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Articles

The Impact of Sustained Immunization Regimens on the Antibody Response to Oligomannose Glycans

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ABSTRACT: The high mannose patch (HMP) of the HIV envelope protein (Env) is the structure most frequently targeted by broadly neutralizing antibodies; therefore, many researchers have attempted to use mimics of this region as a vaccine immunogen. In our previous efforts, vaccinating rabbits with evolved HMP mimic glycopeptides containing Man_9 resulted in an overall antibody response targeting the glycan core and linker rather than the full glycan or Manα1→2Man tips of Man_9 glycans. A possible reason could be processing of our immunogen by host serum mannosidases. We sought to test whether more prolonged dosing could increase the antibody response to intact glycans, possibly by increasing the availability of intact Man_9 to germinal centers. Here, we describe a study investigating the impact of immunization regimen on antibody response by testing immunogen delivery through bolus, an exponential series of mini doses, or a continuously infusing mini-osmotic pump. Our results indicate that, with our glycopeptide immunogens, standard bolus immunization elicited the strongest HIV Env-binding antibody response, even though higher overall titers to the glycopeptide were elicited by the exponential and pump regimens. Antibody selectivity for intact glycan was, if anything, slightly better in the bolus-immunized animals.

After decades of research, very little HIV vaccine efficacy has been observed in clinical trials.[1−3] The challenges of HIV vaccine design include (1) the high mutation rate of HIV, which leads to vast phylogenetic and antigenic diversity, (2) the metastable nature of the HIV envelope protein (Env), a trimer of gp41/gp120 heterodimers that can adopt several functional conformational states, (3) the fact that gp120 can be shed from Env on the viral surface, (4) the low immunogenicity of the most conserved structural features on Env, leading to preferential formation of antibodies against strain-specific epitopes, and (5) the shielding of many conserved epitopes by the dense array of ∼70−80 N-glycans on the Env trimer. Most early generation HIV vaccine approaches utilizing recombinant protein or vectors encoding Env subunits (e.g., monomeric gp120) have elicited antibodies that bind misfolded Env or strain-specific Env structures and do not neutralize a broad range of viral strains.[1] However, stable native-like Env trimers (SOSIP trimers) have been engineered that are correctly folded and elicit neutralizing antibodies to the strain used in the immunogen.[4−8]

Despite these challenges, continued optimism for design of a protective vaccine stems from the fact that broadly neutralizing antibodies (bnAbs) naturally arise in a surprisingly high fraction of infected individuals (∼20%).[9] Although bnAbs typically arise too late after infection (usually >2 years) and viral diversification to be protective in the infected individual, several monoclonal bnAbs have been shown to confer protection if administered prior to encounter with virus.[10−15] Hundreds of bnAb clones have now been isolated from patient cohorts, and their neutralizing breadth and binding epitopes have been characterized.[16−18] These antibodies have stimulated vaccine design efforts because they are proof that an immunological solution exists to broad cross-reactivity with HIV Env; more specifically, structural studies of bnAbs in complex with Env have revealed

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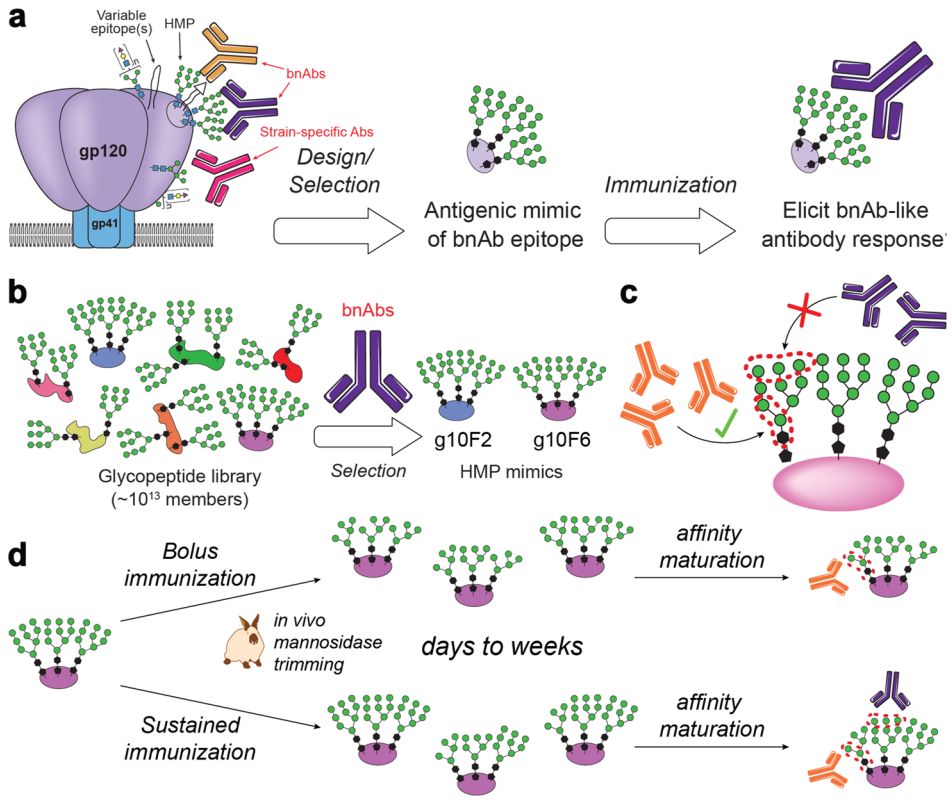

Figure 1. Glycopeptide mimics of HIV epitopes and the glycan trimming hypothesis. (a) The high-mannose patch (HMP) on HIV's gp120 protein is a target of broadly neutralizing antibodies (bnAbs), and glycopeptides that mimic the HMP are attractive vaccine candidates. (b) Our method for *in vitro* selection of glycopeptides that mimic the HMP. (c) Antibodies elicited by glycopeptide HMP mimics have so far preferentially targeted the glycan core rather than the Manα1→2Man "tips" targeted by many bnAbs. (d) Immunization kinetics may influence glycan microspecificity: in bolus immunization, serum mannosidase trimming likely truncates most glycans before the bulk of affinity maturation; we hypothesize that, by contrast, continuous or repetitive immunization will provide fresh intact Man_9 glycan, against which Manα1→2Man-specific antibodies could develop in germinal centers that were originally established by intact Man_9 earlier in the immunization.

which conserved motifs on Env are targeted in bnAb responses.[19,20]

The Env region most commonly targeted[21] by bnAbs (~40%) is the high-mannose patch (HMP, Figure 1a), a region of gp120 containing a dense array of N-linked glycosylation sites (N332, N339, N392, N295, N262, N448, N363) largely populated by $Man_{9/8}GlcNAc_2$ glycans.[22] bnAbs targeting this region bind to combinations of these glycans and conserved polypeptide residues (e.g., PGT121- and PGT128 bnAb families)[23−25] or exclusively to glycans (e.g., bnAb 2G12).[26,27] In all cases, these bnAb epitopes contain multiple glycans; thus, carbohydrate chemists have been very interested in the design and synthesis of carbohydrate clusters to mimic these epitopes.[28−46] In these epitope-focused vaccine strategies, it is reasoned that a glycopeptide or carbohydrate mimic of the HMP might elicit antibodies that bind the HMP and are broadly neutralizing (Figure 1a).

In our attempts to mimic the HMP, we have developed directed evolution methods that enable us to select multivalent Man_9 clusters that bind bnAb 2G12 from extraordinarily diverse libraries of up to 10^{13} glycopeptides[40,47] or glycoDNAs.[41−43,48] Antibody 2G12 binds our evolved glycopeptides with nanomolar to subnanomolar affinity, at least as tightly as it binds to gp120, and in a glycan-dependent manner (Figure 1b). As conjugates to CRM197 carrier protein, our glycopeptides exhibited strong immunogenicity, eliciting robust binding titers (ELISA $EC_{50} \approx 20\,000$) against autologous glycopeptide.[38] Encouragingly, two of these glycopeptide immunogens (g10F6 and g10F2) elicited detectable HIV binding or neutralizing antibodies; however, the anti-HIV titers were very weak, suggesting modest binding to gp120 or its high-mannose glycans. Glycan microspecificity studies indicated that the glycan-dependent antibodies were primarily directed at two core mannose residues of the glycan, and its hydrophobic linker, rather than the Manα1→2Man tips (Figure 1c). Mass spectrometric studies then showed that incubation of immunogen in serum readily trims mannose residues from the glycans, suggesting that mannosidase activity[49−52] *in vivo* may interfere with development of antibodies against these structures.

Serum mannosidase trimming of glycopeptides could also influence the specificity of the antibody response, depending on the relative kinetics of vaccination and immunogen degradation. In natural HIV infection, high-mannose-binding bnAbs arise after months of affinity maturation in the presence of continuously produced viral glycoprotein.[53] By contrast, immunizations are traditionally administered in several bolus doses spaced weeks apart. As we have shown that glycans of relatively dense glycopeptides can be trimmed by serum mannosidase on a time scale of days, germinal center formation and affinity maturation likely occurs primarily in the presence of truncated glycans (Figure 1d). Thus, one might ask, could the glycan microspecificity of vaccine-elicited antibodies be modulated by altering the kinetics of the vaccine dose, with prolonged administration of intact immunogen? In fact, for the antibody response to HIV Env protein, it has been shown that sustained delivery of antigen not only increases the overall

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antibody titer but also improves selectivity for intact versus misfolded or degraded HIV Env.[54]

RESULTS

To test the effect of antigen delivery kinetics on the anti-glycan response, we conducted rabbit immunizations with our g10F6 glycopeptide conjugated to CRM197 carrier protein,[38−40] administered in three different regimens (Figure 2). In the control group, a standard bolus dose was tested at 4-week intervals. In the second group, we tested an exponentially increasing series of minidoses, designed to mimic the kinetics of antigen produced by a replicating pathogen, as recently described.[54] In the third group, we tested a continuous two-week infusion of half the glycopeptide conjugate via a subcutaneous mini-osmotic pump, followed by the second half of the dose as a bolus at the end of the 2 weeks.[54−57] Each type of immunization regimen was repeated at t = 4 and 8 weeks, and all groups of animals received the same total dose of conjugate containing 50 μg of glycopeptide, together with 50 μg of QS-21 saponin/cholesterol liposomes (SI Figure S3). For this study, g10F6 glycopeptide was conjugated to CRM197 through cysteine−bromoacetamide substitution rather than the cysteine−maleimide addition used in our previous immunogenicity studies,[38] as we found that our original cysteine−maleimide conjugates shed glycopeptide upon storage at 4 °C in water after 1 week (Figure 3a and SI Figure S4). The reversibility of the cysteine−maleimide linkage is well-known,[58−61] but we found it to be especially evident in the case of our bulky (~12.5 kDa) glycopeptides conjugated to CRM197. Although this shedding could be prevented by storage of the conjugates in lyophilized form, the use of a nonreversible linkage would rule out shedding after redissolution. The use of potentially labile maleimide conjugates was out of the question for this study because the conjugates would be held at body temperature in the osmotic pump reservoir for 2 weeks in one of our rabbit groups. The cysteine−bromoacetamide CRM197 conjugations proceeded with good efficiency (median loading of 5), and the resulting thioether linkages lack the capacity for a reverse reaction to shed glycopeptide thiol (Figure 3b and SI Figures S1 and S2).[62,63]

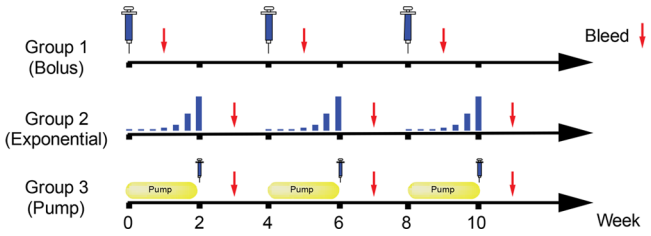

Figure 2. Standard vs sustained immunization regimens. Groups of six rabbits were immunized with the g10F6−CRM197 conjugate by three different regimens. In addition to the group that received standard bolus immunizations, one group ("exponential") received seven exponentially increasing small doses over 2 weeks, and another group ("pump") received half of the dose continuously over 2 weeks by mini-osmotic pump, followed by the second half dose at the end of the 2 weeks. All groups received the same total 50 μg of antigen, and the cycle of immunization was repeated at four-week intervals for all groups. Blood was collected 1 week after the conclusion of each immunization cycle.

Rabbit polyclonal sera were collected 1 week after each immunization and assayed by ELISA to assess IgG binding to test antigens. Consistent with prior studies of sustained immunization that used SOSIP trimeric HIV gp140 Env proteins,[54−57] we observed the strongest antibody binding titers against glycopeptide using the most sustained delivery method, with ELISA EC_{50} titers for glycopeptide−BSA increasing in the order of bolus < exponential < pump (Figure 4a). Differences were greatest after the first dose, with the bolus group partially catching up to the other two groups by the postdose 3 titer measurement (Figure 4b). For all groups, the IgG titers to glycopeptide−BSA were higher than to either peptide−BSA or CRM carrier (Figure 5), consistent with our previous studies.[38] Although significant titers to CRM carrier were observed, measurement of glycopeptide-binding antibodies with glycopeptide−BSA ensured that carrier-independent antibodies were detected. Moreover, our previous studies had shown that antibodies elicited by CRM alone did not cross-react to either glycopeptides or HIV Env.[38]

We then investigated the ability of the antisera to bind to native-like trimeric HIV Env (BG505T332N SOSIP.664 gp140

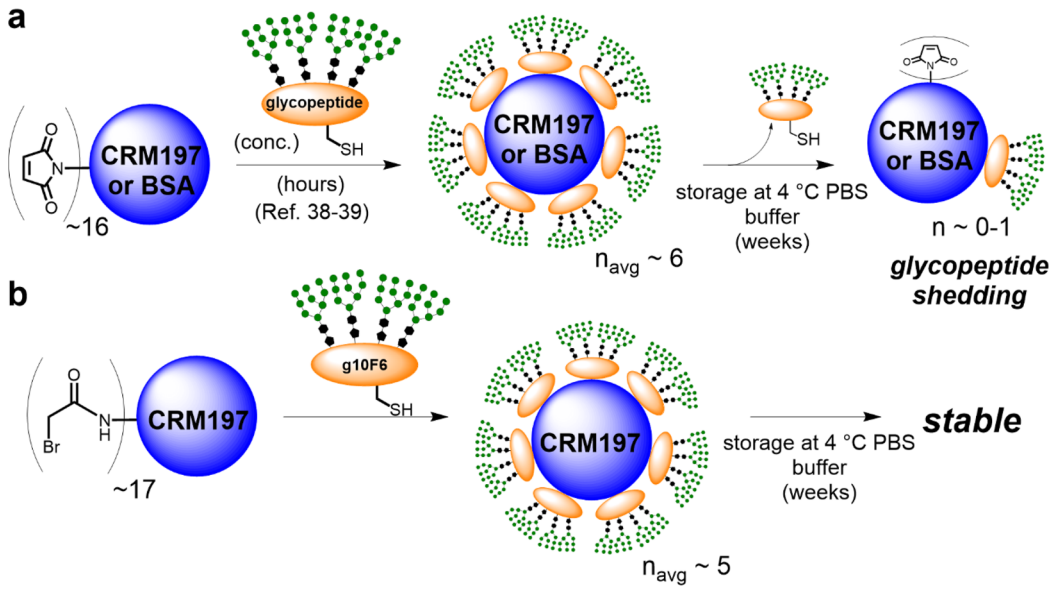

Figure 3. Shedding of glycopeptides from maleimide conjugates versus stability of thioether conjugates.

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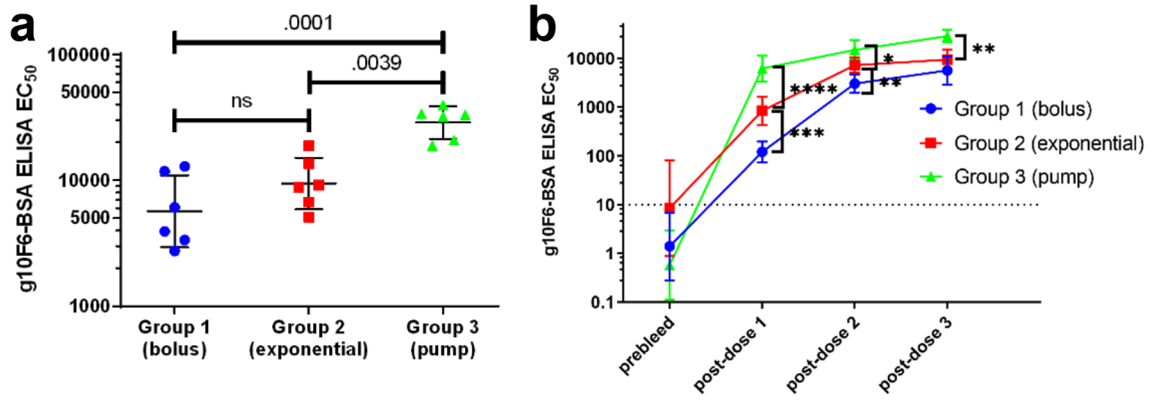

Figure 4. Dependence of g10F6-binding antibodies on immunization regimen. (a) ELISA IgG EC_{50} titers for rabbit serum binding to g10F6−BSA after three cycles of immunization with g10F6−CRM. Geometric means and standard deviations are shown. Numbers displayed above data are *p*-values determined by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons, using log-transformed data; ns denotes $p > 0.1$. (b) Titers as in panle a but plotted versus dose. All data except the prebleed are for sera collected 1 week after the dose: * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$, **** denotes $p < 0.0001$. The dotted line indicates the lowest serum dilution tested in the ELISA.

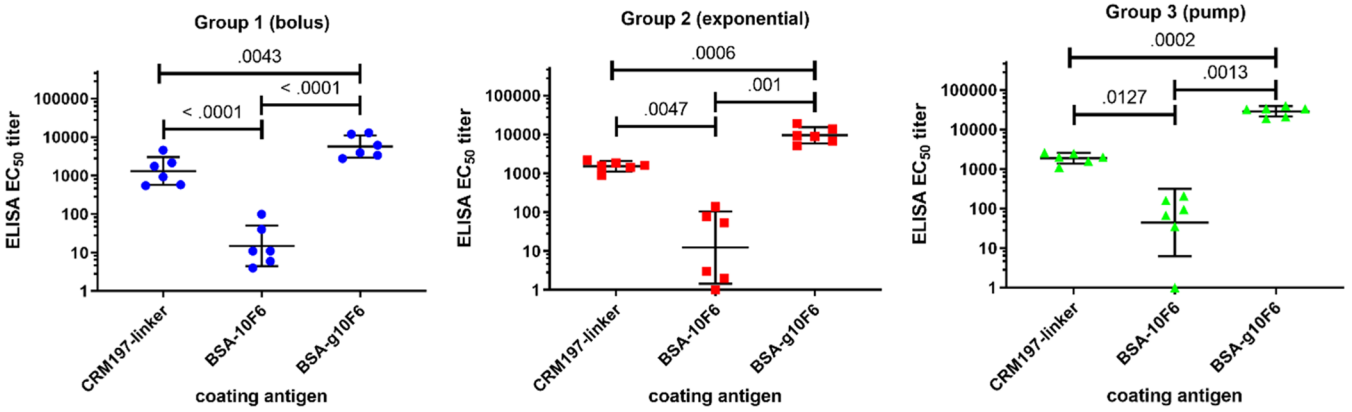

Figure 5. Rabbit serum binding to glycopeptide versus peptide versus carrier. ELISA IgG EC_{50} titers are shown for postdose 3 sera. Glycopeptide g10F6 and peptide 10F6 are both conjugated to BSA in the coating antigen. CRM197 used as coating antigen is functionalized with the linker for conjugation. Numbers displayed above data are *p*-values determined by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons, using log-transformed data.

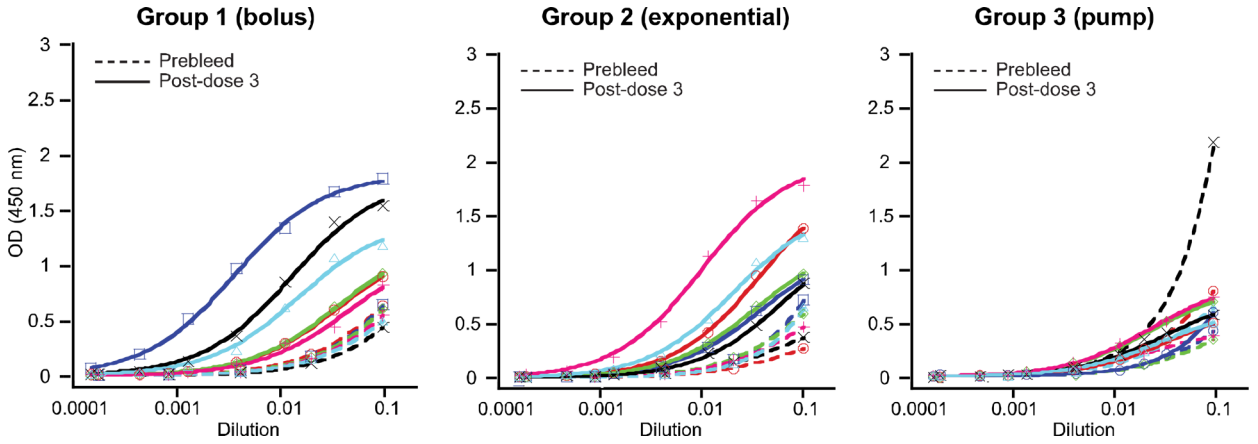

Figure 6. Postdose 3 serum binding to trimeric HIV Env. Data are ELISA for rabbit IgG binding to plates coated with 200 ng/well BG505T332N SOSIP trimers. Solid lines are postdose 3 data, and dotted lines are prebleed data. One animal in Group 3 exhibited a high ELISA absorbance in the preimmunization bleed, at the highest serum concentration (right graph, black dotted line). Because that animal showed no SOSIP binding after glycopeptide immunizations (right graph, black solid line), the prebleed was not investigated further.

trimers).[4] Bolus group sera showed the most binding to HIV Env, with three animals out of six exhibiting EC_{50} titers substantially above preimmunization baseline levels (Figure 6). Exponential group sera showed somewhat less Env binding, and pump group sera exhibited negligible binding above preimmunization baseline. TZM-bl neutralization assays using an HIV strain sensitive to HMP-directed antibodies showed just one serum from group 1 animals was weakly neutralizing (SI Table

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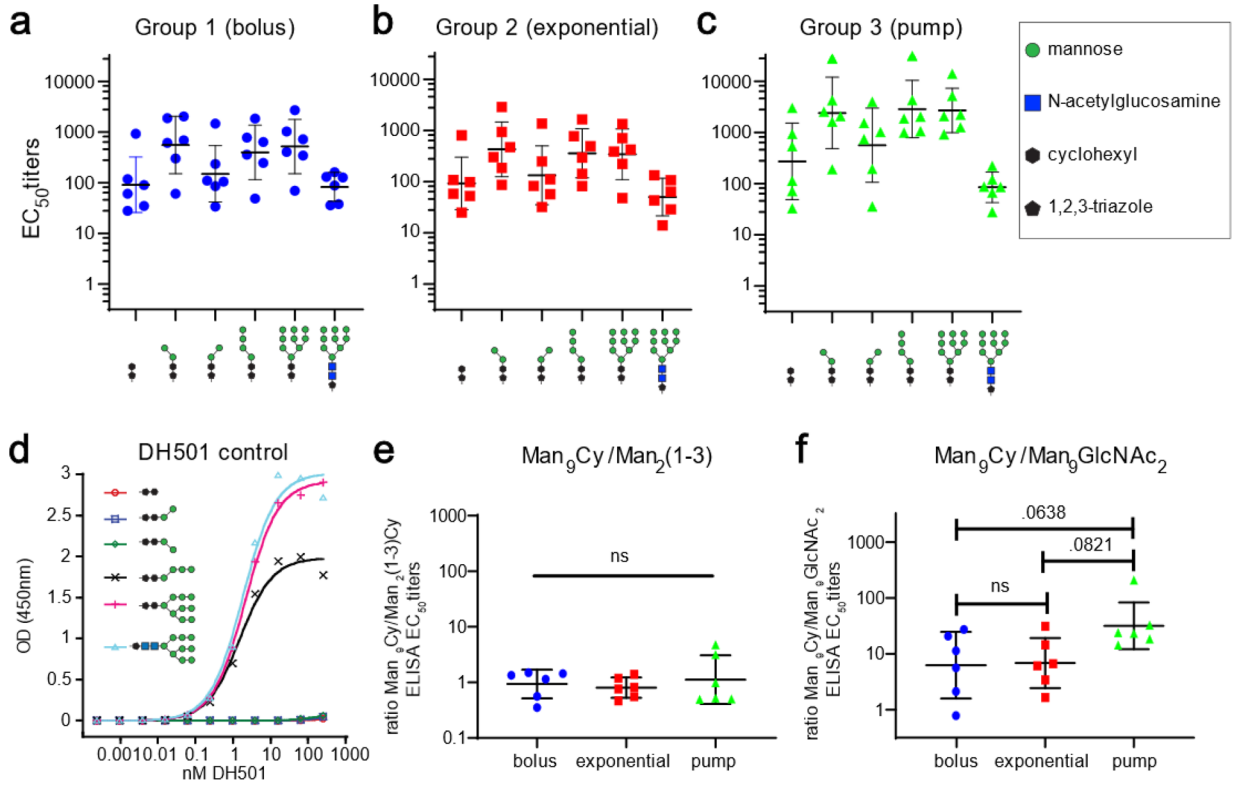

Figure 7. Carbohydrate selectivity of rabbit serum IgGs elicited by g10F6−CRM. (a−c) ELISA EC_{50} IgG titers to different glycans displayed on peptide 10F12M−BSA for animals immunized by (a) group 1 (bolus), (b) group 2 (exponential), and (c) group 3 (pump) regimens. (d) Control ELISA of monoclonal macaque antibody DH501 binding to sugar conjugates from panels a−c. DH501 binds to (Manα1→2Man) termini of glycans, and unlike g10F6−CRM-elicited sera, binds identically to Man_9Cy and $Man_9GlcNAc_2$ conjugates. (e, f) For each rabbit, ratio of serum binding to full vs truncated glycan and full Man_9 glycan with vaccine-derived cyclohexyl linker vs $GlcNAc_2$. Numbers displayed above data are *p*-values determined by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons, using log-transformed data; ns denotes $p > 0.1$. For background binding of rabbit sera to BSA and linker, see Figure S5.

S2). Although elicitation of even weak HIV binding or neutralizing sera without using HIV Env immunogens is encouraging, the greater HIV binding activity among bolus-immunized animals is surprising, as it is the reverse of the trend seen in overall titers to the glycopeptide (cf. Figure 4a); moreover, we had hypothesized that sustained immunization regimens would lead to more antibodies against the intact glycan structures present in the HMP of Env, compared with bolus immunization.

To examine glycan specificity in more detail, we assayed the sera by ELISA against a panel of truncated or altered glycans clustered on a different peptide, 10F12M (Figure 7a−c and SI Figure S6).[38] An antibody response that targets primarily Manα1→2Man tips of the Man_9 structure should bind more strongly in ELISA to Man_9-Cy (Cy = cyclohexyl) than to Man_2(1→3)-Cy conjugates, which are truncated to remove Manα1→2Man linkages. As observed in our previous immunizations with g10F6 conjugate, within each rabbit group, sera bound similarly to Man_9-Cy and Man_2(1→3)-Cy, but more weakly to the Cy linker alone, suggesting that these core two mannose residues together with the cyclohexyl linker are sufficient to account for the bulk of serum reactivity. Moreover, the rabbit antibodies generally bound much better to Man_9-Cy than to $Man_9GlcNAc_2$ structures, suggesting that the core and linker of the glycan was most important and that the Manα1→2Man motif was not the major determinant of binding, as we observed in our previous study.[38] By contrast, a monoclonal macaque antibody DH501 with crystallographically observed Manα1→2Man binding specificity[64] exhibited identical low nanomolar binding EC_{50} values to Man_9-Cy, Man_4-Cy, and $Man_9GlcNAc_2$ but did not bind at all to either the (1→3) or (1→6) isomer of Man_2-Cy (Figure 7d). When the ratio of binding EC_{50} values for Man_9/Man_2(1→3) was calculated for each vaccinated rabbit, no significant difference was observed between groups (Figure 7e). Nearly all animals exhibited binding selectivity for Man_9-Cy vs $Man_9GlcNAc_2$, with apparently the strongest selectivity among pump-immunized animals (Figure 7f). Although the difference between this group and the others was of borderline statistical significance ($p = 0.06$ and 0.08 for comparison with bolus and exponential groups, respectively), it suggests that the most gradual release of the glycoconjugate is, if anything, detrimental to the development of (Manα1→2Man)-focused antibodies, in the context of these immunizations.

We next assessed whether these three glycopeptide immunization regimens might differentially prime responses to an HIV Env boost. The minimal epitope prime/SOSIP protein boost strategy has recently shown some promise in HIV vaccine approaches to focus the antibody response on bnAb epitopes.[46,65] Thus, all groups were boosted with three bolus doses of native-like trimeric BG505T332N SOSIP.664 gp140 protein. Consistent with previous rabbit immunization studies using this trimeric Env,[7] all animals developed binding to this Env (ELISA EC_{50} titers of 400−1500) and most developed BG505T332N neutralizing activity (IC_{50} titers of 40−800, Figure 8 and SI Table S3). However, no statistically significant

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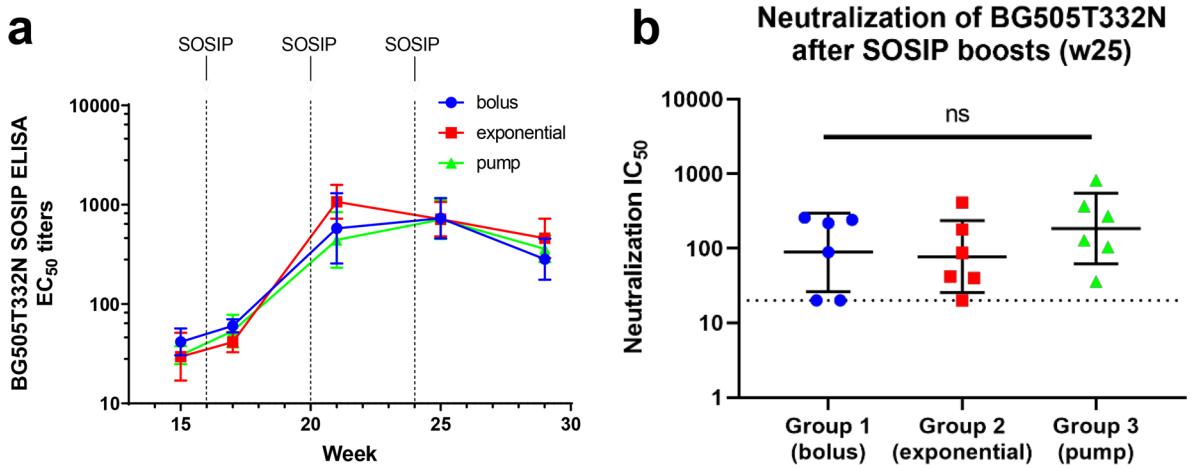

Figure 8. HIV binding and neutralization after SOSIP boosts. (a) Rabbit serum ELISA EC_{50} IgG titers to BG505T332N SOSIP gp140 trimeric HIV Env protein (12 ng/well), the same strain used for the boost immunizations. Dotted lines indicate the time points of the boost immunizations. (b) TZM-bl neutralization assays against BG505T332N pseudovirus. The dotted line denotes the lowest (20:1) dilution tested in the assay. The three data points on this line represent less than half-maximal inhibition at this dilution. ns denotes $p > 0.1$ in one-way ANOVA.

differences were observed between groups. In our previous work,[38] the SOSIP boost immunizations did not increase titers against the g10F6 glycopeptide, and they elicited similar SOSIP binding titers in animals primed with either CRM−g10F6 or CRM alone; therefore, these effects were not investigated in the present study.

DISCUSSION AND CONCLUSIONS

This study was designed to test the hypothesis that slow release immunization regimens could be used to increase the selectivity of vaccine elicited antibodies for the nonreducing "tips" (Manα1→2Man moieties) of oligomannose glycans. Because these glycans are trimmed by serum mannosidase on a time scale (hours to days) competitive with germinal center formation (days) and affinity maturation (weeks), it was reasoned that a constant supply of fresh immunogen would increase the exposure of germinal centers to intact glycans, thereby resulting in a stronger antibody response to the Manα1→2Man tips of the Man_9. Instead, we observed similar results from standard bolus immunizations versus exponential and continuous immunization, with, if anything, more response to intact glycans in the bolus-immunized animals. A better understanding of this result would require more detailed data about the relative kinetics of (1) mannosidase trimming and (2) activation of the B cells that lead to this antibody response. Although we have observed[38] ∼50% cleavage of our immunogen glycans from Man_9 to $Man_{8/7/6}$ within ∼17 h in serum *ex vivo* at a high concentration (100 μL/mL), we do not know the rates *in vivo* and at relevant concentrations; moreover, it is not known what concentrations might saturate the mannosidase activity *in vivo*. If the bolus dose were to saturate mannosidase activity, intact oligomannose would be more likely to encounter B cells; by contrast, the low rate of immunogen release in pump immunizations might allow for more extensive mannosidase trimming prior to B cell encounter. Future dose or kinetics studies might address these questions, although a more straightforward route to vaccine optimization would be to eliminate the degradation of the glycan, using mannosidase inhibitors or chemical modification of the glycans.

Independent of immunization regimen, the lack of antibody response to the Manα1→2Man termini may also be due to a failure to engage appropriate germline precursors of Manα1→2Man-binding antibodies. A great deal of HIV vaccine research is currently devoted to approaches that utilize immunogens designed to bind to inferred germline precursors of bnAbs, under the supposition that vaccine-elicited bnAbs with the same specificity are more likely to arise from the same germline Ab lineage.[66] To address this question experimentally would require the design of immunogens that bind to the human germline precursors of 2G12 or other HMP bnAbs and to test them in germline bnAb knock-in animals.[67−69]

Another potential barrier to elicitation of Manα1→2Man-binding Abs or bnAbs could be insufficient somatic hypermutation. Nearly all HIV bnAbs are highly somatically mutated. This includes the high-mannose-patch antibodies, which generally bear ∼20% nucleotide mutation in the heavy chain.[70] In contrast, antibodies from vaccination have been reported to average ∼6% nucleotide mutation.[71,72] DH501, which binds oligomannose but neutralizes only kifunensine-treated virus (bearing exclusively $Man_{8/9}$ glycans), is also highly mutated (23% of amino acids in the heavy chain).[73] DH501 arose during a four-year course of 17 immunizations using both Env protein and Env DNA in monkeys, which is the only vaccine regimen to date that has definitively elicited antibodies that bind to the Manα1→2Man motif.[64] To recapitulate and improve on those findings with a more practical immunization regimen remains an important goal.

METHODS

Detailed information for all methods used can be found in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.0c00053.

Materials and methods, including preparation and mass spectral data of glycopeptide conjugates, adjuvant preparation and characterization, immunization procedures, ELISA protocol, neutralization assay protocol and data, supplementary ELISA data, and data on glycoconjugate stability (PDF)

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Notes

The authors declare no competing financial interest.

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