V2 glycan) | 0.53 | 1.01 | >10 | 0.87 | 0.21 | 0.66 | 1.02 |
| PGT128 (V3 glycan) | 0.03 | 0.02 | 0.02 | 0.03 | 7.32 | 0.02 | 0.04 |

**B**

| | ID50 (ug/ml) in TZM-bl cells[1] | | | | |
|---|---|---|---|---|---|
| | 25710-2.43 | 25710-2.43.N88A | 25710-2.43.N160K | 25710-2.43.N332A | 25710-2.43.N625A |
| | Clade C | Clade C | Clade C | Clade C | Clade C |
| | Tier 1A | Tier 2 | Tier 2 | Tier 2 | Tier 2 |
| 1D (week 0) | <30 | <30 | <30 | <30 | <30 |
| 1D (week 22) | 114 | 65 | 43 | 115 | 94 |
| VRC34.01 (gp120-gp41) | 2.77 | >16.66 | >16.66 | 0.35 | 1.39 |
| PG16 (V2 glycan) | 0.02 | 0.02 | >6.66 | 0.02 | 0.02 |
| PGT128 (V3 glycan) | 0.03 | 0.02 | 0.03 | >6.66 | 0.02 |



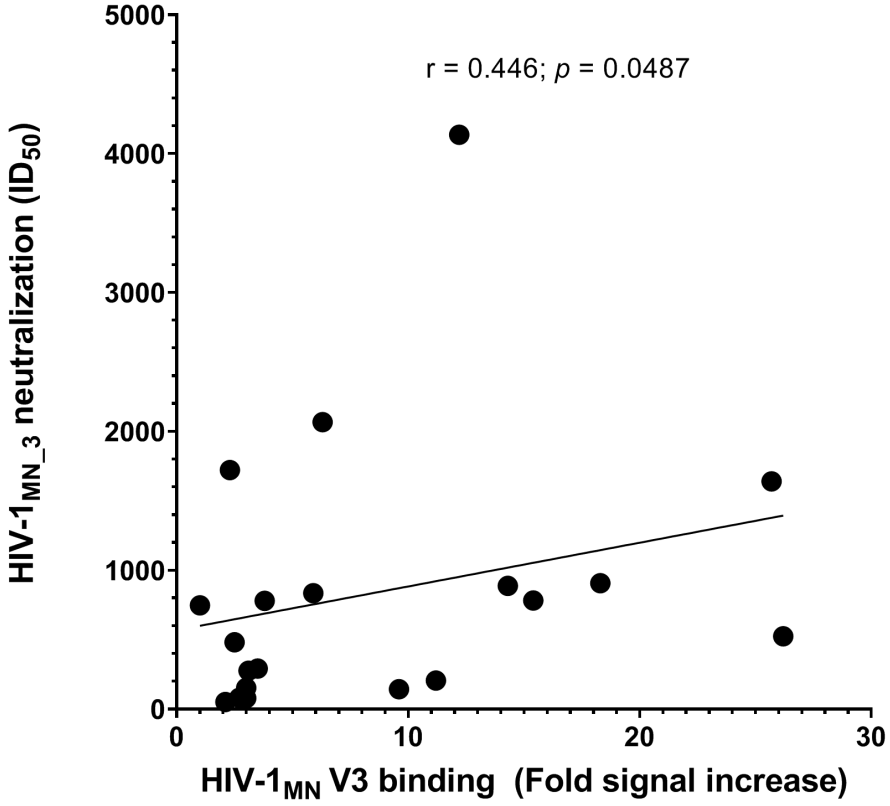



Table 1. Vaccination groups and monoclonal antibodies used to make SOSIP-immune complexes*

| **Group (n = 5 animals /group)** | **Antibody** | **Specificity** | **Kd (M)** | **Kon (1/Ms)** | **Koff (1/s)** |
|---|---|---|---|---|---|
| A | 11A | Glycan hole; C2; centered at S241 | $4.6 \times 10^{-10}$ | $8.5 \times 10^{4}$ | $3.8 \times 10^{-5}$ |
| B | 11B | Glycan hole; C2; centered at S241 | $4.5 \times 10^{-10}$ | $4.6 \times 10^{4}$ | $2.1 \times 10^{-5}$ |
| C | 12A | Overlap with mAb PGT151 determinants | $7.9 \times 10^{-8}$ | $5.6 \times 10^{3}$ | $4.5 \times 10^{-4}$ |
| D | None | -- | -- | -- | -- |

*Animals received SOSIP.664 gp140 trimers as an immune complex (groups A, B and C) or SOSIP trimer alone (group D). Details on antibody specificities are available in references [10] and [34].