JVI Accepted Manuscript Posted Online 24 May 2017
J. Virol. doi:10.1128/JVI.00677-17
Copyright © 2017 Ringe et al.
This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

Reducing V3 antigenicity and immunogenicity on soluble, native-like HIV-1 Env SOSIP trimers

Rajesh P. Ringe[1], Gabriel Ozorowski[2], Kimmo Rantalainen[2], Weston B. Struwe[3], Katie Matthews[1,*] Jonathan L. Torres[2], Anila Yasmeen[1], Christopher A. Cottrell[2], Thomas J. Ketas[1], Celia C. LaBranche[5], David C. Montefiori[4], Albert Cupo[1], Max Crispin[3,4], Ian A. Wilson[2], Andrew B. Ward[2], Rogier W. Sanders[1,6], P.J. Klasse[1], John P. Moore[1,#]

[1] Department of Microbiology and Immunology, Weill Medical College of Cornell University, New York, NY 10021, USA

[2] Department of Integrative Structural and Computational Biology, International AIDS Vaccine Initiative (IAVI) Neutralizing Antibody Center and the Collaboration for AIDS Vaccine Discovery (CAVD), The Scripps Research Institute, La Jolla, CA 92037, USA

[3] Oxford Glycobiology Institute, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK

[4] Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, California, USA

[5] Department of Surgery, Duke University Medical Center, Durham, NC 27710, USA

[6] Department of Medical Microbiology, Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands

 # Corresponding author's mailing address:
John P. Moore, Department of Microbiology and Immunology, Weill Medical College of Cornell University, New York, NY 10065, USA

*** Current address:** Innovimmune Biotherapeutics Inc, New York, NY, USA.

Short title: V3 antigenicity and immunogenicity on SOSIP trimers

Keywords: HIV-1 vaccine; Env trimers; V3 region

Abstract word count: 214

Text word count: 7825

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

39 **ABSTRACT**

40 Native-like trimers of the SOSIP design are being developed as immunogens in human
41 immunodeficiency virus type 1 (HIV-1) vaccine development programs. These trimers display
42 the epitopes for multiple broadly neutralizing antibodies (bNAbs), but can also expose binding
43 sites for some types of non-neutralizing antibodies (non-NAbs). Among the latter are epitopes in
44 the gp120 V3 region that are highly immunogenic when SOSIP trimers are evaluated in animal
45 models. It is presently uncertain whether antibodies against V3 can interfere with the induction
46 of NAbs, but there are good arguments in favor of suppressing such “off-target” immune
47 responses. Accordingly, we have assessed how to minimize the exposure of V3 non-NAb
48 epitopes and thereby reduce their immunogenicity by introducing N-glycans within the V3
49 region of BG505 SOSIP trimers. We found that inserting glycans at positions 306 and 314
50 (termed M1 and M7) markedly reduced V3 antigenicity while improving the presentation of
51 trimer apex bNAb epitopes. Both added glycans were shown to be predominantly of the
52 $Man_6GlcNAc_2$ form. The additional introduction of the E64K ground-state stabilizing
53 substitution markedly reduced or ablated sCD4-induction of non-NAb epitopes in V3 and/or
54 associated with the co-receptor binding site. When a V3 glycan- and E64K-modified trimer
55 variant, BG505 SOSIP.664-E64K.M1M7, was tested in rabbits, V3 immunogenicity was
56 eliminated while the autologous NAb response was unchanged.

57

58 **IMPORTANCE**

59 Trimeric proteins are being developed for future HIV-1 vaccine trials in humans, with the goal of
60 eliciting broadly active neutralizing antibodies (NAbs) that are active against a wide variety of
61 circulating strains. In animal models, the present generation of native-like trimer immunogens,
62 exemplified by the BG505 SOSIP.664 construct, induces narrow specificity antibodies against
63 the neutralization-resistant (Tier-2), sequence-matched virus, and more broadly active antibodies
64 against sequence-divergent atypically neutralization-sensitive (Tier-1) viruses. A concern in the
65 trimer immunogen design field has been whether the latter “off-target” antibodies might interfere
66 with the induction of the more desired responses to Tier-2 epitopes. Here, we have inserted two
67 glycans into the dominant site for Tier-1 NAbs, the gp120-V3 region, to block the induction of
68 “off-target” antibodies. We characterized the new trimers, tested them as immunogens in rabbits
69 and found that the blocking glycans eliminated the induction of Tier-1 NAbs to V3-epitopes.

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Accepted Manuscript Posted Online

INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein trimer is a key element of vaccine development strategies aimed at inducing neutralizing antibodies (NAbs) (1-9). A now widely used immunogen design platform involves SOSIP trimers, which have been engineered for increased Env stability and can be produced and purified in practical quantities (1-3, 5, 6, 10-12). SOSIP trimers of multiple sub-designs and genotypes have been shown to mimic native, virion-associated trimers both antigenically and structurally, including by displaying the epitopes for many different broadly neutralizing antibodies (bNAbs) (1-3, 5, 6, 9, 11, 13-20). However, SOSIP trimers can also expose epitopes for some non-neutralizing antibodies (non-NAbs), particularly those associated with the V3 region of gp120. The extent of V3 exposure *in vitro* varies with the assay used to measure antibody binding, and is far more pronounced in a capture enzyme-linked immunosorbent assay (ELISA) than in other methods such as surface plasmon resonance (SPR) or negative-stain electron microscopy (NS-EM) (5, 21). Of greater relevance is that V3 is clearly immunogenic when SOSIP trimers are tested as immunogens in animal models, with anti-V3 antibodies dominating the neutralization of Tier-1 viruses (6, 15, 22, 23). In the same experiments, antibodies able to neutralize the autologous Tier-2 viruses are induced, which is the more desired response (6, 15, 22, 23). A key question is whether the anti-V3 response is an irrelevant side effect of SOSIP trimer immunogenicity, or whether it could, under certain circumstances, be an immunological distraction that compromises the induction of autologous or heterologous Tier-2 NAbs.

The processes by which initial antibody responses can be driven to evolve towards bNAbs are likely to be highly complex, and to require the sequential use of more than one immunogen. Arguments can be made that "off-target" antibody responses could interfere with the ones that are needed, for example via immunogen complexing and sequestration or epitope competition events within germinal centers (24). For example, naïve B cells with specificity for non-NAb epitopes such as V3 have been shown to outcompete naïve B cells for bNAb epitopes (25). It is prudent, therefore, to explore ways to reduce the immunogenicity of V3 and other non-NAb epitopes on SOSIP trimers, so as to focus the immune response elsewhere. We have already shown that the introduction of two sequence changes in the C1 (at residue 64 or 66) and V3 (at residue 316) regions of the prototypic SOSIP.664 trimer design can further stabilize the resulting SOSIP.v4.1 or SOSIP.v4.2 variants *in vitro* and reduce the immunogenicity of V3 in rabbits by

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Accepted Manuscript Posted Online

101 several-fold. Nonetheless, the immunized rabbits still produced some anti-V3 antibodies that
102 neutralized Tier-1 viruses (15). An alternative BG505 SOSIP trimer design strategy led to
103 broadly similar findings in guinea pigs (23). In neither study was the reduction in the tier-1 NAb
104 response accompanied by a consistent increase in the autologous tier-1 NAb titers (15, 23).

105 Here, we have further addressed the question of V3 antigenicity and immunogenicity on
106 SOSIP trimers. Our approach involved the addition of N-linked glycans at various positions
107 within V3, to occlude the underlying non-NAb epitopes. The masking of potential epitopes by
108 adding glycan sites is an established concept that has been used successfully to mask
109 immunodominant epitopes in the V3 region of HIV-1 gp120 and in the variable head domain of
110 the Influenza virus hemagglutinin (26, 27). The same approach was also used to dampen the
111 immune responses to heterologous trimerization domains added to subunit vaccine antigens (28).
112 The outcome of our own studies was the identification of double glycan-mutant trimers of the
113 BG505 and B41 genotypes that remained fully native-like but were no longer reactive with V3
114 non-NAbs *in vitro*. These glycan substitutions, at positions 306 (designated as M1) and 314
115 (M7), could be combined with the E64K change in C1 that helps to prevent CD4-mediated
116 induction of non-NAb epitopes associated with the coreceptor-binding site (15). When such a
117 V3-masked and prefusion-stabilized trimer, BG505 SOSIP.664-E64K.M1M7, was tested as an
118 immunogen in rabbits, NAb titers against various Tier-1 viruses were reduced by 3- to 22-fold
119 without compromising or improving the induction of autologous Tier-2 NAbs. Additional
120 analyses showed that the residual neutralization of the Tier-1 viruses was not attributable to
121 antibodies reactive with a cyclized BG505 V3 peptide. The V3-glycan masking method may be
122 useful when designing more complex trimer-based strategies aimed at the eventual elicitation of
123 bNAbs in humans.

Journal of Virology
JVI
Journal of Virology
Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Accepted Manuscript Posted Online

Journal of Virology

JVI

124 **RESULTS**

125 **Masking V3 epitopes via added glycans.** We assessed whether one or more epitope-masking
126 glycans could be inserted into the V3 region of BG505 SOSIP.664 trimers. Because this project
127 was initiated before latest high-resolution structures of the trimer were available, we chose to
128 insert glycans at multiple positions in V3 and to assess the properties of the resulting modified
129 trimers empirically. Accordingly, potential N-linked glycosylation sites (PNGS) were introduced
130 at 21 different positions in V3 via an NXT strategy; i.e., the first and third residues of each triplet
131 were changed to N and T, respectively, to create a sequon (Table 1). The resulting single glycan-
132 mutant proteins (D7324-tagged) were then transiently expressed in 293T cells and the unpurified
133 culture supernatants used to assess Env antigenicity by capture ELISA (Table 2). The test panel
134 included bNAbs to trimer-specific or trimer–sensitive epitopes (PG16, PGT145, PGT151), as
135 well as V3 non-NAbs (19b and 14e). Although non-trimer forms of Env with well-exposed V3
136 regions, such as dimers and monomers, contribute to the signals, this assay format is useful for
137 screening multiple constructs and flagging the most promising variant for further evaluation. The
138 outcome was identification of constructs M1 (S306N, R308T) and M7 (G314N, A316T) with
139 reduced binding to V3 non-NAbs, but with the epitopes for the trimer-specific bNAbs PG16,
140 PGT145 and PGT151 retained (Table 2). As the M7 construct had a particularly promising
141 overall antigenicity profile, we then combined the relevant G314N and A316T sequence changes
142 with those present in the M1, M3 or M6 constructs to make three double glycan mutants (M1M7,
143 M3M7 and M6M7) that were similarly expressed and evaluated. Among them, the M1M7
144 mutant had the best antigenicity profile with a marked reduction in V3 non-NAb binding
145 combined with retention of trimer-specific bNAb epitopes (Table 2).

146 We studied the BG505 SOSIP.664-M7 and -M1M7 constructs in greater detail by
147 expressing them in 293F cells and using the 2G12/SEC method to purify the resulting D7324-
148 tagged trimers and, for comparison, the parental BG505 SOSIP.664 trimer (Fig. 1). As assessed
149 by capture ELISA, the antigenicity profiles of the M7 and M1M7 mutants were generally
150 comparable to the parental trimer except for a substantial reduction in V3 non-NAb reactivity.
151 More specifically, the M7 trimer was completely non-reactive with 14e but did still bind 19b and
152 39F, whereas the M1M7 trimer had no reactivity with 14e and 39F and bound 19b to only a
153 minimal extent (Table 2, Fig. 1). The outcome of these exploratory studies was the adoption of
154 the BG505 SOSIP.664-M1M7 design, which contains masking glycans at positions 306 and 314.

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Journal of Virology

Accepted Manuscript Posted Online
Journal of Virology
JVI
Journal of Virology
Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

155 The E64K change in the gp120 C1 domain has been shown to reduce the baseline and
156 sCD4-induced exposure of the co-receptor binding site and its associated non-NAb epitopes (15).
157 We therefore introduced this change into the M1M7-modified trimers to make the SOSIP.664-
158 E64K.M1M7 construct. We also produced the corresponding mutants of the clade B B41
159 SOSIP.664 trimer. The D7324-tagged versions of both genotypes of trimer variants were
160 expressed by transient transfection of 293F cells and purified on either 2G12/SEC or
161 PGT145/SEC columns. The SEC profiles were broadly similar for both methods, with a slightly
162 lower content of non-trimer proteins in the PGT145-purified preparations (Fig. 2A). The overall
163 yields of trimers were comparable for each genotype and purification method (within ±2-fold),
164 implying that addition of the two V3 glycan sites did not adversely affect the overall biochemical
165 properties of the trimers.
166 A site-specific analysis of the glycosylation of the B41 and BG505 SOSIP.664-
167 E64K.M1M7 trimers, produced in 293F cells and purified via the PGT145/SEC method, showed
168 that the introduced N306 (M1) and N314 (M7) sites were both modified by glycosylation. The
169 dominant glycan present at both positions was $Man_6GlcNAc_2$ (Fig. 2B). The dominance of the
170 Man_6 glycan suggests that the presence of the neighboring, wild type glycans impedes the further
171 processing of the newly inserted ones (29, 30).
172 **Antigenicity and conformation of V3 glycan-modified trimers.** An SPR analysis using
173 PGT145-purified, His-tagged proteins showed that the double glycan-modified BG505 and B41
174 trimers had almost unchanged 2G12 reactivity, which serves as a frame of reference for other
175 epitopes (Fig. 3A). The SPR antigenicity profiles of the SOSIP.664, SOSIP.664-M1M7 and
176 SOSIP.664-E64K.M1M7 variants of each genotype were broadly similar with respect to bNAbs
177 against V3-glycan (2G12, PGT122 and PGT128), interface (PGT151) and quaternary apical
178 (PG16, PGT145) epitopes; if anything, the binding of PG16 was somewhat enhanced for the
179 mutants, particularly for SOSIP.664-E64K.M1M7. In contrast, the binding of the 14e and 19b
180 non-NAbs to their V3 epitopes was reduced for the SOSIP.664-M1M7 and SOSIP.664-
181 E64K.M1M7 trimers compared with SOSIP.664. The differences in 14e binding were less
182 marked for these glycan-modified BG505 trimers compared to their B41 counterparts (Fig. 3A).
183 However, we note that the unmodified BG505 SOSIP.664 trimers bound these V3 non-NAbs
184 more weakly than B41 SOSIP.664, as shown here and in previous SPR assays (5, 21).

Accepted Manuscript Posted Online

185 We also compared the antigenicity of the His-tagged BG505 and B41 SOSIP.664 and
186 SOSIP.664-E64K.M1M7 trimers by capture ELISA (Fig. 3B). For both genotypes, binding of
187 the PG16 and PGT145 bNAbs to the modified trimers was modestly improved, similar to the
188 observations made with PG16 in the SPR analysis (Fig. 3A). Thus, the introduction of the V3-
189 glycans certainly does not impair, but rather modestly improves, the presentation of the highly
190 conformational bNAb epitopes at the trimer apex. The non-NAbs 14e, 19b and 17b bound
191 markedly less well. In addition, the ability of sCD4 to induce the exposure of the 19b and 17b
192 non-NAb epitopes was greatly diminished or even fully ablated on the modified trimers (Fig.
193 3B).

194 NS-EM imaging of the BG505 and B41 SOSIP.664-M1M7 and SOSIP.664-E64K.M1M7
195 trimers confirmed that they were all fully native-like (Fig. 3C). Clearly, the two new V3 glycans,
196 with or without the additional ground state-stabilizing E64K change in C1, are fully consistent
197 with the retention of an appropriate trimer conformation while greatly reducing or even ablating
198 the presentation of non-NAb epitopes associated with V3 and the co-receptor binding site.

199 A model of the BG505 SOSIP.664-E64K.M1M7 trimer suggests that there is enough
200 solvent-exposed area to accommodate both glycans near the respective mutation sites. The N306
201 glycan fills a gap between the existing N156 and N301 glycans, but is constrained by the N197
202 glycan from a neighboring protomer (Fig. 3D, 3E). The new glycan at position N314 is more
203 constrained than the one at N306 because of its location at the tip of V3. The most likely
204 orientation of the N314 glycan on one gp120 protomer would result in a clash with the
205 neighboring protomer (Fig. 3D, 3E). However, the substantial occupancy of the N314 site shown
206 by the site-specific analysis (Fig. 2B) and the retention of native-like conformation judged by
207 NS-EM (Fig. 3C) together suggest that some conformational change must occur in this region of
208 the M1M7 trimer. We speculate that accommodation of the N314 glycan must necessitate some
209 rearrangement of the V3 region, at least at the tip, but in a manner whereby the closed
210 conformation of the trimer apex is retained.

211 **Immunogenicity of BG505 SOSIP.664-E64K.M1M7 trimers in rabbits.** To assess
212 whether M1M7 glycans reduce the immunogenicity of V3 non-NAb epitopes *in vivo*, we
213 immunized groups of 5 rabbits three times with the BG505 SOSIP.664-E64K.M1M7 trimers and
214 SOSIP.664 trimers and quantified the NAb titers in sera from 2 weeks after the third
215 immunization (Fig. 4A). The Tier-2 autologous NAb titers were statistically indistinguishable for

Journal of Virology JVI Journal of Virology

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Accepted Manuscript Posted Online

216 the two groups of rabbits (p = 0.69, NS). Thus, although the range of titers for the SOSIP.664
217 group was somewhat narrower than for SOSIP.664-E64K.M1M7, the titers for the individual
218 animals fell within the same titer range. In marked contrast, NAb titers against the 6 Tier-1
219 viruses were 3-22 fold lower for the SOSIP.664-E64K.M1M7 trimer group compared to
220 SOSIP.664. The differences were significant for five of the viruses (p = 0.0079), but not for
221 TH023.6 CRF01_AE (p = 0.30) (Fig. 4A).

222 In a Tzm-bl cell assay at WCMC, none of the ten sera neutralized any of the heterologous
223 Tier-2 viruses, 92UG037.8, Q23env17, Q46envE2 (each clade A) and 25710-2.43 (clade C), at
224 titers >20. In a similar assay at DUMC, the same sera were tested against a panel of nine
225 heterologous Tier-2 viruses: Ce1176_A3 (clade C), 246-F3_C10_2 (clade A/C), CH119.10
226 (circulating recombinant form 07-BC), Ce703010217_B6 (clade A), CNE55 (circulating
227 recombinant form 01_AE), 25710-2.43 (clade C), TRO.11 (clade B), BJOX002000.03.2
228 (circulating recombinant form 07_BC) and X1632-S2-B10 (clade B). Compared to a cut-off titer
229 value of 20, only 2 of the ten sera yielded any positive titers: One serum from the SOSIP.664
230 group neutralized 25710-2.43 (titer of 82), Ce1176_A3 (titer of 29), 246-F3_C10_2 (titer of 29)
231 and CNE55 (titer of 31); and one serum from the SOSIP.664-E64K.M1M7 group neutralized
232 25710-2.43 (titer of 23). Thus, there is no evidence that suppressing the V3 response to
233 SOSIP.664 trimers is sufficient to increase the breadth of the neutralization response against
234 Tier-2 viruses, which is consistent with earlier reports (15, 22, 23).

235 We used a cyclized BG505 V3-peptide as a soluble competitor in the neutralization assay
236 to deplete V3-targeted antibodies active against Tier-1 viruses. Previous studies indicated that
237 this method reduced the Tier-1 NAb response to SOSIP trimers, but not the autologous Tier-2
238 NAb response (6, 22). When the cyclized V3 peptide was mixed with sera from the BG505
239 SOSIP.664 trimer-immunized animals, the median titers against the MN.3, MW965.26,
240 CH0505.w4.3 and TH023.6 Tier-1 viruses were reduced by 11-, 30-, 2.5- and 5.3-fold,
241 respectively. A sequence-scrambled version of an MN.3-V3 peptide, used as a negative control,
242 had no detectable effect (Fig. 4B). In contrast, the cyclized BG505 V3-peptide did not detectably
243 reduce neutralization of any of the four Tier-1 viruses by sera from the SOSIP.664-E64K.M1M7
244 trimer recipients (Fig. 4B). This outcome shows that the modifications made to this trimer
245 ablates its ability to induce any antibodies that cross-react with the cyclized V3-peptide (Fig.
246 4B). For each virus, the residual level of Tier-1 virus neutralization by the SOSIP.664 sera in the

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

JVI Journal of Virology Journal of Virology

Accepted Manuscript Posted Online

247 presence of the BG505 V3-peptide was similar to or lower than that mediated by the SOSIP.664-
248 E64K.M1M7 sera. The residual neutralization mediated by the SOSIP.664 trimer sera in the
249 presence of the V3-peptide, as well as by the SOSIP.664-E64K.M1M7 sera in the absence of the
250 peptide, might be directed against other Tier-1 epitopes such as those associated with the CD4
251 binding site (CD4bs) or CD4-induced sites. It is possible that antibodies to glycan-dependent V3
252 epitopes might also be induced by the modified trimer, but such epitopes on HIV-1 Env proteins
253 are generally considered to be very poorly immunogenic, and hence not likely to be generated in
254 the context of this type of immunization procedure. We conclude, therefore, that adding the
255 M1M7 glycans effectively eliminates the induction of cross-reactive, Tier-1 NAbs by the V3
256 region of the modified trimer, and without compromising the ability of the trimers to induce
257 autologous Tier-2 NAbs to other epitopes.

258 Reductions in V3 immunogenicity were previously found when V3-stabilized BG505
259 SOSIP.v4.1 trimers were compared with SOSIP.664 trimers in rabbits (15). Here, we tested sera
260 from that SOSIP.v4.1 *vs.* SOSIP.664 rabbit experiment and from the present SOSIP.664-
261 E64K.M1M7 *vs.* SOSIP.664 experiment for neutralization of the MN.3 and MW965.26 Tier-1
262 viruses in the same assay (Fig. 4C). Compared to the corresponding SOSIP.664 group, the
263 suppression of Tier-1 NAb titers was greater for SOSIP.664-E64K.M1M7 (~20-fold) than for
264 SOSIP.v4.1 (non-significant differences, with overlapping ranges) for MN.3 and MW965.26,
265 respectively.

266 **Mapping the target for NAbs against the BG505.N332 autologous virus.** We have
267 previously shown that a frequent, but not the only, target for the autologous NAb response in
268 BG505 SOSIP.664 trimer-immunized rabbits is a hole in the glycan shield formed by the
269 absence of glycans from positions 241 and 289 (3). Thus, sera from ~60% of the immunized
270 rabbits completely fail to neutralize BG505.T332N virus mutants with a glycan knocked-in at
271 positions 241 and/or 289. The remaining sera usually also target this same glycan hole but in a
272 way that is less sensitive to the knocked-in glycans, or they also recognize additional
273 neutralization-relevant epitopes. The use of clones of the maternal MG505 virus, with and
274 without specific sequence changes, provides additional information (3). To see whether the
275 introduction of the M1M7 glycans had fundamentally changed the immunogenicity of the
276 BG505 trimers, we used the same panel of mutant viruses to test the week-22 sera from both
277 immunization groups (Table 3). Overall, we found no major difference between the SOSIP.664

Journal of Virology

JVI

Journal of Virology

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Accepted Manuscript Posted Online

278 and SOSIP.664-E64K.M1M7 immunogen groups. Some sera from these groups contained NAbs
279 that targeted only the 241/289 glycan hole (e.g., sera 2111 and 2116), whereas the NAbs in the
280 remaining sera were less affected by the presence of these knocked-in glycans (Table 3). This
281 outcome is generally consistent with what we have previously reported for other sera from
282 BG505 SOSIP.664 trimer-immunized rabbits (3).
283

284 **DISCUSSION**

285 When native-like SOSIP trimers are used as immunogens in animals, they induce NAbs against
286 both autologous Tier-2 viruses and heterologous Tier-1 viruses. These two responses are non-
287 correlated within individual animals and among test groups (3, 6, 15). Moreover, the Tier-1
288 NAbs generally emerge relatively early in the immunization course (3, 6). The Tier-1 NAbs are
289 substantially attributable to V3 peptide-reactive antibodies, whereas the autologous Tier-2 NAbs
290 that have been mapped to date recognize more complex epitopes involving holes in the glycan
291 shield (3). The latter type of response may be a necessary first step towards the development of
292 bNAbs (6, 11, 24). It is not known whether “off-target” V3-antibodies interfere with the
293 elicitation or evolution of bNAbs against the more relevant Tier-2 epitopes, but there are various
294 mechanisms by which this could happen, as discussed further below. Here, our goal was to
295 identify ways to reduce the antigenicity of the V3 region on SOSIP trimers, and assess whether
296 there was a concomitant reduction in V3-dependent non-NAbs or Tier-1 NAbs when the
297 modified trimers were used as immunogens.

298 The prototypic BG505 SOSIP.664 native-like trimer, and others with similar *in vitro*
299 properties, expose multiple bNAb epitopes, but very few for non-NAbs. Among the latter, V3
300 epitopes are the most prominently exposed, but the extent depends on the assay used to measure
301 antibody binding. Thus, V3 non-NAb reactivity with SOSIP.664 trimers is much stronger in
302 capture ELISAs than when measured by SPR or Octet (BLI) methods (5, 14). Moreover, V3
303 exposure is now considered to be a consequence of trimer breathing; i.e. reversible transitions
304 between alternative conformations of the trimer that predominantly involve the V1V2 loop
305 structure at the apex but that also affect the orientation and exposure of V3(31, 32). Although
306 what happens to trimers under *in vivo* conditions is not known, the exposure of highly
307 immunogenic V3 epitopes elicits Tier-1 NAb or non-NAb responses in rabbits, guinea pigs and
308 mice (3, 6, 11, 15, 22, 23, 33). Several methods to reduce the immunogenicity of the V3-region

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Accepted Manuscript Posted Online

309 on BG505 SOSIP trimers have now been described (6, 11, 15, 22, 23). Thus, a point substitution,
310 A316W, that helps lock the V3 region into the body of the trimer, was made in concert with
311 another in C1, either E64K or H66R, that reduces the spontaneous and CD4-driven exposure of
312 CD4i non-NAb epitopes. The resulting BG505 SOSIP.v4.1 (A316W + E64K changes) and
313 SOSIP.v4.2 (A316W + H66R changes) trimers were more stable, had superior antigenicity
314 profiles, and elicited lower titers of V3-dependent Tier-1 NAbs in rabbits, compared to the
315 SOSIP.664 prototype (15). Broadly similar reductions in V3 responses were achieved by
316 complexing a BG505 SOSIP.664 trimer via a PGT145 Fab to occlude the V3 region (22), and by
317 making several sequence changes that individually or collectively stabilize the trimer and reduce
318 V3 exposure (23). However, in each of these studies, as here, the autologous Tier-2 NAb
319 responses to the variously modified trimers were generally similar to those elicited by the
320 SOSIP.664 prototype (6, 11, 15, 22, 23).

321 The focus of this study was to assess whether the addition of masking glycans within V3
322 could further suppress the immunogenicity of this region of SOSIP trimers. There are precedents
323 for the used of this method with earlier generation Env proteins. Thus, the addition of glycans to
324 the V3 region of a monomeric gp120 protein reduced the induction of anti-V3 antibodies in
325 immunized guinea pigs, while increasing the elicitation of anti-V1 antibodies (26). Otherwise
326 antigenic V3 epitopes were successfully masked when four glycans were introduced into this
327 region of an uncleaved, non-native YU2 gp140 protein (34). However, when wild-type and
328 glycan-masked gp140s were tested in mice, the reduced immunogenicity of the glycan-masked
329 V3 region did not divert the B-cell response to other epitopes on the gp140 protein (34). The
330 same glycan-immunosilencing method has also been applied successfully to other protein
331 immunogens (27, 28).

332 After conducting exploratory studies on the BG505 SOSIP.664 construct, we identified
333 residues 306 and 314 as positions where two glycans could be added without compromising the
334 native-like conformation and bNAb epitope presentation of the resulting SOSIP.664-M1M7
335 trimers. A further improvement to antigenicity was achieved by the additional introduction of the
336 E64K change in C1 that helps maintain the trimer in the pre-fusion ground state (15). The final
337 glycan-modified BG505 and B41 trimers were fully native-like as judged by NS-EM, and site-
338 specific glycan analysis showed that both of the inserted glycan sites were modified by
339 glycosylation.

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

JVI Journal of Virology

Journal of Virology

Accepted Manuscript Posted Online

340 As noted earlier, this project was initiated without the guidance of high-resolution
341 structural information. Had we in fact used the now available structures to design the mutants,
342 we may have decided to not make the M7 and thence the M1M7 constructs that turned out to
343 yield the most promising trimer variants. A model of the glycan-modified BG505 trimer shows
344 that the N306 glycan fills a hole between two existing glycans at positions N156 and N301,
345 while the N314 glycan at the V3 tip shields the remainder of V3. We do not know exactly how
346 the N314 glycan is accommodated from the structural perspective, but it seems likely that some
347 such process must take place to minimize a clash between the glycan on one protomer with the
348 neighboring protomer. As residue-314 lies at the interprotomer interface it might be anticipated
349 that introducing a glycan into such an environment would create a steric clash with the adjacent
350 protomer and destabilize the trimer. However, the antigenicity and low resolution EM data
351 together show that no such destabilization occurs. If and when a high-resolution structure of the
352 M1M7 trimer is generated, we may better understand the orientation of the glycan-modified V3
353 region. Until then, our prevailing hypothesis is that the somewhat plastic trimer apex may open
354 up sufficiently to the N314 glycan to be accommodated. Whatever static or dynamic events are
355 taking place, the SOSIP.664-E64K.M1M7 trimers are fully native-like when viewed by NS-EM,
356 and they retain (and even modestly improve) their presentation of bNAb epitopes, including the
357 highly conformationally sensitive ones located at the trimer apex. In contrast, non-NAbs to V3
358 are minimally or non-reactive with the glycan-modified trimers. The latter outcome is entirely
359 consistent with the model of the modified trimer, which shows that very little of the surface of
360 the V3 region is now accessible, but without disrupting the antigenically native, pre-fusion
361 structure of Env.

362 A rabbit immunogenicity study showed that the glycan-modified SOSIP.664-
363 E64K.M1M7 and parental SOSIP.664 trimers induced comparable titers of autologous Tier-2
364 NAbs, but the Tier-1 NAb titers elicited by the modified trimers were reduced by 3- to 22-fold
365 depending on the test virus. A comparative analysis showed that reduction in the Tier-1 NAb
366 response was greater than that conferred by the stabilizing change used to create the SOSIP.v4.1
367 trimer. The residual Tier-1 NAb responses in the sera from the SOSIP.664-E64K.M1M7 group
368 were not reduced by a competing cyclized BG505 V3-peptide, unlike the sera from the
369 SOSIP.664-immunized rabbits. Thus, the Tier-1 NAb response to the modified trimers is not
370 directed at V3 epitopes, or at least not at ones that can be mimicked by a cyclized peptide. Other

Journal of Virology
JVI
Journal of Virology
Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Accepted Manuscript Posted Online

371 epitopes for Tier-1 NAbs, such as those associated with the CD4bs or the CD4-inducd co-
372 receptor-binding site are the most likely targets for the residual Tier-1 NAbs. Overall, we
373 conclude that the masking glycans added to V3, alone or together with the E64K substitution,
374 have ablated the immunogenicity of the V3 region on the modified BG505 SOSIP trimers. As the
375 autologous Tier-2 NAb titer was not increased, we again find no evidence that V3-dependent
376 Tier-1 NAbs interfere with the overall immunogenicity of SOSIP trimers under these
377 experimental conditions in which the autologous Tier-2 NAb is a strong and probably
378 immundominant response (15, 22, 23). These findings are also generally consistent with the
379 observation that ablating the V3 response to an uncleaved gp140 protein in mice did not divert
380 the antibody response to other epitopes on the same immunogen (34).

381 We consider it possible that "off-target" non-NAb responses could impede the initiation
382 or evolution of bNAbs driven by germline-targeting Env proteins, including appropriately
383 designed SOSIP trimers (11, 24, 25, 35, 36). Decreasing the immunodominance of non-NAb
384 epitopes such as those in V3 may be particularly important in this context. Thus, the human
385 antibody repertoire appears to contain an abundant array of germline precursors for V3 and other
386 non-NAb epitopes, and this subset of naive B-cells is readily activated by Env immunogens;
387 once initiated, the non-NAb lineages evolve to acquire high affinity without undergoing
388 extensive or unusual somatic hypermutation (SHM) events (24, 25, 35). The germline precursors
389 of bNAbs are, however, far rarer, and they are not so easily activated by Env proteins; the initial
390 acquisition of long CDR H3 regions and multiple, and often atypical, SHM events are involved
391 in the bNAb maturation process (24, 25, 35, 37, 38). The higher affinity of germline non-NAb
392 precursors for Env proteins over their bNAb counterparts may give them a selection advantage
393 within the germinal center (GC), based on the outcome of B-cell activation experiments *in vitro*
394 (39). Competition within the GC for the same resources implies that higher-affinity B cell clones
395 may have a selective advantage during the critical early stages of Env protein immunization (25,
396 37, 39, 40). There is, then, a sound rationale for preventing the activation of high-affinity non-
397 NAb precursors to give their lower affinity bNAb counterparts a better chance of becoming
398 activated and then undergoing the SHM process (25, 35, 40, 41). Reducing the immunogenicity
399 of V3 and other non-NAb epitopes on germline-targeting SOSIP trimers may, therefore, be
400 worthwhile, including by using the glycan-masking method that we have described here.

401 MATERIALS AND METHODS

Journal of Virology JVI Journal of Virology

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Accepted Manuscript Posted Online

Journal of Virology

JVI

Journal of Virology

402 **Env construct design.** The BG505 clade A and B41 clade B SOSIP.664 trimers have
403 been described previously (5, 14). Point substitutions in these *env* genes were made using the
404 QuickChange site-directed mutagenesis kit (Agilent Technologies). Briefly, a primer pair was
405 designed for each mutation or two adjacent mutations that could be accommodated in a single
406 primer without compromising its melting temperature. To allow trimers to be used in SPR and
407 ELISA antigenicity assays, either a GSGSGGSG spacer connected to an 8-histidine (His) tag, or
408 a GS spacer followed by a D7324 epitope-tag, were added immediately C-terminal to residue
409 664 in $gp41_{ECTO}$. From hereon, we only refer to the presence of the tags when it is relevant to
410 understanding the experiment. Non-tagged versions of trimers were used for rabbit
411 immunizations.

412 **Env protein expression and trimer purification.** All SOSIP.664 and derivative trimers
413 were produced by transient transfection of 293F cells in serum-free medium, essentially as
414 previously described (5, 13, 14). Env proteins were purified from culture supernatants using
415 affinity columns of bNAbs 2G12 or PGT145, which were made using a CNBr-activated
416 Sepharose 4B resin (GE Healthcare) as previously described (5, 13, 14). In each case, the culture
417 supernatant was flowed through the column at a constant rate of 1 ml/min, the beads were
418 washed with buffer (20 mM Tris-HCl, 500 mM NaCl, pH 8), and the Env proteins were eluted
419 with 3M $MgCl_2$ (pH 7.2). The eluted proteins were immediately buffer-exchanged into trimer-
420 storage buffer (10 mM Tris-HCl, 75mM NaCl, pH 8) and concentrated using a 100-kDa cut-off
421 Vivaspin column (GE Healthcare). A Superdex 200 26/60 size-exclusion chromatography (SEC)
422 column and the same buffer were then used to isolated trimer fractions, which were pooled,
423 concentrated and stored at -80°C.

424 To produce culture supernatants containing unpurified SOSIP.664 Env proteins for pilot-
425 scale assessments by ELISA (see Results), the transfection procedures involved 293T cells and
426 the culture medium contained 5% fetal bovine serum (FBS).

427 **Blue Native (BN)-PAGE.** The affinity purified Env proteins or fractions derived from
428 SEC column runs were analyzed on BN-PAGE gels (Invitrogen), which were stained with
429 Coomassie blue to visualize protein bands (5, 13, 14).

430 **Site-specific N-glycosylation analysis.** Approximately 100 µg of the BG505 and B41
431 SOSIP-E64K.M1M7 trimers were reduced and alkylated, and then digested in solution using
432 trypsin (Promega, Madison, Wisconsin) as described previously (42). Briefly, trypsin was added

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Accepted Manuscript Posted Online
Journal of Virology
JVI
Journal of Virology

433 to trimers at a 1:30 ratio (w/w) and the mixture was incubated for 12 h at 37°C. The resulting
434 glycopeptides were enriched using the ProteoExtract Enrichment Kit according to the
435 manufacturer's instructions (Merck Millipore, Darmstadt, Germany). They were then dried,
436 reconstituted in 1% formic acid and analysed by reversed phase LC-MS/MS using a Fusion
437 Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, California) coupled to an EASY
438 nLC 1200 with a PepMap C18 75 µm × 50 cm column. Data interpretation and quantification
439 procedures were performed using Byonic and Byologic software (Protein Metrics, San Carlos,
440 California) followed by manual assessment (42).

441 **Antibodies.** Monoclonal antibodies (MAbs) were obtained as gifts, or purchased from,
442 the following sources: VRC01 (John Mascola); PG9, PG16, PGT122, PGT128, PGT145,
443 PGT151, b6, b12, (International AIDS Vaccine Initiative); 2G12 (Polymun Scientific); 39F, 17b,
444 19b, 14e (James Robinson).

445 **Surface plasmon resonance (SPR).** SPR assays were carried out as described previously
446 (21). Briefly, purified His-tagged trimers were captured onto CM5 chips (GE Healthcare) by
447 anti-His antibodies. The anti-His antibodies were covalently immobilized in amounts yielding
448 15,000 response units (RU), The trimers were then captured to immobilizations levels of 500 RU
449 (= R_L). Test MAbs were injected at a concentration of 500 nM, with a flow rate was 50 µl/min
450 throughout the association and dissociation phases. Control-channel and 0-analyte subtractions
451 were performed throughout. After each MAb association and dissociation cycle, the antibody-
452 conjugated surface was regenerated by injecting a single pulse of 10 mM glycine (pH 2.0) for 60
453 s at a flow rate of 30 µl/min.

454 **Enzyme-linked immunoabsorption assay (ELISA).** Briefly, purified trimers were
455 captured via their His-tags onto pre-blocked Ni-NTA 96-well plates (Qiagen) by incubation for 2
456 h at 0.5 µg/ml in Tris-buffered saline (TBS) containing 5% FBS. Culture supernatants from
457 transfected 293T cells, which contain 5% FBS, were diluted 1:4 in TBS before addition to the
458 ELISA wells. After the capture stage and washing, test MAbs or related reagents were added for
459 1 h in the same buffer. Bound MAbs were detected using an appropriate horseradish peroxidase
460 (HRP)-conjugated secondary antibody and the 3, 3', 5, 5'-Tetramethylbenzidine (TMB) substrate
461 system with an optical density endpoint at 450 nm (BioRad). The 50% binding values (EC_{50}) for
462 MAb binding were calculated by plotting the nonlinear regression curves using Prism software,
463 version 5.0.

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Accepted Manuscript Posted Online
Journal of Virology
JVI
Journal of Virology

464 **Negative stain electron microscopy.** Purified trimers were prepared for NS-EM analysis
465 by adsorbing 3 μl of sample onto a glow discharged, carbon-coated Cu400 grid. Following
466 blotting, the samples were stained using either 2% (w/v) uranyl formate (3 μl of stain for 60 s) or
467 the Nano-W system (NanoProbes, Inc; 3 μL of stain for 7 s, followed by blotting and a second 3
468 μl of stain for 15 s). Data collection, processing, and particle analysis methods have been
469 previously described (5, 14, 15).

470 **Rabbit immunization procedures and ethics statement.** The various rabbit
471 experiments were all approved and carried out in accordance with protocols provided to the
472 Institutional Animal Care and Use Committee (IACUC) at Covance Research Products (CRP)
473 Inc. (Denver, PA), approval number C0080-16. The rabbits were kept, immunized and bled at
474 Covance in compliance with the Animal Welfare Act and other federal statutes and regulations
475 relating to animals, and adhered to the Guide for the Care and Use of Laboratory Animals,
476 National Research Council, 1996.

477 Rabbit immunizations and blood sampling were carried out under contract at CRP
478 essentially as described previously (3, 15). Female New Zealand White rabbits (5 per group)
479 were immunized intramuscularly three times at weeks 0, 4 and 20, each time with 30 μg of
480 trimers formulated with 75 Units of Iscomatrix™, a saponin-based adjuvant obtained from CSL
481 Ltd. (Parkville, Victoria, Australia) via the International AIDS Vaccine Initiative (43).

482 **Viruses and neutralization assays.** NAbs in rabbit sera were detected and quantified
483 with Env-pseudotyped viruses in the TZM-bl cell assay as described previously (n.b. Tzm-bl
484 cells are derived from the HeLa cell line and supplied by the NIH AIDS Reagents Program,
485 Catalog Number 8129) (44). For additional information on this assay and all supporting
486 protocols see: http://www.hiv.lanl.gov/content/nab-reference-strains/html/home.htm. NAb assays
487 were carried out at either Duke University Medical Center (DUMC)(44) or the Weill Cornell
488 Medical College (WCMC) (5, 14). Env-pseudotyped viruses at DUMC were made with the
489 SG3Δenv backbone (45); at WCMC, the NL-Luc-AM vector was used (46). The viruses bore
490 either full-length BG505.T332N (at WCMC) or cytoplasmic-tail-deleted BG505.T332NΔCT (at
491 DUMC) envelope glycoproteins. When the two variants were directly compared, no differences
492 in neutralization sensitivity were observed.

493 Sera from a subset of time points were also tested at WCMC against three Tier-1A Env-
494 pseudotyped viruses, MN and SF162 (clade B) and MW965.27 (clade C) and four heterologous

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Accepted Manuscript Posted Online

495 Tier-2 viruses; and at DUMC against a panel of six Tier-1 viruses. An amphotropic murine
496 leukemia virus (MLV) Env-pseudotyped virus was used as a negative control at both sites to
497 determine non-specific inhibition of infection by rabbit sera. In each assay, all serum dilutions
498 were tested in duplicate. In some experiments, a Cys-cyclized peptide based on the BG505 V3
499 sequence (CTRPNNNTRKSIRIGPGQAFYATGDIIGDIRQAHC) was used as a soluble
500 competitor for NAbs against Tier-1 viruses. The peptide (10 μg/ml) was incubated with serial
501 dilutions of the test sera for 30 min prior to addition of virus and cells according to the standard
502 TZM-bl cell neutralization assay protocol. The negative control peptide was a scrambled version
503 of the MN V3-sequence, HTGKYTYPTNIAIRGRGNKFRNKKI.
504 Neutralization was defined as the reduction (%) of the infectivity obtained in the presence
505 of serum. The serum dilution factors reducing infectivity by 50% were calculated from nonlinear
506 regression fits of a sigmoid function (with maximum constrained to ≤100% and minimum
507 unconstrained) to the normalized inhibition data by the use of Prism software (Graphpad). For
508 convenience, the resulting reciprocal titers, i.e., inhibitory dilution factors (ID_{50}), are henceforth
509 referred to simply as "titers". The NAb titers in the groups of rabbits were compared by two-
510 tailed Mann-Whitney U tests (Prism, GraphPad).
511

512

513 **ACKNOWLEDGEMENTS**

514 The non-animal research was supported by NIH grants P01 AI110657, R37 AI36082, R21
515 AI112389 and the NIH-NIAID NHP Humoral Immunology Laboratory Contract
516 HHSN27201100016C. The rabbit immunization experiment and the NS-EM imaging research
517 were supported by the Bill and Melinda Gates Foundation Collaboration for AIDS Vaccine
518 Development grants OPP1132237 and OPP1115782, respectively. RWS is a recipient of a Vidi
519 grant from the Netherlands Organization for Scientific Research (NWO) and a Starting
520 Investigator Grant from the European Research Council (ERC-StG-2011–280829-SHEV). MC is
521 supported by the International AIDS Vaccine Initiative. We thank Heather Arendt (IAVI) for
522 facilitating the provision of Iscomatrix adjuvant. We are grateful to Pavel Pugach for helpful
523 advice.

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Accepted Manuscript Posted Online

524 **FIGURE LEGENDS**

525 FIG 1. Comparative antigenicity of BG505 trimers assessed by ELISA. D7324-tagged BG505
526 SOSIP.664, -M7 and -M1M7 trimers were purified via the 2G12/SEC method and assessed for
527 antibody binding by capture ELISA.

528

529 FIG 2. Glycan masking of V3 epitopes on BG505 and B41 SOSIP trimers. (**A**) SEC profiles of
530 2G12-purified or PGT145-purified, D7324-tagged BG505 or B41 SOSIP.664 and SOSIP-
531 E64K.M1M7 Env proteins. The dotted vertical lines indicate the trimer peaks. The yields of
532 trimers purified by the various methods are tabulated below the profiles. (**B**) Site-specific glycan
533 analysis of the B41 SOSIP-E64K.M1M7 trimer. The glycan profiles for the corresponding
534 BG505 trimer were similar (not shown). The bars show the distribution and relative abundance
535 of N-linked glycans displayed by type; oligomannose in green and complex/hybrid in pink.
536 Glycans are categorized as oligomannose series (M5 to M9; $Man_5GlcNAc_2$ to $Man_9GlcNAc_2$),
537 hybrids (H), and fucosylated hybrids (FH), and also by the number of branching antennae (A) of
538 complex-type glycans. An, number (n) of antennae; B, bisected GlcNAc; F, presence of a core
539 fucose.

540

541 FIG 3. The V3 glycan-masked BG505 and B41 SOSIP trimers retain a native conformation as
542 assessed by bnAb binding using SPR and ELISA. (**A**) The indicated PGT145/SEC-purified, His-
543 tagged SOSIP.664, SOSIP.664-M1M7 and SOSIP.E64K-M1M7 trimers were analyzed by SPR
544 (top, BG505; bottom, B41). The test antibodies were used at 500 nM. The response difference
545 (RU) is given on the y-axis as a function of time (s) on the x-axis. (**B)** ELISA analysis of the
546 same purified SOSIP.664 and SOSIP.664-E64K.M1M7 trimers used in panel-A to assess bNAb
547 and non-NAb reactivity, with sCD4 also present when noted. The amount of each B41 and
548 BG505 trimer comparator tested was adjusted to give equal binding of 2G12 bNAb, for
549 normalization purposes. (**C**) The indicated SOSIP.664-M1M7 (2G12/SEC-purified) and
550 SOSIP.664-E64K.M1M7 trimers (2G12/SEC or PGT145/SEC-purified), all D7324-tagged, were
551 visualized by NS-EM. The percentages of the total trimers with native-like (NL) conformation
552 are recorded below each collage. (**D**) Model of a single, wild type BG505 gp120 protomer. The
553 sites of glycan addition at residues S306 and G314 are colored in pale blue and
554 magenta, respectively, with the geometrically favorable directions of the mutated asparagine side

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

JVI Journal of Virology

Accepted Manuscript Posted Online

555 chains represented by arrows in the same colors. (**E**) Model of the BG505 SOSIP.664-
556 E64K.M1M7 trimer. The estimated positions of inserted N306 (M1) and N314 (M7) glycans are
557 again colored pale blue and magenta, respectively, with no indication of their detailed
558 composition. In both panels D and E, the wild-type glycans are colored green, with selected
559 ones labeled, and both models are based on PDB 5FYL.
560
561 FIG 4. NAb responses to BG505 SOSIP.664 and SOSIP.664-E64K.M1M7 trimers in rabbits. (**A**)
562 NAb titers (ID_{50} values on the y axes) of the two groups of rabbits are plotted for each test virus,
563 which all have the Tier-1 neutralization phenotype except for the autologous BG505.T332N
564 virus (Tier-2). Open triangles, SOSIP.664; closed circles, SOSIP.664-E64K.M1M7. (**B**) The
565 NAb titers for the same groups of rabbits against the indicated Tier-1 viruses were measured
566 after pre-incubation of the sera with or without the indicated cyclized V3 peptide. In both plots,
567 each data point represents an individual rabbit, with the median value for each group of 5
568 indicated by the horizontal bar. Significant titer differences are indicated by the p-values. (**C**)
569 NAb titers for the autologous Tier-2 virus BG505.T332N and the Tier-1 viruses MN and
570 MW965.26 are shown for the SOSIP.664 and SOSIP.664-E64K.M1M7 comparator groups
571 describe in the present study (left panels) and for the SOSIP.664 and SOSIP.v4.1 comparator
572 groups described previously (right panels) (15). The data in panels A and B were generated at
573 DUMC, and in panel C at WCMC. There was no overlap between the titer values in the two
574 immunogen groups, and the p values from the non-parametric Man-Whitney U test were
575 identical and minimal (p=0.0079).
576
577
578
579
580
581
582
583
584
585

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

JVI Journal of Virology

Journal of Virology

586

587

588 **TABLES**

589 Table 1. Design of BG505 SOSIP.664 V3-glycan mutants

V3 Position	Designation	Amino acid substitution	V3 sequence
	HxBc2		NCTRPNNNTRKRIRIQRGPGRAFVTIG.KIGNMRQAHCNIS
	BG505-WT		NCTRPNNNTRKSIRI..GPGQAFYATGDIIGDIRQAHCNVS
306	M1	S306N R308T	NCTRPNNNTRKNITI..GPGQAFYATGDIIGDIRQAHCNVS
307	M2	I307N I309T	NCTRPNNNTRKSNRT..GPGQAFYATGDIIGDIRQAHCNVS
308	M3	R308N G312T	NCTRPNNNTRKSINI..TPGQAFYATGDIIGDIRQAHCNVS
309	M4	I309N P313T	NCTRPNNNTRKSIRN..GTGQAFYATGDIIGDIRQAHCNVS
312	M5	G312N P313G G314T	NCTRPNNNTRKSIRI..NGTQAFYATGDIIGDIRQAHCNVS
313	M6	P313N Q315T	NCTRPNNNTRKSIRI..GNGTAFYATGDIIGDIRQAHCNVS
314	M7	G314N A316T	NCTRPNNNTRKSIRI..GPNQTFYATGDIIGDIRQAHCNVS
315	M8	Q315N F317T	NCTRPNNNTRKSIRI..GPGNATYATGDIIGDIRQAHCNVS
316	M9	A316N Y318T	NCTRPNNNTRKSIRI..GPGQNFTATGDIIGDIRQAHCNVS
317	M10	F317N A319T	NCTRPNNNTRKSIRI..GPGQANYTTGDIIGDIRQAHCNVS
318	M11	Y318N	NCTRPNNNTRKSIRI..GPGQAFNATGDIIGDIRQAHCNVS
319	M12	A319N G321T	NCTRPNNNTRKSIRI..GPGQAFYNTTDIIGDIRQAHCNVS
320	M13	T320N D321aT	NCTRPNNNTRKSIRI..GPGQAFYANGTIIGDIRQAHCNVS
321	M14	G321N I322T	NCTRPNNNTRKSIRI..GPGQAFYATNDTIGDIRQAHCNVS
321a	M15	D321aN I323T	NCTRPNNNTRKSIRI..GPGQAFYATGNITGDIRQAHCNVS
322	M16	I322N G324T	NCTRPNNNTRKSIRI..GPGQAFYATGDNITDIRQAHCNVS
323	M17	I323N D325T	NCTRPNNNTRKSIRI..GPGQAFYATGDINGTIRQAHCNVS
324	M18	G324N I326T	NCTRPNNNTRKSIRI..GPGQAFYATGDIINDTRQAHCNVS
325	M19	D325N R327T	NCTRPNNNTRKSIRI..GPGQAFYATGDIIGNITQAHCNVS
326	M20	I326N Q328T	NCTRPNNNTRKSIRI..GPGQAFYATGDIIGDNRTAHCNVS
327	M21	R327N A329T	NCTRPNNNTRKSIRI..GPGQAFYATGDIIGDINQTHCNVS
306, 314	M1M7	S306N R308T G314N A316T	NCTRPNNNTRKNITI..GPNQTFYATGDIIGDIRQAHCNVS
308, 314	M3M7	R308N G312T G314N A316T	NCTRPNNNTRKSINI..TPNQTFYATGDIIGDIRQAHCNVS
313, 314	M6M7	P313N G314N Q315T A316T	NCTRPNNNTRKSIRI..GNNTTFYATGDIIGDIRQAHCNVS

590 The designations of the glycan mutants at V3 residues from 306 to 327 are outlined in the first
591 two columns. Residues were numbered using the HxBc2 system. Each construct was based on
592 the BG505 SOSIP.664-D7324 background (designated BG505-WT). The specific substitutions
593 in each mutant are listed in the third column, and the complete V3 sequence of the mutants, as
594 well as wild-type BG505-WT and HxBc2, are in the fourth column. The introduced glycan sites
595 are colored blue and underlined; the naturally present glycan sites at 295 and 301 are highlighted
596 in green and underlined; the non-V3 but proximal glycan site at 332 is shown in red and
597 underlined. Each of the introduced *N*-glycan motifs was of the NXT type, not NXS, to enhance
598 the probability of glycan attachment (47-49). For construct M5, the P313G substitution was also
599 made to increase the probability of glycan addition at position-312. For construct M15, inserting
600 one additional residue at BG505 position-321 lead to this residue being numbered 321a.

Accepted Manuscript Posted Online
Journal of Virology
JVI
Journal of Virology
Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Accepted Manuscript Posted Online

Journal of Virology

JVI

Table 2. Antigenicity of BG505 SOSIP.664 V3-glycan mutants

V3 Glycan Position	Mutant designation	Antibody								
		bNAbs							Non-NAbs (v3)	
		2G12	PG9	PG16	PGT145	PGT151	PGT121	VRC01	14e	19b
	WT	+++	++	++	+++	++	++	++	+++	+++
	WT*	+++	+++	+++	+++	+++	+++	+++	+++	+++
306	M1	+++	+++	++	++	++	++	+++	+++	+
307	M2	+++	–	–	–	++	+	+++	++	–
308	M3	+++	++	+	+	++	++	+++	+++	–
309	M4	+++	–	–	–	++	++	+++	–	–
312	M5	+++	++	–	–	++	++	+++	–	–
313	M6	+++	++	–	–	++	++	+++	–	–
314	M7	+++	+++	++	++	+++	++	+++	–	++
314	M7*	+++	+++	+++	+++	+++	+++	+++	–	++
315	M8	+++	–	–	–	++	++	+++	–	–
316	M9	+++	+	–	–	++	++	+++	–	–
317	M10	+++	–	–	–	++	++	+++	–	–
318	M11	+++	–	–	–	++	+	++	–	–
319	M12	+++	–	–	–	++	+	++	–	–
320	M13	+++	–	–	–	++	–	++	+++	+++
321	M14	+++	–	–	–	++	–	++	+++	+++
321a	M15	+++	+++	–	+	++	+	+++	+++	+++
322	M16	+++	–	–	–	++	–	++	+++	+++
323	M17	+++	+++	++	++	++	+	+++	+++	+++
324	M18	+++	–	–	–	+	–	++	+++	+++
325	M19	+++	+++	+	++	++	–	+++	+++	+++
326	M20	+++	–	–	–	++	–	+++	+++	+++
327	M21	+++	+++	+	++	++	+	+	+++	+++
306, 314	M1M7	+++	+++	++	++	+++	++	+++	–	+
306, 314	M1M7*	+++	+++	+++	+++	+++	+++	+++	–	–
308, 314	M3M7	+++	+++	–	+	+	++	++	++	+
313, 314	M6M7	+++	+++	+	+	++	++	++	++	+++

The various mutants outlined in the first two columns contain one, or sometimes two, glycan sites inserted into various positions in V3 from residue 306 to 327 (see Table 1 for additional details). Unless indicated by *, the antigenicity data were derived by D7324-capture ELISA using unpurified culture supernatants from 293T cell transient transfections. * D7324-tagged trimers based on the M7 and M1M7 constructs were also purified by the 2G12/SEC method, with the data (see Fig. 1) summarized here for comparison. For each antibody in the test panel, the magnitude of reactivity with the indicated Env proteins is recorded semi-quantitatively on a scale from – (no binding, red) to +++ (strong binding, in green).

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Journal of Virology

Accepted Manuscript Posted Online

615 Table 3. Neutralization of variant BG505 and MG505 viruses by rabbit sera

Rabbit number	Trimer immunogen	BG505.T332N						MG505		
		WT (%)	Q130N (%)	S241N (%)	S241K (%)	P291T (%)	S241N + P291T (%)	MG505.A2 (%)	MG505.A2 K241S (%)	MG505.H3 (%)
2115	SOSIP.664	100	98 ± 19	99 ±10	92 ± 7	80 ± 3	70 ± 22	<10	78 ± 4	<10
2116	SOSIP.664	100	140 ± 38	<10	<10	<10	<10	<10	128 ± 16	<10
2117	SOSIP.664	100	91 ± 12	103 ± 0	105 ± 2	97 ± 4	87 ± 17	<10	88 ± 11	<10
2118	SOSIP.664									
2119	SOSIP.664	100	77 ± 21	86 ± 13	85 ± 2	58 ± 5	71 ± 17	<10	90 ± 4	<10
2110	E64K.M1M7	100	81 ± 18	101 ± 3	103 ± 4	82 ± 18	76 ± 1.5	<10	81 ± 15	<10
2111	E64K.M1M7	100	18± 65	<10	<10	<10	<10	<10	107 ± 5	<10
2112	E64K.M1M7									
2113	E64K.M1M7	100	63 ± 20	69 ± 10	46 ± 1	45 ± 14	46 ± 12	<10	103 ± 0	<10
2114	E64K.M1M7									

616

617 The rabbit sera tested were from week-22, which is 2-weeks after the third and final
618 immunization with the BG505 SOSIP.664 or SOSIP.664-E64K.M1M7 trimer. The Q130N,
619 S241N and P291T changes introduce N-linked glycans at BG505.T332N virus positions 130,
620 241 and 289, respectively, in Env. The MG505.A2 and MG505.H3 viruses are different clones of
621 the maternal MG505 isolate. The K241S substitution introduces a sequence change within the
622 241/289 glycan hole of the MG505.A2 clone. The values recorded for various mutant viruses are
623 the percentage neutralization at a dilution of 1/50, relative to the BG505.T332N parental virus
624 (labeled WT and defined as 100%), and are the averages of 2 or 3 replicates ± s.e.m. Red and
625 yellow boxes indicate strong (>75%) and partial (25-75%) loss of neutralizing activity compared
626 to the wild type virus, respectively, with green boxes indicating no meaningful change. For
627 rabbits #2118, #2112 and #2114, the titers against the WT virus were not high enough to allow
628 mapping of the epitope (grey boxes).

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Journal of Virology JVI Journal of Virology

Accepted Manuscript Posted Online

629 **REFERENCES**

630 1. **Julien JP, Lee JH, Ozorowski G, Hua Y, Torrents de la Pena A, de Taeye SW,**
631 **Nieusma T, Cupo A, Yasmeen A, Golabek M, Pugach P, Klasse PJ, Moore JP,**
632 **Sanders RW, Ward AB, Wilson IA.** 2015. Design and structure of two HIV-1 clade C
633 SOSIP.664 trimers that increase the arsenal of native-like Env immunogens. Proc Natl
634 Acad Sci U S A **112:**11947-11952.
635 2. **Julien JP, Cupo A, Sok D, Stanfield RL, Lyumkis D, Deller MC, Klasse PJ, Burton**
636 **DR, Sanders RW, Moore JP, Ward AB, Wilson IA.** 2013. Crystal structure of a
637 soluble cleaved HIV-1 envelope trimer. Science **342:**1477-1483.
638 3. **Klasse PJ, LaBranche CC, Ketas TJ, Ozorowski G, Cupo A, Pugach P, Ringe RP,**
639 **Golabek M, van Gils MJ, Guttman M, Lee KK, Wilson IA, Butera ST, Ward AB,**
640 **Montefiori DC, Sanders RW, Moore JP.** 2016. Sequential and simultaneous
641 immunization of rabbits with HIV-1 envelope glycoprotein SOSIP.664 trimers from
642 clades A, B and C. PLoS Pathog **12:**e1005864.
643 4. **McGuire AT, Hoot S, Dreyer AM, Lippy A, Stuart A, Cohen KW, Jardine J, Menis**
644 **S, Scheid JF, West AP, Schief WR, Stamatatos L.** 2013. Engineering HIV envelope
645 protein to activate germline B cell receptors of broadly neutralizing anti-CD4 binding site
646 antibodies. J Exp Med **210:**655-663.
647 5. **Sanders RW, Derking R, Cupo A, Julien JP, Yasmeen A, de Val N, Kim HJ,**
648 **Blattner C, de la Pena AT, Korzun J, Golabek M, de Los Reyes K, Ketas TJ, van**
649 **Gils MJ, King CR, Wilson IA, Ward AB, Klasse PJ, Moore JP.** 2013. A next-
650 generation cleaved, soluble HIV-1 Env trimer, BG505 SOSIP.664 gp140, expresses
651 multiple epitopes for broadly neutralizing but not non-neutralizing antibodies. PLoS
652 Pathog **9:**e1003618.
653 6. **Sanders RW, van Gils MJ, Derking R, Sok D, Ketas TJ, Burger JA, Ozorowski G,**
654 **Cupo A, Simonich C, Goo L, Arendt H, Kim HJ, Lee JH, Pugach P, Williams M,**
655 **Debnath G, Moldt B, van Breemen MJ, Isik G, Medina-Ramirez M, Back JW, Koff**
656 **WC, Julien JP, Rakasz EG, Seaman MS, Guttman M, Lee KK, Klasse PJ,**
657 **LaBranche C, Schief WR, Wilson IA, Overbaugh J, Burton DR, Ward AB,**
658 **Montefiori DC, Dean H, Moore JP.** 2015. HIV-1 neutralizing antibodies induced by
659 native-like envelope trimers. Science **349:**aac4223.
660 7. **Crooks ET, Tong T, Chakrabarti B, Narayan K, Georgiev IS, Menis S, Huang X,**
661 **Kulp D, Osawa K, Muranaka J, Stewart-Jones G, Destefano J, O'Dell S, LaBranche**
662 **C, Robinson JE, Montefiori DC, McKee K, Du SX, Doria-Rose N, Kwong PD,**
663 **Mascola JR, Zhu P, Schief WR, Wyatt RT, Whalen RG, Binley JM.** 2015. Vaccine-
664 elicited tier 2 HIV-1 neutralizing antibodies bind to quaternary epitopes involving
665 glycan-deficient patches proximal to the CD4 binding site. PLoS Pathog **11:**e1004932.
666 8. **Tong T, Crooks ET, Osawa K, Binley JM.** 2012. HIV-1 virus-like particles bearing
667 pure env trimers expose neutralizing epitopes but occlude nonneutralizing epitopes. J
668 Virol **86:**3574-3587.
669 9. **Pancera M, Zhou T, Druz A, Georgiev IS, Soto C, Gorman J, Huang J, Acharya P,**
670 **Chuang GY, Ofek G, Stewart-Jones GB, Stuckey J, Bailer RT, Joyce MG, Louder**
671 **MK, Tumba N, Yang Y, Zhang B, Cohen MS, Haynes BF, Mascola JR, Morris L,**
672 **Munro JB, Blanchard SC, Mothes W, Connors M, Kwong PD.** 2014. Structure and
673 immune recognition of trimeric pre-fusion HIV-1 Env. Nature **514:**455-461.

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Accepted Manuscript Posted Online

674 10. **Chung NP, Matthews K, Kim HJ, Ketas TJ, Golabek M, de Los Reyes K, Korzun J,**
675 **Yasmeen A, Sanders RW, Klasse PJ, Wilson IA, Ward AB, Marozsan AJ, Moore**
676 **JP, Cupo A.** 2014. Stable 293 T and CHO cell lines expressing cleaved, stable HIV-1
677 envelope glycoprotein trimers for structural and vaccine studies. Retrovirology **11:**33.
678 11. **Sanders RW, Moore JP.** 2017. Native-like Env trimers as a platform for HIV-1 vaccine
679 design. Immunol Rev **275:**161-182.
680 12. **Ringe RP, Ozorowski G, Yasmeen A, Cupo A, Cruz Portillo VM, Pugach P,**
681 **Golabek M, Rantalainen K, Holden LG, Cottrell CA, Wilson IA, Sanders RW,**
682 **Ward AB, Klasse PJ, Moore JP.** 2017. Improving the expression and purification of
683 soluble, recombinant native-like HIV-1 envelope glycoprotein trimers by targeted
684 sequence changes. J Virol doi: 10.1128/JVI.00264-17.
685 13. **Ringe RP, Yasmeen A, Ozorowski G, Go EP, Pritchard LK, Guttman M, Ketas TA,**
686 **Cottrell CA, Wilson IA, Sanders RW, Cupo A, Crispin M, Lee KK, Desaire H,**
687 **Ward AB, Klasse PJ, Moore JP.** 2015. Influences on the design and purification of
688 soluble, recombinant native-like HIV-1 envelope glycoprotein trimers. J Virol **89:**12189-
689 12210.
690 14. **Pugach P, Ozorowski G, Cupo A, Ringe R, Yasmeen A, de Val N, Derking R, Kim**
691 **HJ, Korzun J, Golabek M, de Los Reyes K, Ketas TJ, Julien JP, Burton DR, Wilson**
692 **IA, Sanders RW, Klasse PJ, Ward AB, Moore JP.** 2015. A native-like SOSIP.664
693 trimer based on an HIV-1 subtype B env gene. J Virol **89:**3380-3395.
694 15. **de Taeye SW, Ozorowski G, Torrents de la Pena A, Guttman M, Julien JP, van den**
695 **Kerkhof TL, Burger JA, Pritchard LK, Pugach P, Yasmeen A, Crampton J, Hu J,**
696 **Bontjer I, Torres JL, Arendt H, DeStefano J, Koff WC, Schuitemaker H, Eggink D,**
697 **Berkhout B, Dean H, LaBranche C, Crotty S, Crispin M, Montefiori DC, Klasse PJ,**
698 **Lee KK, Moore JP, Wilson IA, Ward AB, Sanders RW.** 2015. Immunogenicity of
699 stabilized HIV-1 envelope trimers with reduced exposure of non-neutralizing epitopes.
700 Cell **163:**1702-1715.
701 16. **Do Kwon Y, Pancera M, Acharya P, Georgiev IS, Crooks ET, Gorman J, Joyce MG,**
702 **Guttman M, Ma X, Narpala S, Soto C, Terry DS, Yang Y, Zhou T, Ahlsen G, Bailer**
703 **RT, Chambers M, Chuang GY, Doria-Rose NA, Druz A, Hallen MA, Harned A,**
704 **Kirys T, Louder MK, O'Dell S, Ofek G, Osawa K, Prabhakaran M, Sastry M,**
705 **Stewart-Jones GB, Stuckey J, Thomas PV, Tittley T, Williams C, Zhang B, Zhao H,**
706 **Zhou Z, Donald BR, Lee LK, Zolla-Pazner S, Baxa U, Schon A, Freire E, Shapiro L,**
707 **Lee KK, Arthos J, Munro JB, Blanchard SC, Mothes W, Binley JM, McDermott**
708 **AB, Mascola JR, Kwong PD.** 2015. Crystal structure, conformational fixation and entry-
709 related interactions of mature ligand-free HIV-1 Env. Nat Struct Mol Biol **22:** 522-531.
710 17. **Stewart-Jones GB, Soto C, Lemmin T, Chuang GY, Druz A, Kong R, Thomas PV,**
711 **Wagh K, Zhou T, Behrens AJ, Bylund T, Choi CW, Davison JR, Georgiev IS, Joyce**
712 **MG, Kwon YD, Pancera M, Taft J, Yang Y, Zhang B, Shivatare SS, Shivatare VS,**
713 **Lee CC, Wu CY, Bewley CA, Burton DR, Koff WC, Connors M, Crispin M, Baxa**
714 **U, Korber BT, Wong CH, Mascola JR, Kwong PD.** 2016. Trimeric HIV-1-Env
715 structures define glycan shields from clades A, B, and G. Cell **165:**813-826.
716 18. **Harris A, Borgnia MJ, Shi D, Bartesaghi A, He H, Pejchal R, Kang YK, Depetris R,**
717 **Marozsan AJ, Sanders RW, Klasse PJ, Milne JL, Wilson IA, Olson WC, Moore JP,**
718 **Subramaniam S.** 2011. Trimeric HIV-1 glycoprotein gp140 immunogens and native

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Accepted Manuscript Posted Online

719 HIV-1 envelope glycoproteins display the same closed and open quaternary molecular
720 architectures. Proc Natl Acad Sci U S A **108:**11440-11445.

721 19. **Lee JH, Ozorowski G, Ward AB.** 2016. Cryo-EM structure of a native, fully
722 glycosylated, cleaved HIV-1 envelope trimer. Science **351:**1043-1048.

723 20. **Ward AB, Wilson IA.** 2017. The HIV-1 envelope glycoprotein structure: nailing down a
724 moving target. Immunol Rev **275:**21-32.

725 21. **Yasmeen A, Ringe R, Derking R, Cupo A, Julien JP, Burton DR, Ward AB, Wilson**
726 **IA, Sanders RW, Moore JP, Klasse PJ.** 2014. Differential binding of neutralizing and
727 non-neutralizing antibodies to native-like soluble HIV-1 Env trimers, uncleaved Env
728 proteins, and monomeric subunits. Retrovirology **11:**41.

729 22. **Cheng C, Pancera M, Bossert A, Schmidt SD, Chen RE, Chen X, Druz A, Narpala**
730 **S, Doria-Rose NA, McDermott AB, Kwong PD, Mascola JR.** 2015. Immunogenicity
731 of a prefusion HIV-1 envelope trimer in complex with a quaternary-structure-specific
732 antibody. J Virol **90:**2740-2755.

733 23. **Chuang GY, Geng H, Pancera M, Xu K, Cheng C, Acharya P, Chambers M, Druz**
734 **A, Tsybovsky Y, Wanninger TG, Yang Y, Doria-Rose NA, Georgiev IS, Gorman J,**
735 **Joyce MG, O'Dell S, Zhou T, McDermott AB, Mascola JR, Kwong PD.** 2017.
736 Structure-based design of a soluble prefusion-closed HIV-1-Env trimer with reduced
737 CD4 affinity and improved immunogenicity. J Virol doi:10.1128/JVI.02268-16.

738 24. **Havenar-Daughton C, Lee JH, Crotty S.** 2017. Tfh cells and HIV bnAbs, an
739 immunodominance model of the HIV neutralizing antibody generation problem.
740 Immunol Rev **275:**49-61.

741 25. **McGuire AT, Dreyer AM, Carbonetti S, Lippy A, Glenn J, Scheid JF, Mouquet H,**
742 **Stamatatos L.** 2014. Antigen modification regulates competition of broad and narrow
743 neutralizing HIV antibodies. Science **346:**1380-1383.

744 26. **Garrity RR, Rimmelzwaan G, Minassian A, Tsai WP, Lin G, de Jong JJ, Goudsmit**
745 **J, Nara PL.** 1997. Refocusing neutralizing antibody response by targeted dampening of
746 an immunodominant epitope. J Immunol **159:**279-289.

747 27. **Eggink D, Goff PH, Palese P.** 2014. Guiding the immune response against influenza
748 virus hemagglutinin toward the conserved stalk domain by hyperglycosylation of the
749 globular head domain. J Virol **88:**699-704.

750 28. **Sliepen K, van Montfort T, Melchers M, Isik G, Sanders RW.** 2015. Immunosilencing
751 a highly immunogenic protein trimerization domain. J Biol Chem **290:**7436-7442.

752 29. **Behrens AJ, Harvey DJ, Milne E, Cupo A, Kumar A, Zitzmann N, Struwe WB,**
753 **Moore JP, Crispin M.** 2017. Molecular architecture of the cleavage-dependent mannose
754 patch on a soluble HIV-1 envelope glycoprotein trimer. J Virol **91:** e01894-16. doi:
755 10.1128/JVI.01894-16.

756 30. **Behrens AJ, Crispin M.** 2017. Structural principles controlling HIV envelope
757 glycosylation. Curr Opin Struct Biol **44:**125-133.

758 31. **Munro JB, Gorman J, Ma X, Zhou Z, Arthos J, Burton DR, Koff WC, Courter JR,**
759 **Smith AB, 3rd, Kwong PD, Blanchard SC, Mothes W.** 2014. Conformational
760 dynamics of single HIV-1 envelope trimers on the surface of native virions. Science
761 **346:**759-763.

762 32. **Lee JH, Andrabi R, Su CY, Yasmeen A, Julien JP, Kong L, Wu NC, McBride R,**
763 **Sok D, Pauthner M, Cottrell CA, Nieusma T, Blattner C, Paulson JC, Klasse PJ,**
764 **Wilson IA, Burton DR, Ward AB.** 2017. A broadly neutralizing antibody targets the

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Journal of Virology

JVI

Journal of Virology

Accepted Manuscript Posted Online

765 dynamic HIV envelope trimer apex via a long, rigidified, and anionic beta-hairpin
766 structure. Immunity **46:**690-702.

767 33. **Hu JK, Crampton JC, Cupo A, Ketas T, van Gils MJ, Sliepen K, de Taeye SW, Sok**
768 **D, Ozorowski G, Deresa I, Stanfield R, Ward AB, Burton DR, Klasse PJ, Sanders**
769 **RW, Moore JP, Crotty S.** 2015. Murine antibody responses to cleaved soluble HIV-1
770 envelope trimers are highly restricted in specificity. J Virol **89:**10383-10398.

771 34. **Forsell MN, Soldemo M, Dosenovic P, Wyatt RT, Karlsson MC, Karlsson Hedestam**
772 **GB.** 2013. Independent expansion of epitope-specific plasma cell responses upon HIV-1
773 envelope glycoprotein immunization. J Immunol **191:**44-51.

774 35. **Stamatatos L, Pancera M, McGuire AT.** 2017. Germline-targeting immunogens.
775 Immunol Rev **275:**203-216.

776 36. **Medina-Ramirez M, Garces F, Escolano A, Skog P, Del Moral-Sanchez I, Dosenovic**
777 **P, Hua Y, McGuire AT, Gitlin AD, Freund NT, Yasmeen A, Behrens AJ, Ozorowski**
778 **G, de Taeye SW, van den Kerkhof TL, Sliepen K, Blane T, Kootstra NA, van**
779 **Breemen MJ, Pritchard LK, Stanfield RL, Crispin M, Ward AB, Stamatatos L,**
780 **Klasse PJ, Moore JP, Nemazee D, Nussenzweig MC, Wilson IA, Sanders RW.** 2017.
781 Design and crystal structure of a native-like HIV-1 envelope trimer that engages multiple
782 broadly neutralizing antibody precursors *in vivo*. J Exp Med **[In Press]**.

783 37. **Scharf L, West AP, Sievers SA, Chen C, Jiang S, Gao H, Gray MD, McGuire AT,**
784 **Scheid JF, Nussenzweig MC, Stamatatos L, Bjorkman PJ.** 2016. Structural basis for
785 germline antibody recognition of HIV-1 immunogens. Elife **5:** e13783.

786 38. **Escolano A, Dosenovic P, Nussenzweig MC.** 2017. Progress toward active or passive
787 HIV-1 vaccination. J Exp Med **214:**3-16.

788 39. **Victora GD, Nussenzweig MC.** 2012. Germinal centers. Annu Rev Immunol **30:**429-
789 457.

790 40. **Zhang Y, Meyer-Hermann M, George LA, Figge MT, Khan M, Goodall M, Young**
791 **SP, Reynolds A, Falciani F, Waisman A, Notley CA, Ehrenstein MR, Kosco-Vilbois**
792 **M, Toellner KM.** 2013. Germinal center B cells govern their own fate via antibody
793 feedback. J Exp Med **210:**457-464.

794 41. **McGuire AT, Gray MD, Dosenovic P, Gitlin AD, Freund NT, Petersen J, Correnti**
795 **C, Johnsen W, Kegel R, Stuart AB, Glenn J, Seaman MS, Schief WR, Strong RK,**
796 **Nussenzweig MC, Stamatatos L.** 2016. Specifically modified Env immunogens activate
797 B-cell precursors of broadly neutralizing HIV-1 antibodies in transgenic mice. Nat
798 Commun **7:**10618.

799 42. **Behrens AJ, Vasiljevic S, Pritchard LK, Harvey DJ, Andev RS, Krumm SA, Struwe**
800 **WB, Cupo A, Kumar A, Zitzmann N, Seabright GE, Kramer HB, Spencer DI, Royle**
801 **L, Lee JH, Klasse PJ, Burton DR, Wilson IA, Ward AB, Sanders RW, Moore JP,**
802 **Doores KJ, Crispin M.** 2016. Composition and antigenic effects of individual glycan
803 sites of a trimeric HIV-1 envelope glycoprotein. Cell Rep **14:**2695-2706.

804 43. **Chung KY, Coyle EM, Jani D, King LR, Bhardwaj R, Fries L, Smith G, Glenn G,**
805 **Golding H, Khurana S.** 2015. ISCOMATRIX adjuvant promotes epitope spreading and
806 antibody affinity maturation of influenza A H7N9 virus like particle vaccine that
807 correlate with virus neutralization in humans. Vaccine **33:**3953-3962.

808 44. **Montefiori DC.** 2009. Measuring HIV neutralization in a luciferase reporter gene assay.
809 Methods Mol Biol **485:**395-405.

Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Accepted Manuscript Posted Online

810 45. **Wei X, Decker JM, Liu H, Zhang Z, Arani RB, Kilby JM, Saag MS, Wu X, Shaw**
811 **GM, Kappes JC.** 2002. Emergence of resistant human immunodeficiency virus type 1 in
812 patients receiving fusion inhibitor (T-20) monotherapy. Antimicrob Agents Chemother
813 **46:**1896-1905.
814 46. **Marozsan AJ, Kuhmann SE, Morgan T, Herrera C, Rivera-Troche E, Xu S,**
815 **Baroudy BM, Strizki J, Moore JP.** 2005. Generation and properties of a human
816 immunodeficiency virus type 1 isolate resistant to the small molecule CCR5 inhibitor,
817 SCH-417690 (SCH-D). Virology **338:**182-199.
818 47. **Kaplan HA, Welply JK, Lennarz WJ.** 1987. Oligosaccharyl transferase: the central
819 enzyme in the pathway of glycoprotein assembly. Biochim Biophys Acta **906:**161-173.
820 48. **van den Kerkhof TL, Feenstra KA, Euler Z, van Gils MJ, Rijsdijk LW, Boeser-**
821 **Nunnink BD, Heringa J, Schuitemaker H, Sanders RW.** 2013. HIV-1 envelope
822 glycoprotein signatures that correlate with the development of cross-reactive neutralizing
823 activity. Retrovirology **10:**102.
824 49. **Gavel Y, von Heijne G.** 1990. Sequence differences between glycosylated and non-
825 glycosylated Asn-X-Thr/Ser acceptor sites: implications for protein engineering. Protein
826 Eng **3:**433-442.
827

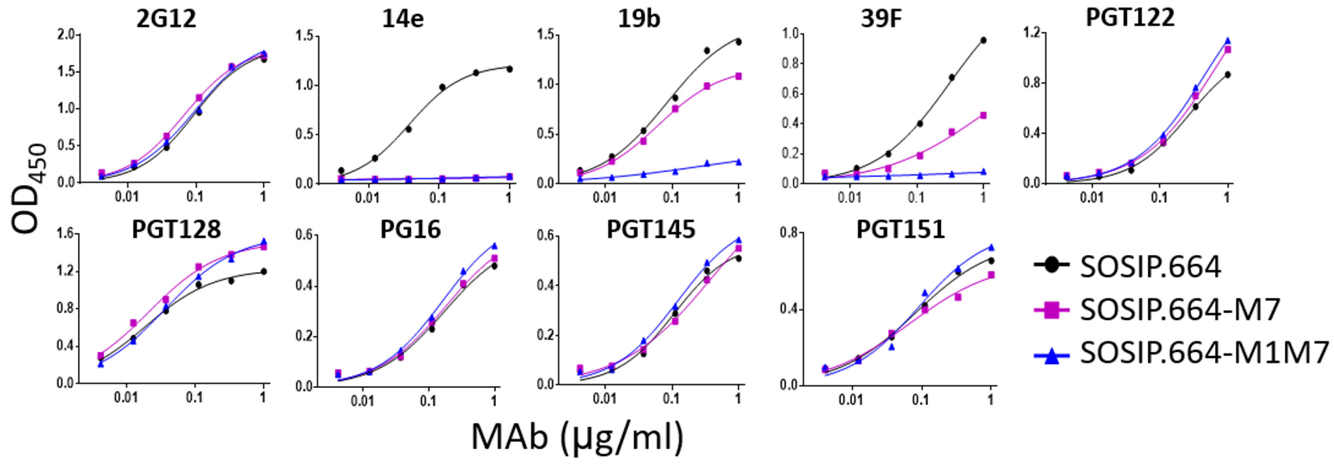
Journal of Virology

JVI

Journal of Virology

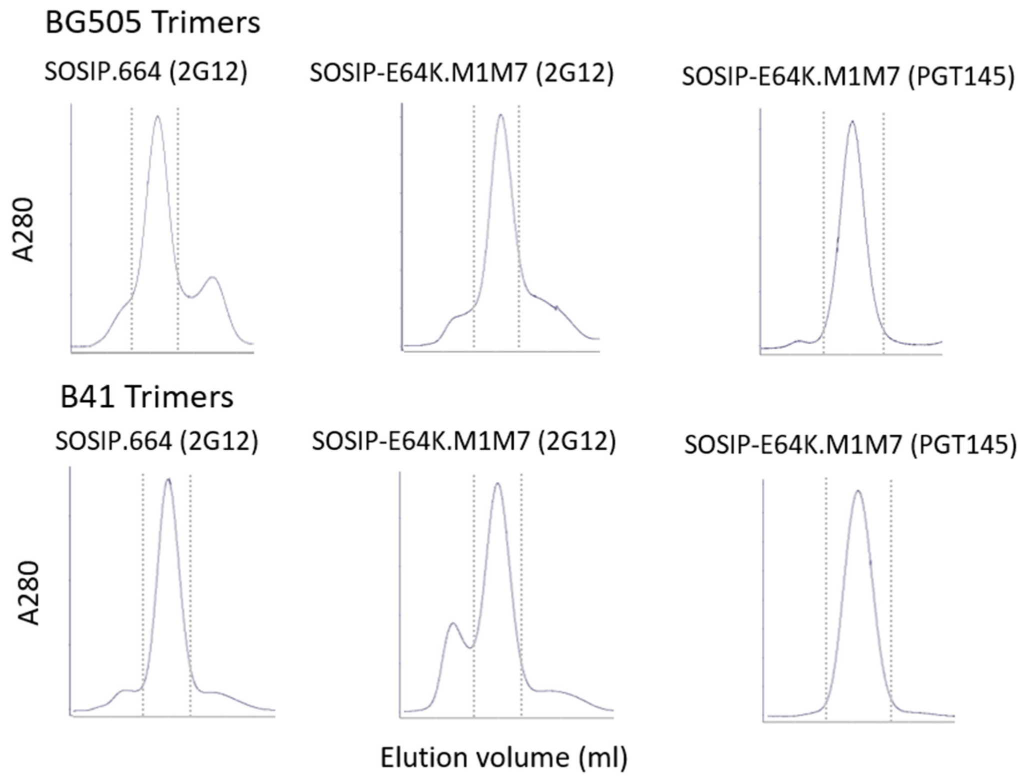
Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Accepted Manuscript Posted Online

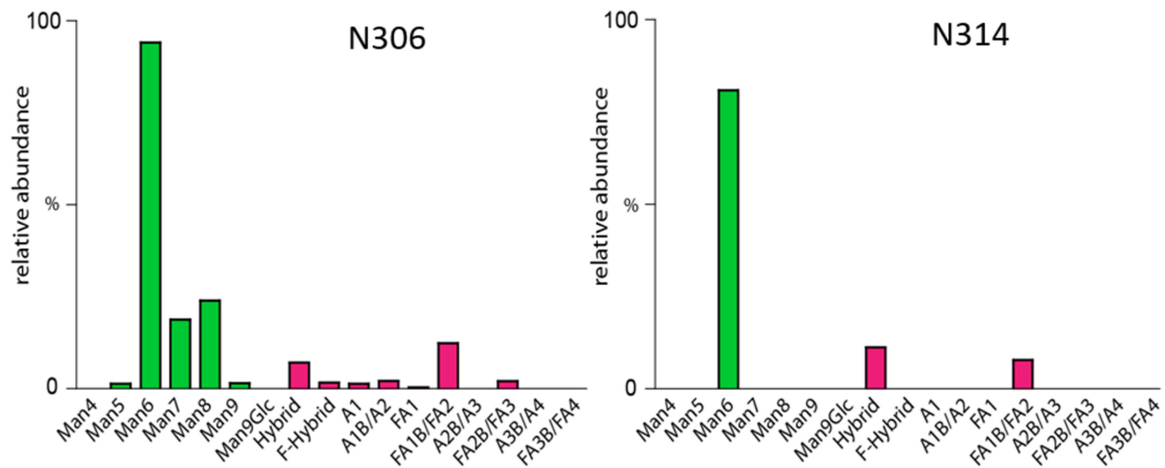


Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Accepted Manuscript Posted Online
Journal of Virology
JVI
Journal of Virology

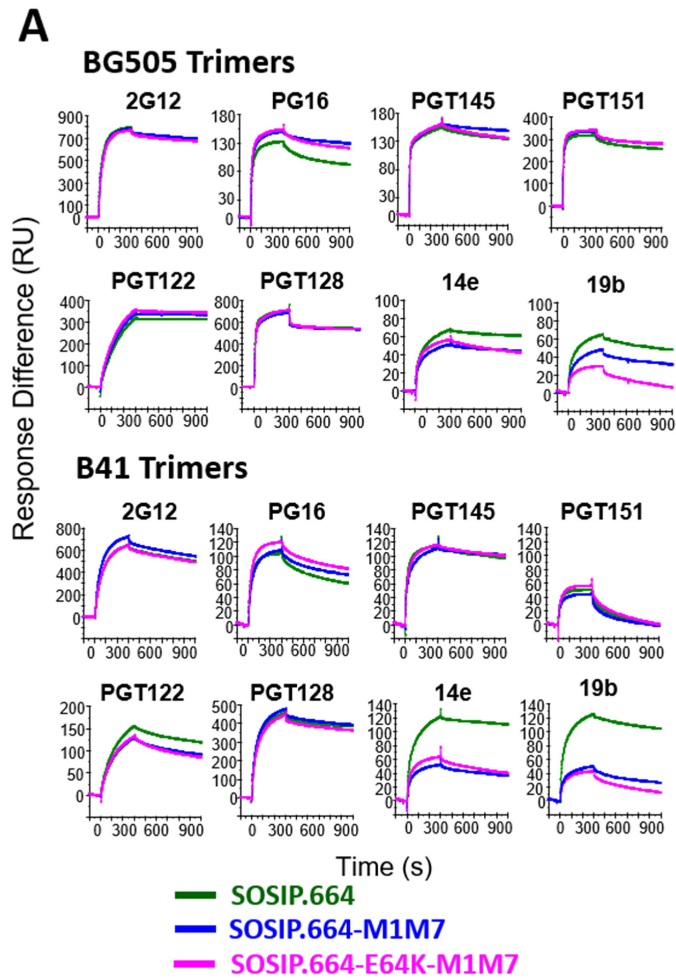


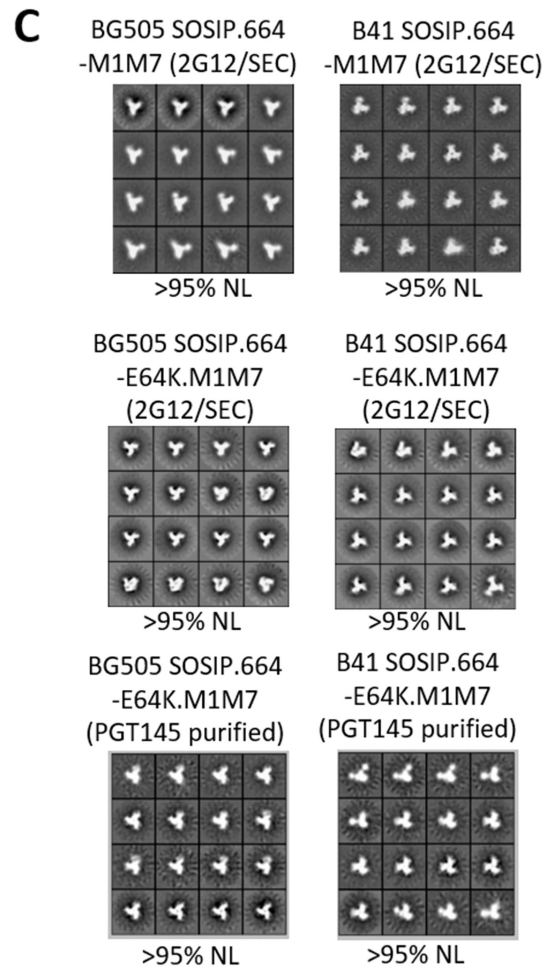
Genotype	Trimer	Purification	Yield (mg/L)
BG505	SOSIP.664	2G12/SEC	1.2
	SOSIP.664	PGT145/SEC	1.0
	SOSIP.664-E64K.M1M7	2G12/SEC	1.0
	SOSIP.664-E64K.M1M7	PGT145/SEC	0.7
B41	SOSIP.664	2G12/SEC	1.8
	SOSIP.664	PGT145/SEC	1.5
	SOSIP.664-E64K.M1M7	2G12/SEC	1.5
	SOSIP.664-E64K.M1M7	PGT145/SEC	1.1

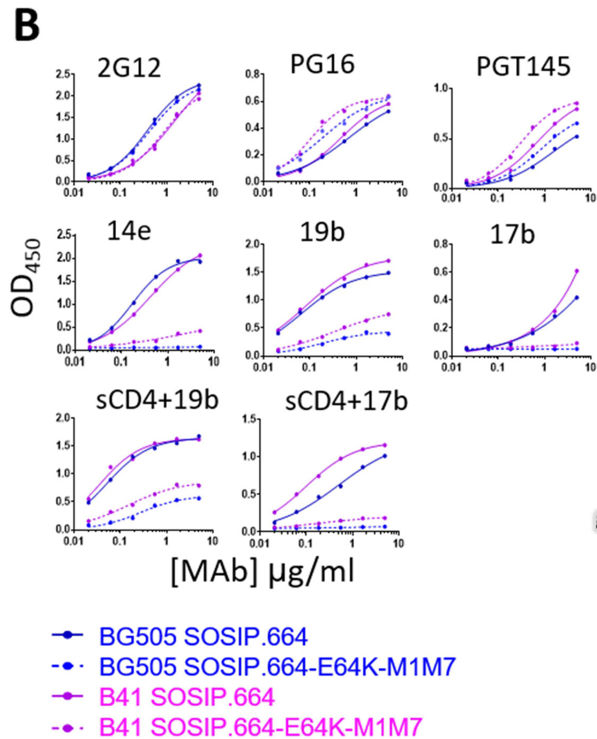


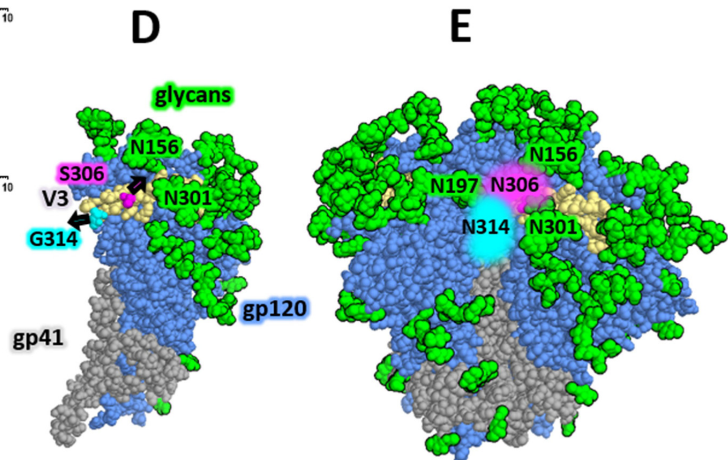
Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Accepted Manuscript Posted Online





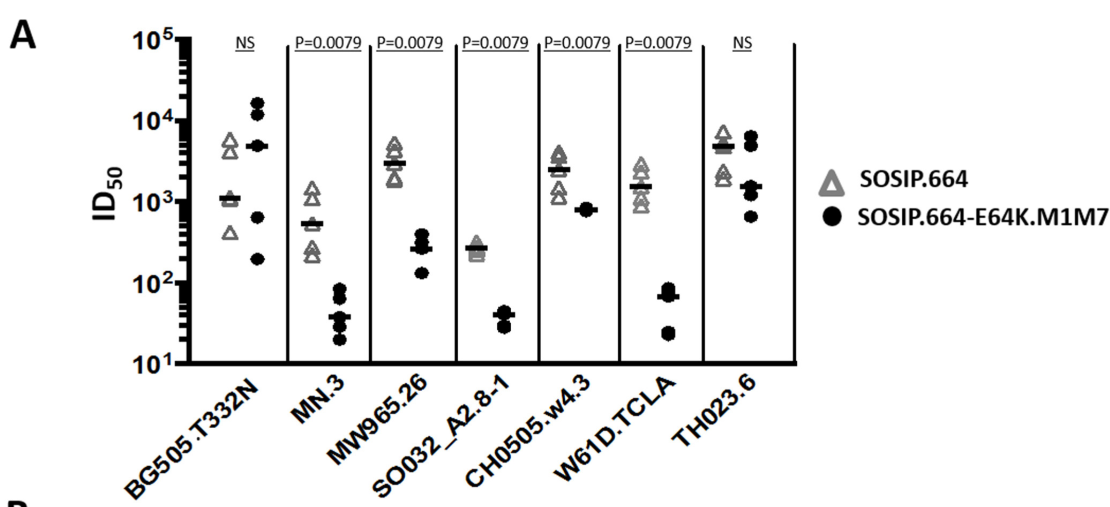


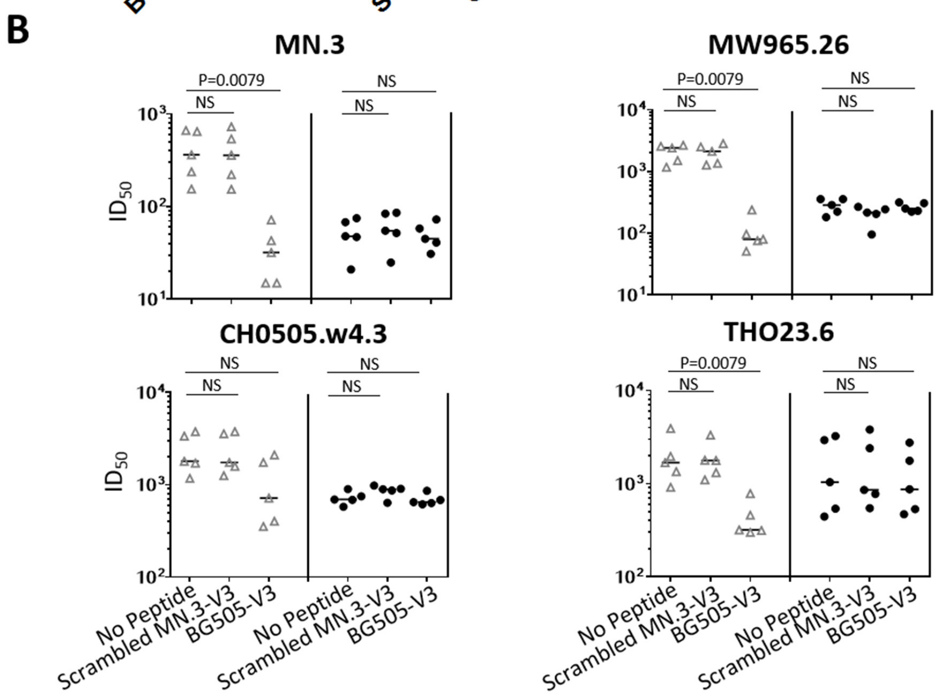


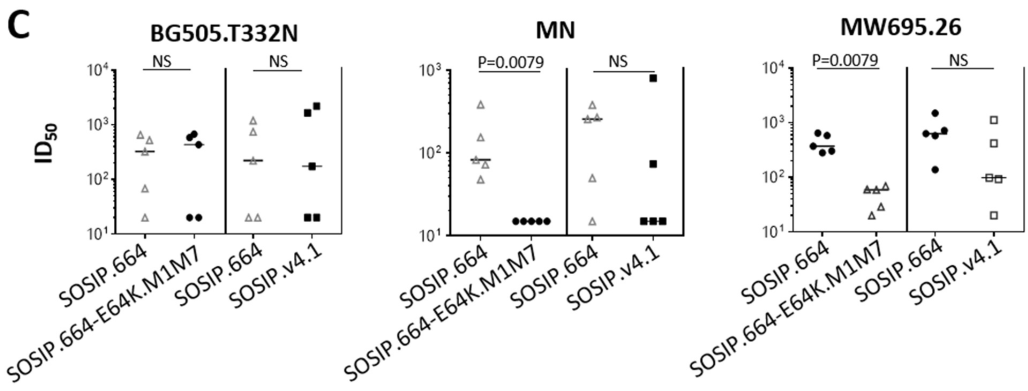
Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest

Journal of Virology
JVI
Journal of Virology

Accepted Manuscript Posted Online







Journal of Virology
JVI
Journal of Virology
Downloaded from http://jvi.asm.org/ on May 25, 2017 by guest