

1 **SARS-CoV-2 spike glycosylation affects function and neutralization sensitivity**

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24 **Abstract**

25 The glycosylation of viral envelope proteins can play important roles in virus biology and
26 immune evasion. The spike (S) glycoprotein of severe acute respiratory syndrome
27 coronavirus-2 (SARS-CoV-2) includes 22 N-linked glycosylation sequons and 17 O-
28 linked glycosites. Here, we investigated the effect of individual glycosylation sites on
29 SARS-CoV-2 S function in pseudotyped virus infection assays and on sensitivity to
30 monoclonal and polyclonal neutralizing antibodies. In most cases, removal of individual
31 glycosylation sites decreased the infectiousness of the pseudotyped virus. For
32 glycosylation mutants in the N-terminal domain (NTD) and the receptor binding domain
33 (RBD), reduction in pseudotype infectivity was predicted by a commensurate reduction
34 in the level of virion-incorporated spike protein. Notably, the presence of a glycan at
35 position N343 within the RBD had diverse effects on neutralization by RBD-specific
36 monoclonal antibodies (mAbs) cloned from convalescent individuals. The N343 glycan
37 reduced overall sensitivity to polyclonal antibodies in plasma from COVID-19
38 convalescent individuals, suggesting a role for SARS-CoV-2 spike glycosylation in
39 immune evasion. However, vaccination of convalescent individuals produced
40 neutralizing activity that was resilient to the inhibitory effect of the N343 glycan.

41

42 **Introduction**

43 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent
44 of the COVID-19 disease and has caused a devastating pandemic (1, 2). SARS-CoV-2
45 encodes a spike (S) glycoprotein which binds angiotensin-converting enzyme 2 (ACE2)
46 and mediates viral entry into host cells (3-6). The S glycoprotein (1273 aa) consists of a
47 signal peptide followed by the S1 subunit (13-685 aa) and the S2 subunit (686-1273
48 aa). These two subunits are separated by a furin cleavage site (PRRAR), abrogation of
49 which can increase virus infectivity in some circumstances (7). The receptor-binding
50 domain (RBD, 319-541 aa) that is responsible for ACE2 binding (6) and the N-terminal
51 domain (NTD), both encoded in S1, are the major targets of neutralizing antibodies. Like
52 other viral envelope glycoproteins including HIV-1 (8), SARS-CoV-2 spike protein is
53 heavily glycosylated (9-11). Indeed, approximately 40% of the surface the SARS-CoV-2
54 S protein expressed in human 293T cells is shielded by glycans (12). The majority of
55 this shield is comprised of N-linked oligomannose-type or complex glycans, linked to 22
56 sites (Asn-X-Ser/Thr) on the S protein (13). Additionally, 17 O-linked glyco-sites have
57 been identified by biochemical methods (14-16).

58

59 Glycosylation of viral envelope proteins can play an important role in virus-host
60 interactions (17). In the case of HIV-1, for example, N-linked glycans are essential for
61 correct folding and processing of gp120 as well as structural rearrangements required
62 for receptor binding (18). The HIV-1 glycan shield also plays a crucial role in preventing
63 neutralizing antibodies from binding to HIV-1 envelope (19). Likewise, S glycosylation
64 affects SARS-CoV-2 infection (20). Blocking N-glycan biosynthesis onto SARS-CoV-2

65 spike protein and, to lesser extent, O-glycan elaboration, reduces viral infectivity (21).

66 Additionally, cryo-electron microscopy studies have revealed that the N-glycan at

67 position N343 in the RBD facilitates transition of the spike protein to the 'open'

68 conformation, which is important for ACE2 binding (22). Accordingly, mutation of this

69 site (N343Q) reduced viral entry into ACE2-expressing cells (23).

70

71 Little is known about how SARS-CoV-2 S glycosylation might affect immune

72 surveillance. It is conceivable that glycans sterically shield underlying epitopes from

73 recognition by antibodies, as is the case in HIV-1 (19). Many SARS-CoV-2 neutralizing

74 antibodies target the RBD and can be divided into 4 broad classes based on the

75 epitopes targeted (24). Class 1 antibodies recognize epitopes overlapping with the

76 ACE2-binding site and bind only to 'up' RBDs. Class 2 antibodies bind both 'up' and

77 'down' RBDs and also block ACE2 binding. In contrast, class 3 antibodies bind epitopes

78 distinct from the ACE2 binding site but can potently neutralize. Class 4 antibodies are

79 generally less potent and recognize epitopes that are distinct from the ACE2 binding

80 site that are shielded in the down conformation. Interestingly, some antibodies, namely

81 S309 and SW186, recognize epitopes that include the N343 glycan (25) (26), raising the

82 possibility that this glycan might play a dual role in antibody recognition, either as shield

83 or as a component of an epitope.

84

85 Co-expression of SARS-CoV-2 S with envelope-deficient viruses such as HIV-1 (human

86 immunodeficiency virus-1) produces pseudotyped viruses capable of infecting ACE2-

87 expressing cells and is widely used as a surrogate to study viral entry and neutralization

88 by antibodies (27, 28). To comprehensively understand the role of spike glycans in viral
89 infectivity and antigenicity, we individually mutated each of the 22 N-linked glycosylation
90 sites in the spike protein as well as two O-linked sites (S323 and T325) in the RBD. We
91 found mutations introduced at many glycosylation sites in the NTD and RBD reduced
92 pseudotype infectivity, and the magnitude of this effect was predicted by the magnitude
93 of the loss of S incorporation into virions. Furthermore, while the S protein levels in
94 virions had little effect on neutralization sensitivity, the presence or absence of a glycan
95 on N343 in RBD governed the sensitivity to some monoclonal antibodies cloned from
96 convalescent individuals. Moreover, the glycan at N343 reduced neutralization
97 sensitivity to polyclonal antibodies from convalescent individuals, but this evasive effect
98 imparted by glycosylation was overcome by plasma antibodies from the same
99 individuals who were subsequently vaccinated.

100

101 **Results**

102 **Levels of virion-incorporated SARS-CoV-2 spike protein and pseudotyped HIV-1
103 particle infectiousness**

104 While SARS-CoV-2 S pseudotyped HIV-1 has been widely utilized to study S-mediated
105 viral entry and neutralizing activities by antibodies, the extent to which the amount of S
106 protein on virions affects particle infectivity and neutralization sensitivity is not fully
107 understood. To address this question, we co-transfected 293T cells with various
108 amounts of an S expression plasmid (pSARS-CoV-2_{Δ19}), along with an envelope-
109 deficient HIV-1 proviral plasmid encoding NanoLuc. The number of SARS-CoV-2 spikes
110 incorporated into HIV-1 pseudotype virions were estimated using purified recombinant

111 purified S protein (S-6P-NanoLuc (29)) and recombinant p24 CA proteins as standards
112 on near-IR fluorescent western blotting (LiCor) and assuming 1500 capsid (CA) protein
113 subunits per mature HIV-1 particle (30, 31) (Fig. S1A). While the amount of S
114 expression plasmid used for transfection had little effect on the amount of virions
115 produced (Fig. 1A), the amount of S protein incorporated into virions correlated with the
116 amount expressed in cells. S incorporation into virions reached a plateau (~30 ng S/ml
117 in virions, or around 300 spike trimers per virion) when 0.125 µg or 0.25 µg of an S
118 expression plasmid was co-transfected with the HIV-1 proviral plasmid (Fig. 1A, 1B,
119 1C).

120

121 To assess the effect of the number of spikes on pseudotype infectiousness, the titers of
122 pseudotyped viruses were measured on 293T cells expressing ACE2 (293T/cl.22). Co-
123 transfection with as little as 2 ng of S expression plasmid produced virions that induced
124 1000x the level of luciferase observed with ‘bald virions’, indicating that small amounts
125 of S protein (0.15 ng/ml or a mean of ~1-2 S trimers per virion) were sufficient to
126 mediate viral entry (Fig. S2, Fig. 1B and 1C). Nevertheless, pseudotype virion infectivity
127 increased with increasing spike numbers. Indeed, infectivity and the number of spikes
128 was approximately linearly correlated, in the range 1 spike to 300 spikes per virion (Fig.
129 1C). Of note, these spike numbers are comparable to the average number of S trimers
130 on authentic SARS-CoV-2 virions (25–127 prefusion spikes per virion) (32) and higher
131 than the numbers of gp120 trimers on HIV-1 virions (33).

132

133 **Reduced infectious virion yield conferred by SARS-CoV-2 spike glycosylation site**

134 **removal**

135 To investigate the contribution of glycosylation to S protein function, we generated 22
136 spike substitution mutants, each containing a single Asn to Asp (N to D) substitution at

137 one of the 22 N-glycosylation sequons. Additionally, we generated a mutant with Ala

138 replacements at potential O-linked sites S323 and T325 in the receptor binding domain

139 (RBD) (13). None of these substitutions affected the levels of the S protein in

140 transfected cells, but some of them reduced S incorporation into virions (Fig. 2A).

141 Specifically, N61D, N122D, N165D, N343D, and S323A T325A that fall within S1, that

142 includes the NTD and RBD, exhibited 10-fold or greater reductions in the levels of

143 virion-associated S protein, suggesting that these glycans affect S protein transport or

144 virion incorporation. Conversely, removal of the glycosylation sites in S2 had either no

145 or minor effects on S protein incorporation into virions (Fig. 2A). Measurements of the

146 yield of infectious pseudotyped particles carrying each of the substitutions indicated that

147 several substitutions in the NTD and RBD markedly reduced infectivity. For example,

148 the N61D substitution reduced infectivity by almost 50-fold (Fig. 2B, Fig. S2) while

149 substitutions at glycosylation sites in the RBD, namely N331D, N343D, and S323A

150 T325A, resulted in 5- to 10-fold reduction in infectivity (Fig. 2B, Fig. S2). Western blot

151 analyses revealed that the amount of S protein in virions was directly correlated with

152 infectivity (Fig. 2B), suggesting a potential role for most glycans during synthesis or

153 folding of spike trimers, or their incorporation into virions, rather than in S protein

154 function after virion incorporation. In contrast, two substitutions (N1194D and N657D) did

155 not change the amount of S protein in virions, but substantially reduced infectivity (Fig.

156 2B, Fig. S2). This finding suggests a functional role for N1194 and N657 glycans in
157 spike conformation or stability on virions, or function during viral entry. Overall, we
158 conclude that a subset of glycosylation sites is important for the incorporation of S
159 protein into virions, while others are required for optimal particle infectivity.

160

161 **Impact of spike density and RBD glycosylation on neutralization sensitivity**

162 The RBD is the major target of neutralizing antibodies. To determine the effect of glycan
163 removal on sensitivity to neutralizing antibodies, we focused on glycosylation sites in the
164 RBD (N331 and N343), and one site adjacent to the RBD (N282). Because these
165 glycosylation sites affected the level of spike incorporation into virions, we first tested
166 the effect of SARS-CoV-2 spike density on neutralization sensitivity, as this parameter
167 could be a potential confounder. We harvested pseudotyped virions from cells
168 transfected with varying amounts (from 2 ng to 1 µg) of wild-type S expression plasmid
169 and tested their sensitivity to C144, a potent class 2 neutralizing human monoclonal
170 antibody cloned from a convalescent individual (34) (Fig. 3A). Notably, varying the
171 levels of WT S protein on virions over a wide range (Fig. 1B) had no discernable effect
172 on neutralization sensitivity to C144.

173 We next tested the neutralization sensitivity of the RBD glycosylation site mutants
174 bearing Asn to Asp mutation at N-linked sites (N331D and N343D) or proximal (N282D)
175 to the RBD or alanine substitutions at O-linked sites S323 and T325. To provide
176 matched control viruses with approximately similar numbers of S trimers and similar

177 levels of infectivity, neutralization of the glycosylation site mutants was compared to
178 virions generated with an appropriate level of the WT spike protein. We compared the
179 neutralization sensitivity of WT and mutant virions to C144 and another potent class 2
180 neutralizing antibody, C121, both of which target the ACE2 binding site. While the
181 N282D, S323/T325A, and N331D mutant pseudotypes exhibited neutralization
182 sensitivities that were similar to the WT pseudotype, the N343D substitution conferred
183 significantly increased neutralization sensitivity to both C144 and C121 (Fig. 3B).
184 Specifically, the half maximal inhibitory concentration (IC_{50}) of C144 was reduced from
185 1.8 to 3.4 ng/ml (against the WT pseudotype) to 0.45 ng/ml (against the N343D
186 pseudotype), while the C121 IC_{50} was reduced from 2.3 to 2.9 ng/ml (against the WT
187 pseudotype) to 0.21 ng/ml (against the N343D pseudotype).

188

189 **Positive and negative effects of RBD glycosylation on sensitivity to human
190 monoclonal antibodies**

191 To determine the effects of glycosylation more broadly on SARS-CoV-2 sensitivity to
192 neutralizing antibodies, we used pseudotyped viruses bearing spike proteins with
193 R683G substitution, which ablates the furin cleavage site. This substitution does not
194 affect S incorporation into virions (Fig. S1B) but enhances particle infectivity (7). The
195 glycosylation site mutations had a smaller effect on spike incorporation into virions in
196 the R683G context (Fig. S3A). Nevertheless, transfection of cells with 1 μ g of N282D,
197 S323A T325A, and N331D S expression plasmids generated pseudotyped viruses with
198 S protein amounts and infectious properties comparable to those generated by

199 transfection of 30-100 ng of WT S expression plasmid (Fig. S3A and S3B). Transfection
200 of cells with 1 μ g of the N343D spike expression plasmid yielded pseudotyped virus
201 particles carrying a similar amount of S protein to those generated by cells transfected
202 with 10 ng of the R683G S protein expression plasmid (Fig. S3A), while the particle
203 infectivity was similar to those from cells transfected with 3 ng of the WT R683G
204 expression plasmid (Fig. S3B). To evaluate the effect of glycosylation site mutations on
205 sensitivity to neutralizing antibodies, we generated WT and mutant virion stocks bearing
206 similar amounts of spike protein and then assessed their susceptibility to neutralization
207 by a panel of RBD-specific human monoclonal antibodies of the various neutralizing
208 classes (24) recovered from convalescent individuals, including antibodies from class 1
209 (C098 (35), C099 (35), C936 (36)), class 2 (C121 (34), C144 (34)), class 3 (C032 (34),
210 C080 (35), C135 (34), C581 (36), C952 (24)), and class 4 (C022 (34) (37), C118 (34)
211 (37)).

212

213 Of class 1 antibodies, C098 had only weak neutralizing activity, whereas its clonal
214 relative C099 was potent ($IC_{50} = 21.3$ ng/ml against WT (10ng) or 24.5 ng/ml against WT
215 (3ng) and its activity was resilient to many naturally occurring mutations in the RBD (7).
216 The N343D mutation decreased the neutralization sensitivity to C099 by 7-fold, ie IC_{50}
217 was increased to 176.9 ng/ml (Fig. 3C). Neutralization sensitivity to a third unrelated
218 class 1 antibody, C936, was reduced by almost 10-fold, and IC_{50} was increased from
219 41.1 ng/ml against WT (10ng) or 54.9 ng/ml against WT (3ng) to 467.1 ng/ml against
220 N343D (Fig. 3C).

221

222 In contrast to the class 1 antibodies, pseudotyped virus sensitivity to two class 2
223 antibodies was increased for the N343D mutant compared to WT. As was the case in
224 the context of the furin-cleavable S protein (Fig. 3B), the N343D mutation in R683G
225 spike increased neutralization sensitivity to C121 and C144. The IC₅₀ of C121 was
226 reduced from 4.8 to 8.1 ng/ml (against the WT) to 2.6 ng/ml (against the N343D), while
227 the C144 IC₅₀ was reduced from 5.7 to 6.1 ng/ml (against the WT) to 1.7 ng/ml (against
228 the N343D) (Fig. 3C).

229

230 Class 3 antibodies, which bind epitopes distinct from the ACE2 binding site, showed a
231 complicated pattern of effects in response to the N343D substitution. Some class 3
232 antibodies, including C032, C080, and C952, inhibited the WT and N343D mutant
233 pseudotypes with approximately the same potency. A different class 3 antibody C135,
234 that exhibited incomplete neutralization of the WT pseudotypes at high concentrations
235 despite exhibiting low IC₅₀ (IC₅₀= 8.3 -11.5ng/ml), was able to achieve almost complete
236 neutralization of the N343D pseudotypes at high concentrations (Fig. 3C). In contrast,
237 another class 3 antibody C581 showed reduced potency against the N343D mutant, ie,
238 the IC₅₀ was >2000 ng/ml against N343D compared to 70.0 to 76.6 ng/ml against the
239 wild-type pseudotype (Fig. 3C).

240

241 For two class 4 antibodies, C022 and C118, the N343D substitution affected the slope
242 of the neutralization curves and conferred partial resistance at high of antibody
243 concentrations (Fig. 3C). For example, C022 at 2000 ng/ml almost completely
244 neutralized the wild-type pseudotypes but only inhibited the N343D pseudotypes by
245 ~50%. A similar trend was seen for a second class 4 antibody, C118.

246

247 Overall, the N343D substitution had a range of positive and negative effects on
248 neutralization sensitivity that varied greatly dependent on the nature of the particular
249 monoclonal antibody tested. To better understand the molecular basis for the impact of
250 N343 glycan on antibody neutralization, we inspected the structures of some of the
251 aforementioned antibodies in complex with spike (Fig. 4A, B, C). The glycosylation site
252 at N343 (shown in red in Fig. 4A, B, C) is distinct from the binding site of the class 1
253 antibody C098 – suggesting that the effects of the glycan on sensitivity to class 1
254 antibodies are mediated through effects of the glycan on RBD conformational dynamics
255 and epitope exposure (Fig. 4B). In contrast, for the class 2 antibodies C121 and C144,
256 N343 is proximal to the antibody bound to the neighboring spike subunit (Fig. 4C). Since
257 removal of the glycan increased sensitivity to these antibodies (Fig. 3C), it is likely that
258 the N343 glycan partly shields these class 2 antibody epitopes. For class 3 antibodies,
259 N343 protrudes towards the C135 binding site on the same S subunit (Fig. 4),
260 potentially explaining incomplete neutralization by this antibody (Fig. 3C). For the class
261 4 antibody C118, N343 is distal to the antibody binding site on spike (Fig. 4C),

262 suggesting that N343 glycan is not involved in direct antibody binding and likely exerts
263 changes in neutralization through effects on RBD conformational dynamics.

264

265 Other RBD substitutions (S323A/T325A and N331D) did not alter the neutralization
266 sensitivity to most monoclonal antibodies tested herein (Fig. S4, Fig. S5). However, the
267 N282D substitution, which lies outside the RBD, had marginal effect on sensitivity to the
268 class I antibodies C099, C936 and the class 3 antibody C581 (Fig. S6).

269

270 **Effect of the N343 glycan on neutralization by polyclonal SARS-CoV-2
271 neutralizing plasma**

272 Given that the N343D substitution exhibited different effects on sensitivity to neutralizing
273 monoclonal antibodies, we next asked whether this substitution affected neutralization
274 by polyclonal antibodies present in convalescent plasma. As with the monoclonal
275 antibodies, we compared the neutralization sensitivity of N343D pseudotyped particles
276 with that of WT pseudotyped particles containing same amount of WT spike protein or
277 showing the same infectivity as the N343D pseudotype, to convalescent plasma from 15
278 patients collected early in the COVID19 pandemic (at 1.3 months after infection) and
279 from the same individuals ~1 year later following subsequent vaccination (34) (36). The
280 N343D mutant pseudotypes were more sensitive than the WT pseudotypes to
281 convalescent plasma collected at 1.3 months after infection (Fig. 5A and Fig. S7).
282 Indeed, the 50% neutralization titers (NT50) were a mean of 5.1-fold greater for the

283 N343D mutant as compared to WT pseudotypes ($p=0.0022$) (Fig. 5B). Notably, the
284 difference in neutralization sensitivity between N343D mutant (mean NT50=26179) and
285 WT S pseudotypes (mean NT50=20853) was negligible ($p=0.2805$) when plasmas
286 collected from the same individuals 1 year later and after subsequent vaccination were
287 tested. Of note, these subsequently collected plasma exhibited higher neutralization
288 potency than those collected shortly after infection (Fig. 5). We conclude that the glycan
289 at N343 confers protection against neutralization by antibodies generated shortly after
290 SARS-CoV-2 infection but that this effect is lost against antibodies from the same
291 individuals who are subsequently vaccinated.

292

293 **Discussion**

294 While SARS-CoV-2 spike pseudotyped viruses have been widely used as a surrogate to
295 study S-mediated viral entry (28) little is known about the varying effect of S level on
296 virions on particle infectivity and neutralization sensitivity. Previously it was reported that
297 approximately 8 HIV-1 trimer-receptor interactions are required for HIV-1 to infect a
298 target cell (38), a result that is broadly consistent with our finding that a small number
299 (minimally an average of 1-2 spike per virion) is sufficient for detection of SARS-CoV-2
300 pseudotype infection. To achieve neutralization, monoclonal antibodies must encounter
301 prefusion spikes and reduce the number of functional spike trimers below the threshold
302 required for infection. We found that an increased level of S on pseudotyped virions is
303 associated with increased infectivity but had little effect on neutralization sensitivity to
304 monoclonal antibodies. This result suggests that monoclonal antibodies are in excess

305 over functional spikes in a typical neutralization assay, and their measured potency is
306 limited by their affinity, rather than by the amount of functional spike protein in a
307 pseudotype neutralization assay.

308

309 In this study, we found that glycosylation at several sites in the spike NTD and RBD,
310 including N61, N122, N165, N331, N343 and S323 T325 are required for full
311 pseudotyped virus infectivity. Substitution of these glycosylation sites resulted in a
312 reduction in pseudotype infectivity of about 10-fold or more and correlated with a
313 reduction in spike incorporation into virions. While it is unclear precisely how
314 glycosylation on these sites drives S incorporation into virions, glycans could in principle
315 affect protein stability and trafficking through the secretory pathway (39). We note,
316 however, that the glycosylation site mutations did not affect steady state levels of S in
317 transfected cells. Compared with other glycosylation sites in the S1 domain, these sites
318 are highly conserved among sarbecovirus S proteins (40). Likewise, the conservation of
319 these glycosylation sites is observed among the major SARS-CoV-2 variant lineages,
320 including Alpha, Beta, Gamma, Delta and Omicron (41). Some studies, largely
321 employing molecular dynamics simulations, have suggested glycans on spike proteins
322 affect the conformational dynamics of the spike's RBD "up" and "down" states (10) (42).
323 In particular, the glycan at N343 stabilizes RBD states in a process termed "glycan
324 gating" (22, 43, 44). Our findings show that the N-glycan at N343 affected incorporation
325 into virions, suggesting that the conformational state, might affect trimer assembly or
326 stability, transit through the secretory pathway or incorporation into pseudotyped virions.

327

328 The N343 glycan also affected neutralization by RBD-targeted monoclonal antibodies,
329 and the effect was largely dependent on the class to which antibodies belong. The N343
330 glycan is positioned on the RBD distal to the ACE2 and class 1 antibody (C098) binding
331 site, but increased sensitivity to class 1 antibodies (C098, C099, C936), which bind only
332 to “up” RBDs (24), suggesting that this effect is mediated through the impact of the
333 glycan on RBD conformation. Conversely, the N343 glycan reduced sensitivity to class
334 2 antibodies, including C121 and C144, both of which can bind both ‘up’ and ‘down’
335 RBDs. Cryo-EM structures (24) (Fig. 4) show that N343 is located proximally to the
336 C121 and C144 antibody bound to the neighboring subunit, in a manner that might
337 interfere with antibody binding. Overall, our results are consistent with a model in which
338 N343 glycan affects sensitivity to class 1 and class 2 antibodies by affecting the RBD
339 conformational dynamics (43) and also potentially by sterically hindering class 2
340 antibody binding into neighboring RBDs in the “down” conformation (Fig. 4).

341
342 The N343 glycan is proximal to the binding sites of class 3 antibodies, and the N343D
343 substitution had a range of effects on sensitivity to class 3 antibodies. Three clonally
344 related antibodies, C032, C080 and C952, were unaffected by the N343D substitution,
345 while substantial but opposing effects were seen for two others, C135 and C581. In the
346 case of C135, removal of the glycan reduced the fraction of virions that resisted
347 neutralization at high antibody concentration. A possible explanation for this
348 phenomenon is that the N343 site is heterogeneously glycosylated, and some
349 subfraction of the glycans occlude the C135 binding site. For C581, removal of the
350 N343 glycan had the opposite effect, reducing neutralization sensitivity. In this case it

351 seems likely C581 mimics the properties of two previously described cross-reactive
352 class 3 antibodies, S309 and SW186, for which the N343 glycan constitutes part of the
353 antibody binding site (25) (26). Finally, for the two class 4 antibodies, the glycan at N343
354 affected the character of the neutralization curve. Since in these cases the antibody
355 binding site is on the opposite face of the RBD to that of the glycan, it is likely that these
356 effects are mediated through alteration of spike conformational dynamics and exposure
357 of the class 4 epitope, which is shielded in the RBD 'down' conformation.

358

359 The N343 glycan reduced neutralization by convalescent plasma collected at 1.3
360 months after infection, echoing the effects on sensitivity to the C121, C144 (class 2) and
361 C135 (class 3) antibodies. Conversely, neutralization by convalescent plasma from the
362 same individuals (collected at 12 months following subsequent vaccination) was
363 unaffected by the N343 glycan. That neutralizing antibodies are relatively sensitive to
364 glycan-mediated protection early after infection may be a reflection of the fact that the
365 initial neutralizing response is based to a large extent on class 2 antibodies that are
366 easily escaped (29, 34). Subsequent increases in antibody affinity and neutralizing
367 potency and a shift in the RBD epitopes that are recognized, following months of affinity
368 maturation and boosting by vaccination (7, 35, 36, 45) results in neutralizing antibodies
369 that are mostly unaffected by the N343 glycan. In sum, while the SARS-CoV-2 N343
370 glycan affects both spike conformation and neutralization sensitivity shortly after
371 infection, antibody evolution confers sufficient potency and breadth to combat glycan-
372 mediated immune evasion.

373

374 **Acknowledgements**

375 We thank members of the Bieniasz and Hatzioannou laboratories for helpful
376 discussions. This work is supported by grants from NIAID: P01 AI165075 (P.D.B. and
377 T.H.), R37AI64003 (P.D.B.), R01AI157809 (P.D.B.) and R01AI78788 (T.H.). P.D.B. and
378 M.C.N. are Howard Hughes Medical Institute (HHMI) Investigators. The funders had no
379 role in study design, data collection and analysis, decision to publish or preparation of
380 the manuscript. This Article is subject to HHMI's Open Access to Publications policy.
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385

386 **Author Contributions**

387 F.Z., T.H. and P.D.B. conceived the study. F.Z. constructed spike mutants, expressed
388 proteins, performed Western Blot analysis and the pseudovirus neutralization assays.
389 F.S. provided the wild-type spike plasmid as well as technical support. F.M., C.G., M.C.
390 and M.C.N provided monoclonal antibodies. C.G., M.C. and M.C.N. provided
391 convalescent plasma from individuals with COVID-19. P.D.B., T.H. and M.C.N.
392 supervised the work. F.Z., T.H. and P.D.B. wrote the manuscript with input from all co-
393 authors.

394

395 **Competing Interests Statement**

396 The authors declare no competing interests.

397

398 **Materials and Methods**

399 **Antibodies and recombinant HIV p24**

400 Antibodies used here are anti-SARS CoV-2 spike antibody [1A9] (Genetax,
401 GTx632604) and anti-HIV capsid p24 (183-H12-5C, NIH AIDS Research and
402 Reference Reagent Program). Secondary antibodies included goat anti-mouse
403 conjugated to IRDye 800CW or IRDye 680 for Western blot analysis. The recombinant
404 HIV p24 protein was purchased from abcam (ab43037).

405 **Plasmid construction**

406 The plasmid expressing C-terminally truncated, human-codon-optimized SARS-CoV-2
407 spike protein (pSARS-CoV-2_{Δ19}) has been previously described (28). Using this plasmid
408 as the template, asparagine at N-linked glycosylation sites was replaced by aspartate
409 by overlap-extension PCR amplification with primers that incorporated the
410 corresponding nucleotide substitutions. O-linked glycosylation sites in the RBD region
411 (S323, T325) were replaced by alanine using the same strategy. The purified PCR
412 products were then inserted into the pCR3.1 expression vector with NEBuilder® HiFi
413 DNA Assembly. Some mutations within or adjacent to the RBD region, including N282D,
414 N331D, N343D, and S323A T325A, were also introduced in spike bearing the R683G
415 substitution which impairs the furin cleavage site and enhances particle infectivity.

416 **Cell lines**

417 Human embryonic kidney HEK-293T cells (ATCC CRL-3216) and the derivative
418 expressing ACE2, ie 293T/ACE2.cl22, were maintained in Dulbecco's Modified Eagle
419 Medium (DMEM) supplemented with 10% fetal bovine serum (Sigma F8067) and
420 gentamycin (Gibco). All cell lines used in this study were monitored periodically to
421 ensure the absence of retroviral contamination and mycoplasma.

422 **Generation of clarified SARS-CoV-2 pseudotyped virions and infectivity**
423 **measurement**

424 To generate HIV-1/NanoLuc-SARS-CoV-2 pseudotyped virions, two million 293T cells
425 in 10-cm dish were transfected with 7.5 µg of HIV-1 proviral plasmids expressing
426 NanoLuc along with increasing amounts (0 ng, 2 ng, 3.9 ng, 7.8 ng, 15.6 ng, 31.2 ng,
427 62.5 ng, 125 ng, 250 ng, 500 ng, or 1000 ng) of WT or 1000 ng of glycosylation site
428 mutant SARS-CoV-2 expression plasmids (pSARS-CoV-2_{Δ19}). In transfection
429 experiment to generate viruses bearing R683G substitution, 1 ng, 3 ng, 10 ng, 30 ng,
430 100 ng, and 300 ng of expression plasmid were used instead. The total amount of DNA
431 was held constant by supplementing the transfection with empty expression vector.
432 Cells were harvested at 48 hr post transfection and subjected to Western blot analysis.
433 Virus-containing supernatant was filtered (0.2 µm), and, to remove cell debris, clarified
434 by Lenti-X (TaKaRa). Particle infectivity was measured as previously described (28).
435 Briefly, viral stocks were three-fold serially diluted and added to 293T/ACE2 cl.22 in 96-
436 well plates. Cells were then harvested at 48 hr post infection for measuring NanoLuc
437 activity (Promega). The number of spike trimers per virion was estimated using the
438 following formula: S ng/ml/78.3 x 1500/(p24 ng/ml/24)/3.

439 **Western blot analysis**

440 Cell lysates were separated on NuPage Novex 4-12% Bis-Tris Mini Gels (Invitrogen).
441 Proteins were blotted onto nitrocellulose membranes. Thereafter, the blots were probed
442 with primary antibodies and followed by secondary antibodies conjugated to IRDye
443 800CW or IRDye 680. Fluorescent signals were detected and quantitated using an
444 Odyssey scanner (LI-COR Biosciences).

445 **S-6P-NanoLuc protein purification**

446 To express S-6P-NanoLuc proteins, Expi293 cells were transfected with S-6P-NanoLuc
447 expression plasmids using ExpiFectamine 293 (ThermoFisher Scientific). Four days
448 later, the supernatant was harvested and loaded on Ni-NTA agarose and, after thorough
449 wash, S-6P-NanoLuc proteins were released after HRV 3C protease treatment.

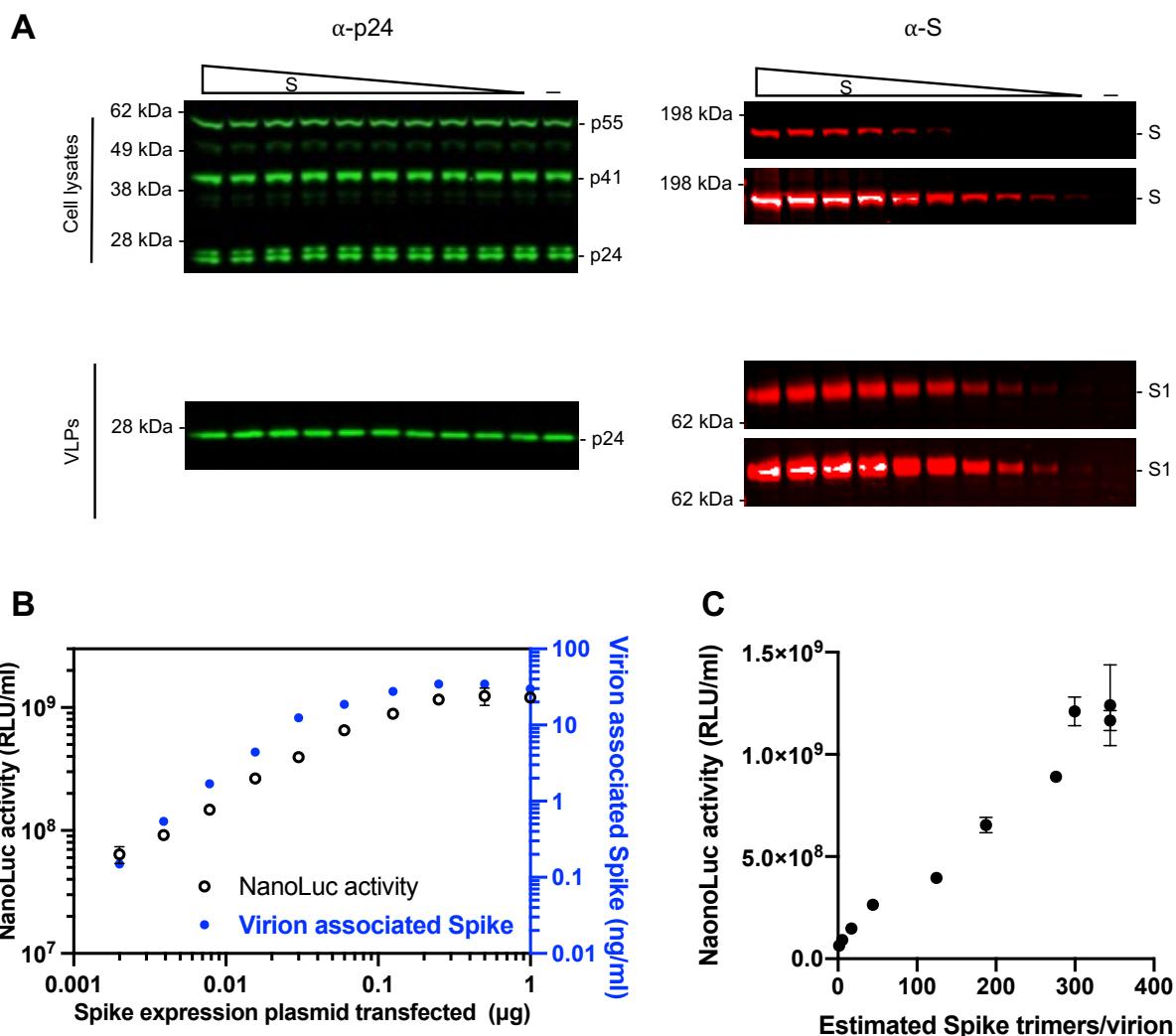
450 **Neutralization assays**

451 To measure neutralizing activity of monoclonal antibodies, serial dilutions of antibodies
452 beginning at 3 µg/ml were four-fold serially diluted in 96-well plates over seven dilutions.
453 To determine the neutralizing activity in convalescent plasma, the initial dilution started
454 at a 1:30 (for plasma at 1.3 months) or a 1:150 (for plasma at 12 months). Thereafter,
455 SARS-CoV-2 spike pseudotyped viruses were incubated with monoclonal antibodies or
456 the convalescent plasma for 1 hr at 37°C in 96-well plates. The mixture was then added
457 to 293T/ACE2cl.22 target cells seeded one day prior to infection so the final starting
458 dilutions were 1.5 µg/ml for monoclonal antibodies and 1:60 or 1:300 for plasma. Cells
459 were then harvested 48 hours post infection for NanoLuc luciferase assays.

460 **Human plasma samples and monoclonal antibodies**

461 Monoclonal antibodies C022, C032, C080, C098, C099, C118, C121, C135, C144,
462 C581, C936, and C952 used in this study were previously reported (24, 34-37). The
463 human convalescent plasma samples (COVs) were obtained under protocols approved
464 by Institutional Review Boards at Rockefeller University.

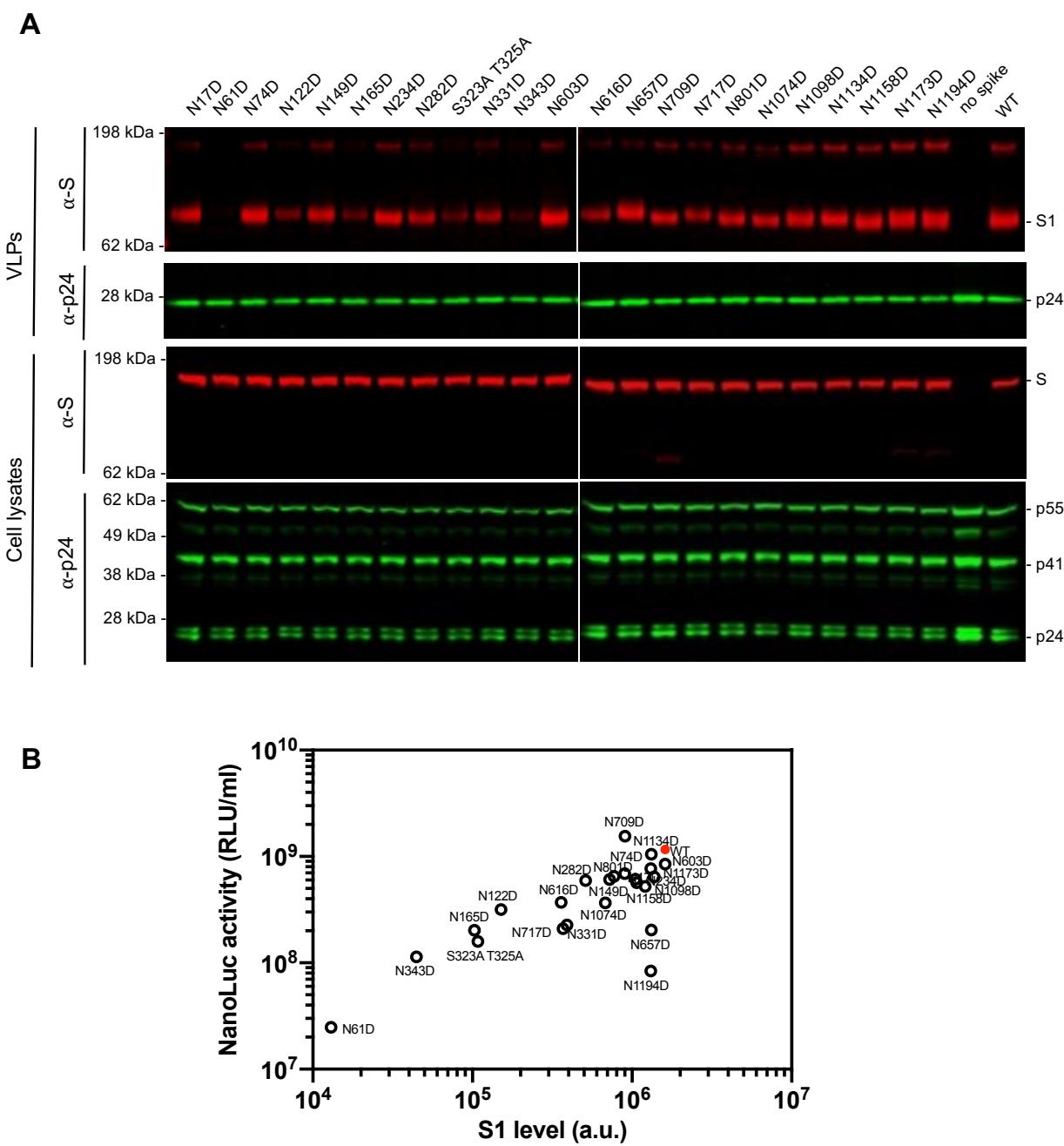
465 **Figures**
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468 **FIG 1 The effect of spike proteins level on pseudotype infectiousness**
469 (A) Western blot analysis of 293T cell lysates (upper panel) or virions (lower panel) at
470 48 hours after transfection with various amounts (0 ng, 2 ng, 3.9 ng, 7.8 ng, 15.6 ng,
471 31.2 ng, 62.5 ng, 125 ng, 250 ng, 500 ng, or 1000 ng) of a WT SARS-CoV-2 spike
472 expression plasmid along with envelope-deficient HIV-1 proviral plasmid expressing
473 NanoLuc luciferase. Each S blot was scanned twice, at low intensity (upper) and high
474 intensity (lower), respectively. Representative of three independent experiments.
475 (B) Characterization of virions pseudotyped with spike expression plasmids.
476 Infectiousness (on left y axis) was quantified by measuring NanoLuc luciferase activity
477 (RLUs) following infection of 293T cells expressing ACE2 (293T/ACE2.cl22) in 96-well
478 plates with pseudotyped viruses. The S1 incorporated into viruses (on right y axis) was
479 determined by quantitative Western Blot, using purified recombinant S-6P-NanoLuc
480 proteins as standard, representative of three independent experiments. The mean and
481 range of two technical replicates are shown.

482 (C) Characterization of virions pseudotyped with spike expression plasmids as in (B).
483 virus infectiousness (on y axis) was plotted against S trimers per virion (on x axis),
484 which was determined by quantitative Western blot, using purified recombinant S-6P-
485 NanoLuc proteins as standard. Representative of three independent experiments.
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489 **FIG 2 The impact of glycosylation site mutations on spike incorporation and**
490 **particle infectivity**

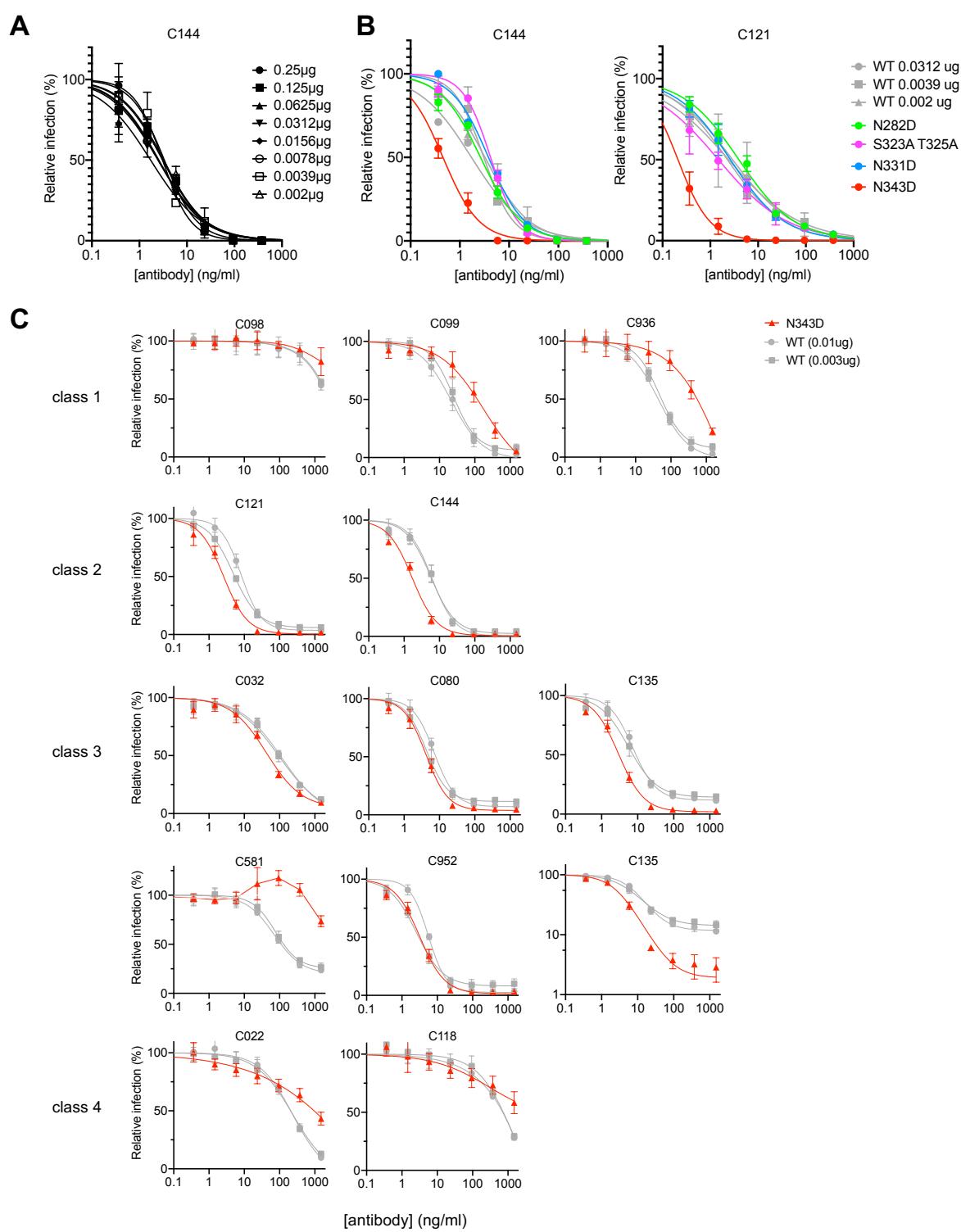
491 (A) Western blot analysis of 293T cell lysates (lower panel) or virions (upper panel) at
492 48 hours after transfection with 1 μ g of glycosylation site mutants or wild-type spike
493 expression plasmid along with envelope-deficient HIV-1 proviral plasmid expressing
494 NanoLuc. Representative of two independent experiments.

495 (B) Characterization of virions pseudotyped with spike expression plasmids.

496 Infectiousness (on y axis) was quantified by measuring NanoLuc luciferase activity

497 (RLUs) following infection of 293T/ACE2.cl22 cells in 96-well plates with pseudotyped
498 viruses. The S1 incorporated into viruses (on x axis) was determined by quantitative
499 Western blot. Glycosylation site mutants are shown in black and wild-type spike is
500 shown in red. The mean and range of two technical replicates are plotted.
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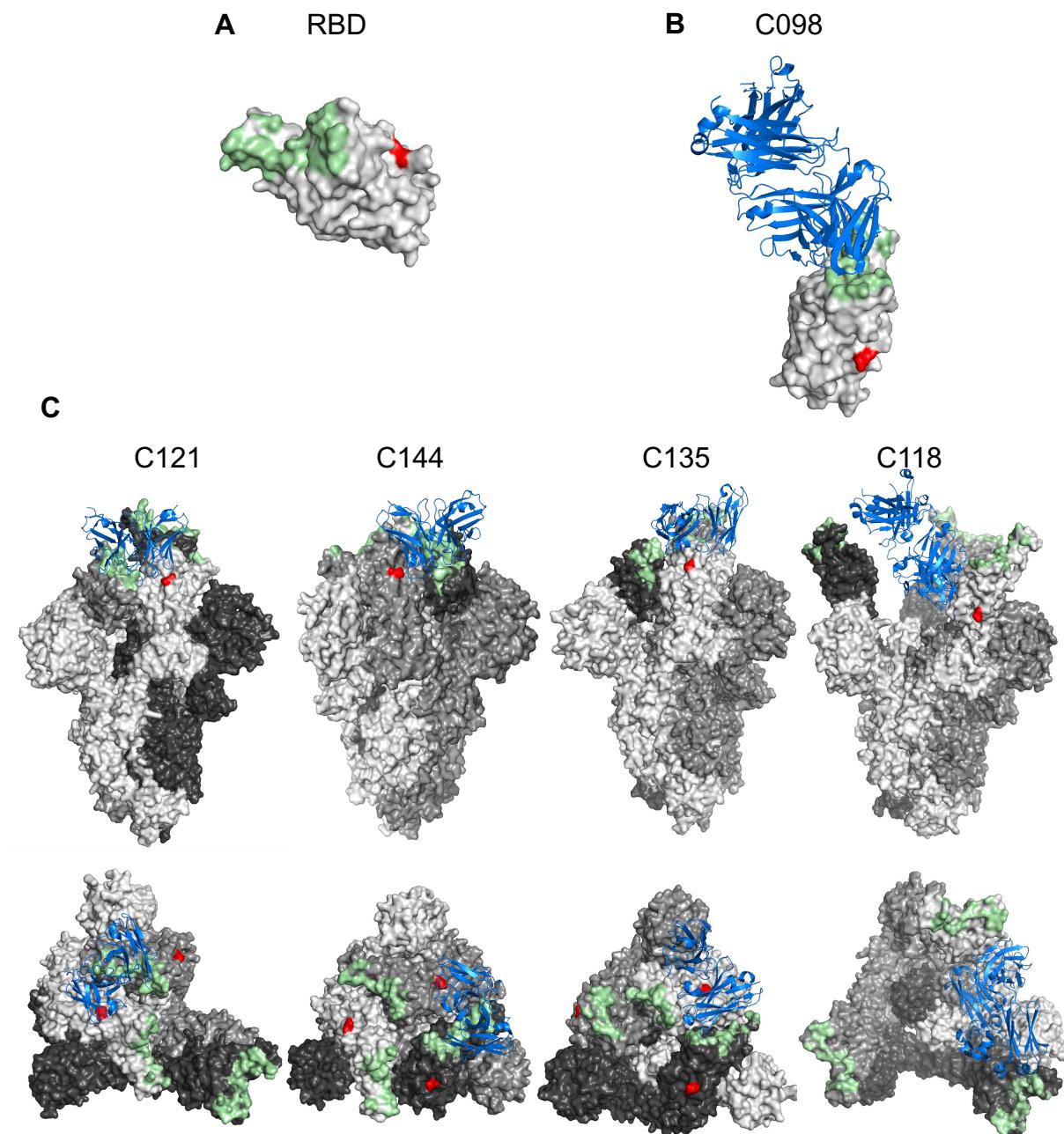
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FIG 3 Neutralization sensitivity of Spike to monoclonal antibodies is affected by N343D substitution

(A) Quantification of pseudotyped virions from cells transfected with various amounts of wild-type spike expression plasmid along with envelope-deficient HIV proviral plasmid expressing NanoLuc in the presence of the indicated concentrations of the class 2

509 neutralizing monoclonal antibody C144. Infectivity was quantified by measuring
510 NanoLuc luciferase levels (RLU). The mean and range of two technical replicates are
511 shown.
512 (B) Quantification of glycosylation site mutant N282D, S323A T325A, N331D, and
513 N343D or wild-type spike pseudotyped virus infection in the presence of the indicated
514 concentrations of the class 2 neutralizing monoclonal antibody C144 (*left*) or C121
515 (*right*). Infectivity was quantified by measuring NanoLuc luciferase levels (RLU). The
516 mean and range of two technical replicates are shown.
517 (C) Quantification of neutralization of glycosylation site mutant N343D in the
518 background of furin uncleavable (R683G) SARS-CoV-2 S pseudotyped virus infection in
519 the presence of the indicated concentrations of a panel of monoclonal antibodies,
520 including class 1 (C098, C099, and C936), class 2 (C121 and C144), class 3 (C032,
521 C080, C135, C581, and C952), and class 4 (C022 and C118) antibodies. As controls,
522 glycosylation intact spike expression plasmid (WT in the R683G background) was
523 transfected at two doses, 10 ng or 3 ng, and the neutralization sensitivity of the resulting
524 viruses were assessed in parallel. The mean and range of two technical replicates are
525 shown. The C135 neutralization graph is depicted with both a linear and logarithmic Y-
526 axis to more clearly show effects of glycosylation on the completeness of neutralization
527 at high antibody concentrations

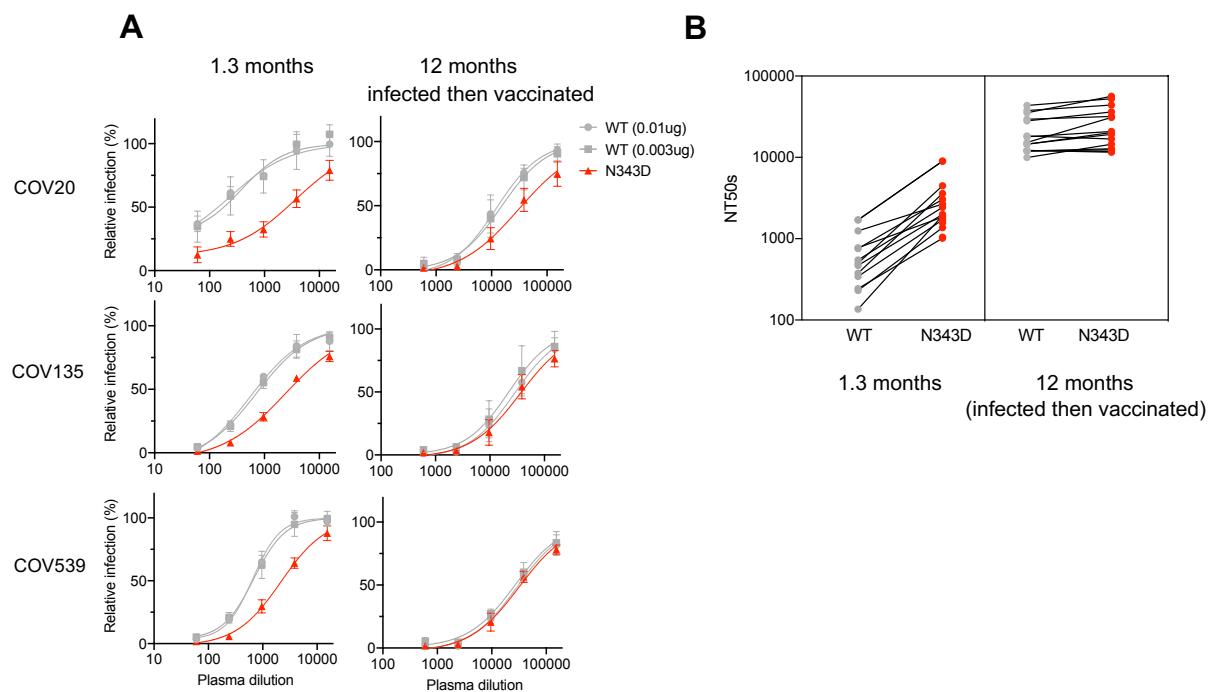


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FIG 4 Proximity of N343 to antibody binding sites

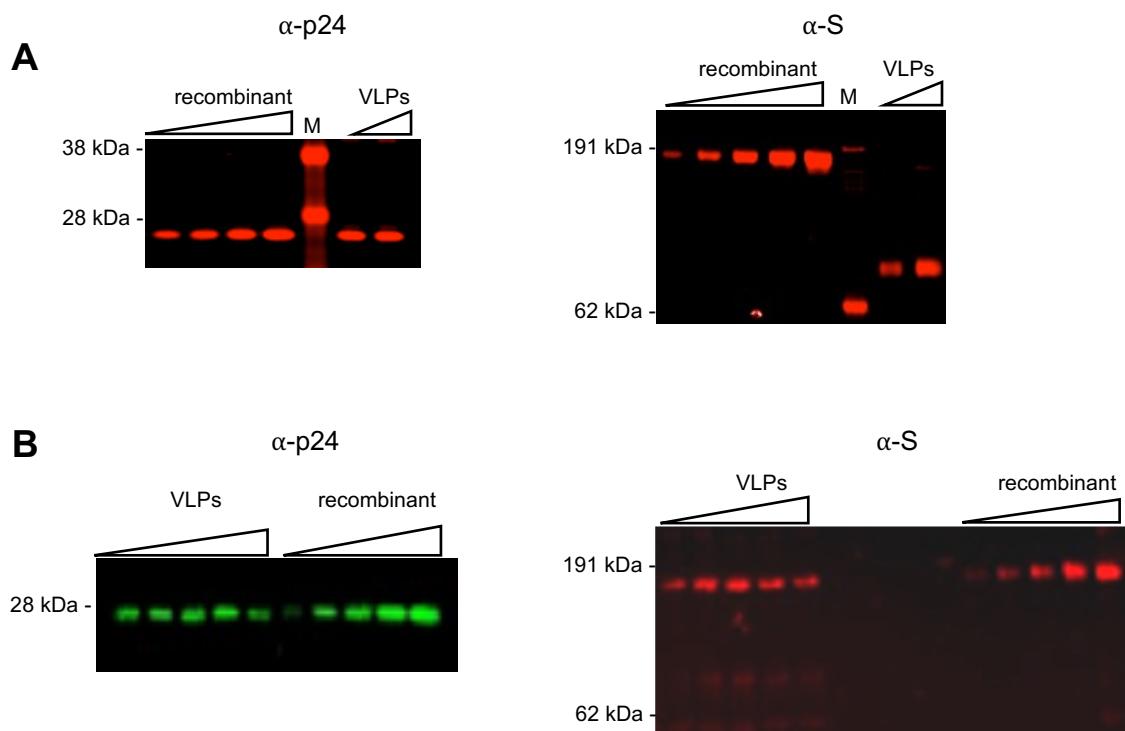
(A) Surface representation of the receptor-binding domain (RBD) in an X-ray crystal structure (PDB ID: 7K8M). ACE2-binding site and the N343 glycosylation site are highlighted in palegreen and red, respectively.

(B) Views of antibody Fab variable domains (blue) binding to spike (grey), in which each trimer subunit, is shown with distinct gray shade. ACE2-binding site and N343 are highlighted in pale green and red, respectively. The structures illustrated herein are C098 (PDB ID: 7N3I), C121 (PDB ID: 7K8X), C144 (PDB ID: 7K90), C135 (PDB ID: 7K8Z) and C118 (PDB ID: 7RKV).

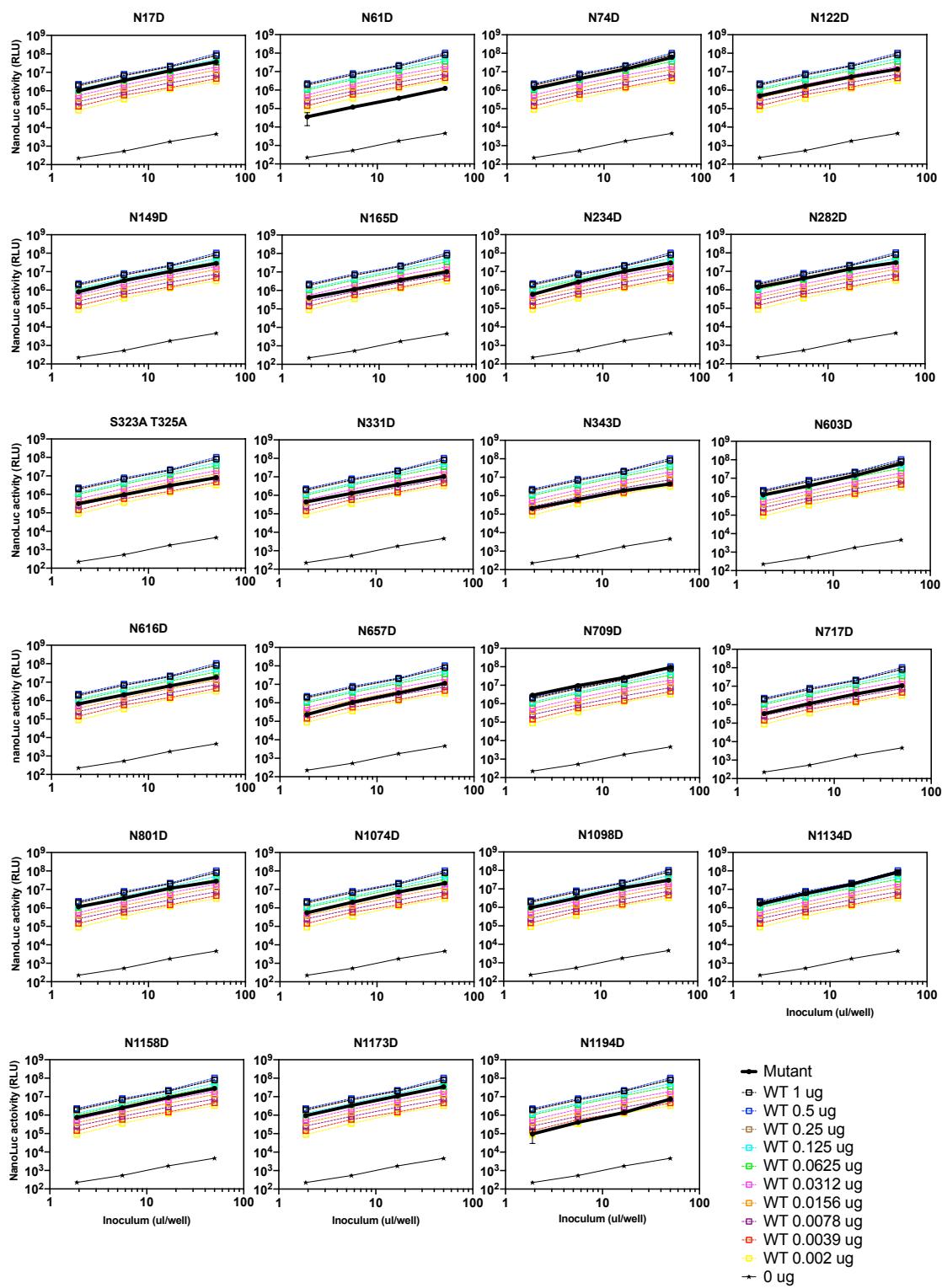


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540 **FIG 5 Neutralization sensitivity of N343D mutant to convalescent plasma**
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(A) Plasma neutralization of N343D or glycosylation site intact spike (in the furin uncleavable (R683G) background) pseudotyped virus using 293T/ACE2.cl22 target cells. The convalescent plasma samples were collected at 1.3 months and 12 months (infected then vaccinated) and diluted four-fold serially followed by incubation with viruses. As controls, glycosylation intact spike expression plasmid (WT in the R683G background) was transfected at two doses, 10 ng or 3 ng, and the neutralization sensitivity of the resulting viruses were assessed in parallel. The mean and range of two technical replicates are shown.
(B) Comparison of NT₅₀ values for each of the 15 convalescent plasma samples collected at 1.3 months and at 12 months (infected then vaccinated) for the N343D or glycosylation intact spike pseudotypes.



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553 **FIG S1 Western blot analysis of virions, using recombinant proteins as standard**
554 (A) Western blot analysis of virions pelleted through 20% sucrose from 100 μ l
555 supernatant harvested at 48 hours after transfection with 0.0625 μ g or 0.5 μ g of wild-
556 type spike expression plasmid along with envelope-deficient HIV-1 proviral plasmid
557 expressing NanoLuc luciferase. The blot was probed with an anti-p24 antibody and
558 recombinant HIV p24 protein was used as a standard (1.0 ng, 2.0 ng, 4.0 ng, or 8.0 ng
559 per lane) on the left, or with anti-Spike antibody using recombinant S-6P-nanoLuc as a
560 standard (0.25 ng, 0.5 ng, 1.0 ng, 2.0 ng, or 4.0 ng per lane) on the right.
561 Representative of two independent experiments.
562 (B) Western blot analysis of virions pelleted through 20% sucrose from 100 μ l
563 supernatant harvested at 48 hours after transfection with 0.008 μ g, 0.024 μ g, 0.073 μ g,
564 0.22 μ g, or 0.67 μ g of wild-type spike expression (furin uncleavable R683G background)
565 along with envelope-deficient HIV-1 proviral plasmid expressing NanoLuc luciferase.
566 The blot was probed with anti-p24 antibody using recombinant HIV p24 protein as
567 standard (0.5 ng, 1.0 ng, 2.0 ng, 4.0 ng, or 8.0 ng per lane) on the left, or with anti-Spike
568 antibody using recombinant S-6P-nanoLuc as standard (0.125 ng, 0.25 ng, 0.5 ng, 1.0
569 ng, or 2.0 ng per lane) on the right. Representative of two independent experiments.



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FIG S2 The impact of glycosylation site mutations on particle infectivity

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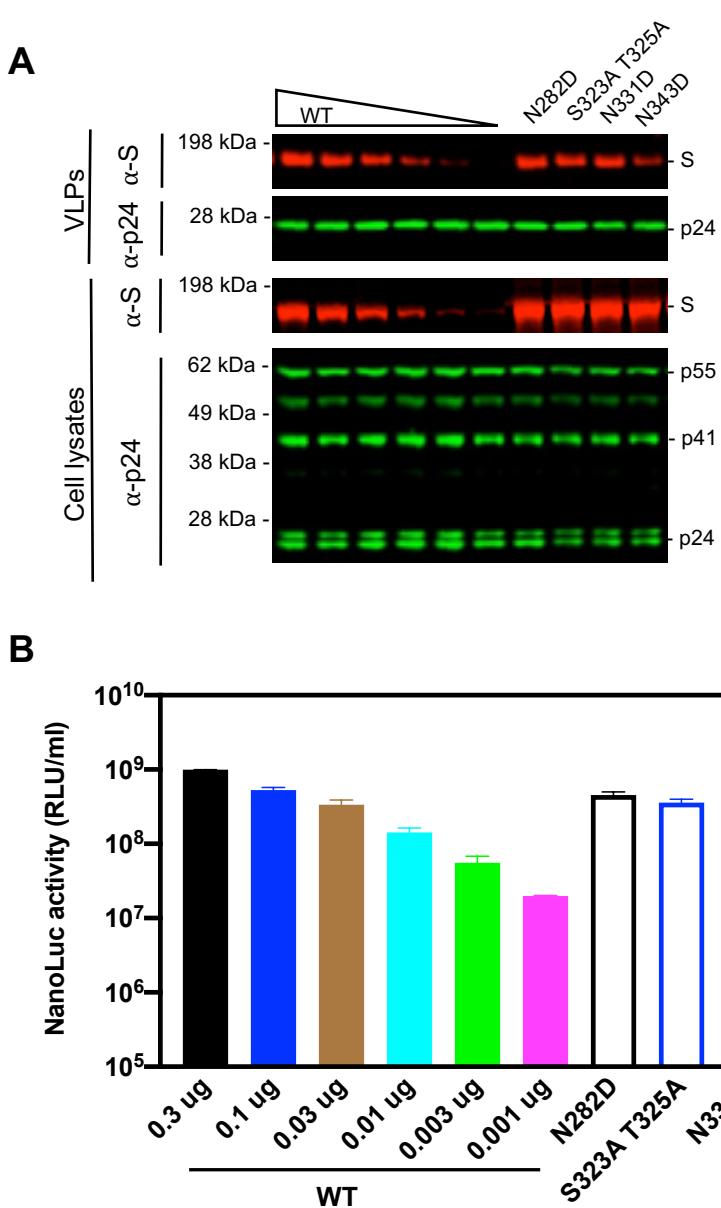
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Infectious virion measurements of for pseudotypes bearing glycosylation site mutant spike proteins (bold black lines, 1 μ g transfected S expression plasmid), compared with as well as WT S pseudotypes (dashed lines) collected from 293T cells transfected with various amounts of spike expression plasmids. Infection was quantified by measuring

576 NanoLuc luciferase activity (RLU). Virus generated in the absence of S (0 µg), shown in
577 thin black line, was used as a background control. 293T/ACE2.cl22, as target cells,
578 were infected with the indicated volumes of pseudotyped viruses in 96-well plates and
579 harvested 48 hours post infection for NanoLuc luciferase assay. The mean and range
580 deviation from two technical replicates are shown.
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FIG S3 The impact of glycosylation site mutations in the furin uncleavable (R683G) background on spike incorporation and particle infectivity

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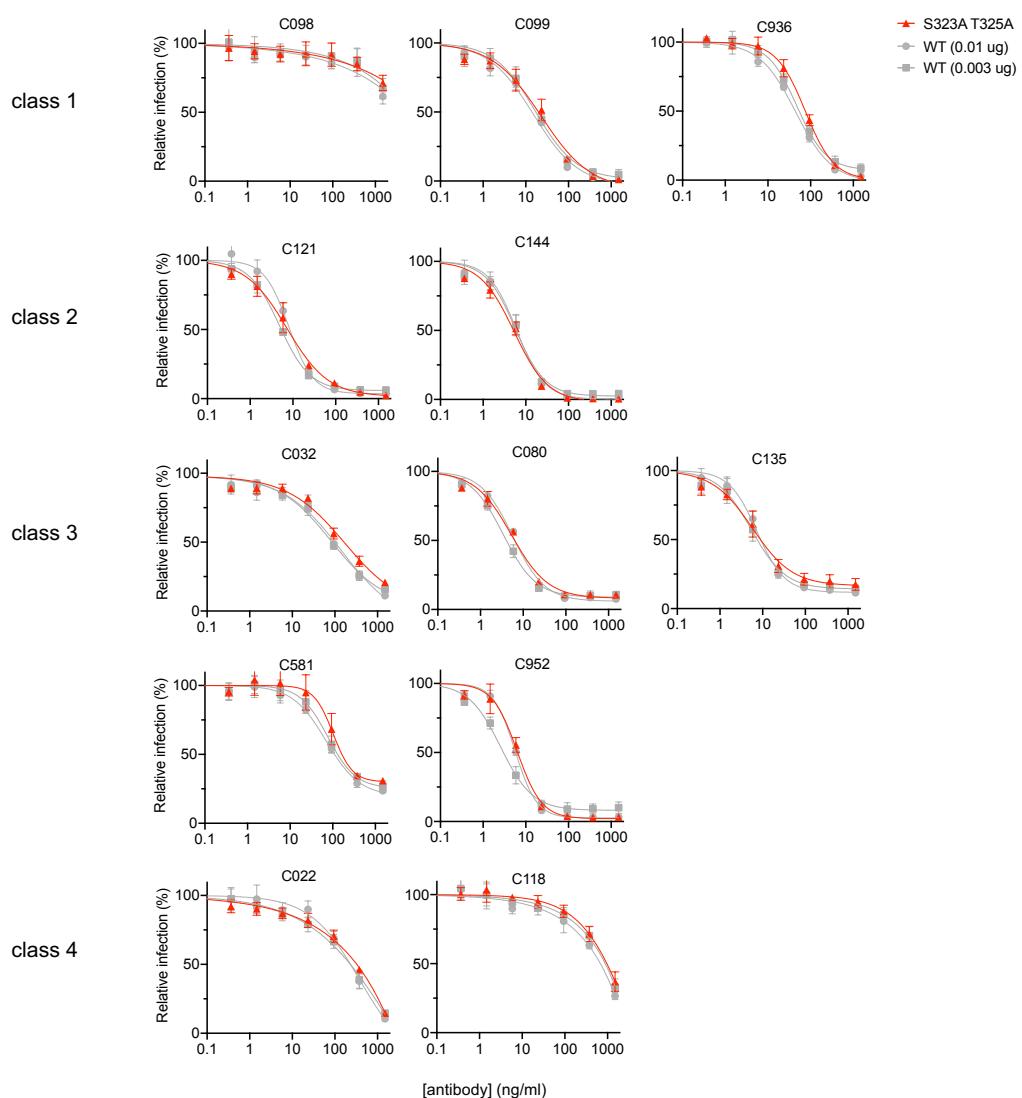
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(A) Western blot analysis of 293T cell lysates (lower panel) or virions (upper panel) at 48 hours after transfection with various amounts of glycosylation site intact spike expression plasmid (R683G background), or 1 μ g of glycosylation site mutants (N282D, S323A T325A, N331D, or N343D) along with envelope-deficient HIV-1 proviral plasmid expressing NanoLuc.

(B) Infectivity was quantified by measuring NanoLuc luciferase activity (RLUs) following infection of 293T expressing ACE2 (293T/ACE2.cl22) in 96-well plates with pseudotyped viruses as depicted in (A). The mean and range of two technical replicates are plotted.

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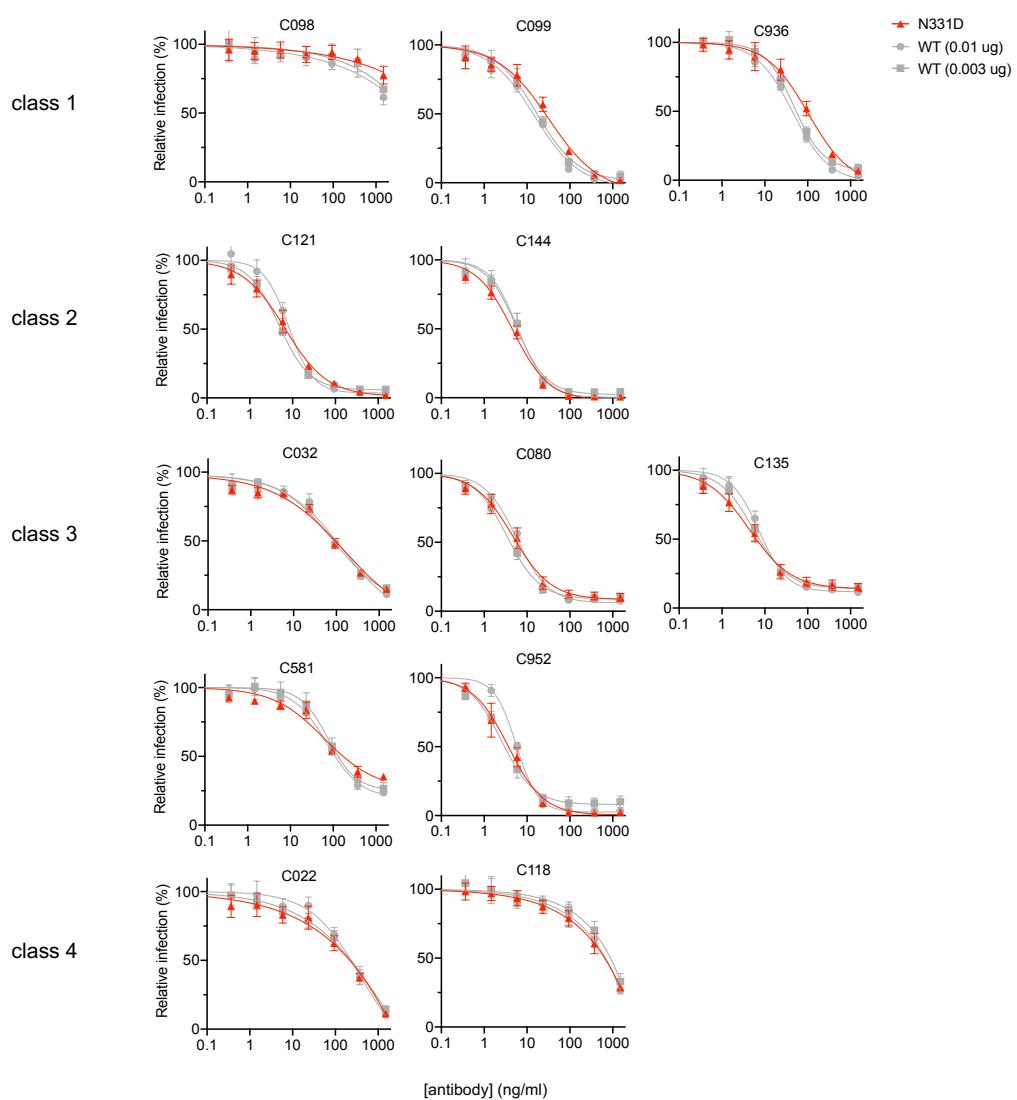


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598 **FIG S4 Mutations of the O-linked glycosylation sites at 323 and 325 in the RBD** 599 **(S323A T325A) have marginal effect on neutralization sensitivity**

600 Neutralization of glycosylation site mutant S323A T325A pseudotyped virus infection in
601 the presence of the indicated concentrations of a panel of monoclonal antibodies,
602 including class 1 (C098, C099, and C936), class 2 (C121 and C144), class 3 (C032,
603 C080, C135, C581, and C952), and class 4 (C022 and C118) antibodies. As controls,
604 glycosylation intact spike expression plasmid (WT in the furin uncleavable R683G
605 background) was transfected at two doses, 10 ng or 3 ng, and the resulting viruses
606 were assessed for neutralization sensitivity in parallel. The mean and range of two
607 technical replicates are shown.
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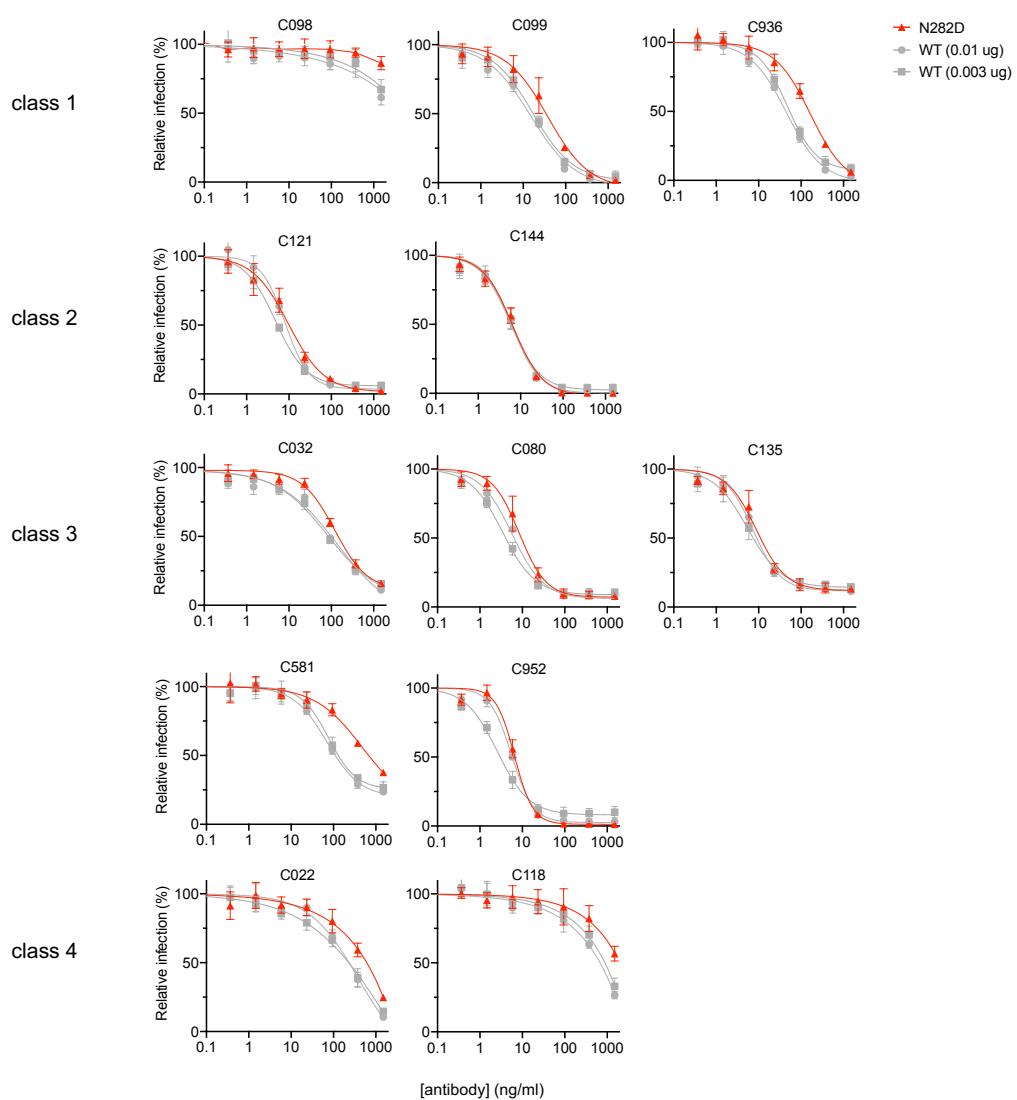


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611 **FIG S5 Effect of glycosylation at N331 on neutralization sensitivity**

612 Neutralization of glycosylation site mutant N331D pseudotyped virus infection in the
613 presence of the indicated concentrations of a panel of monoclonal antibodies, including
614 class 1 (C098, C099, and C936), class 2 (C121 and C144), class 3 (C032, C080, C135,
615 C581, and C952), and class 4 (C022 and C118) antibodies. As controls, glycosylation
616 intact spike (WT in the furin uncleavable R683G background) was transfected at two
617 doses, 10 ng or 3 ng, and the resulting viruses were assessed for neutralization
618 sensitivity in parallel. The mean and range of two technical replicates are shown.
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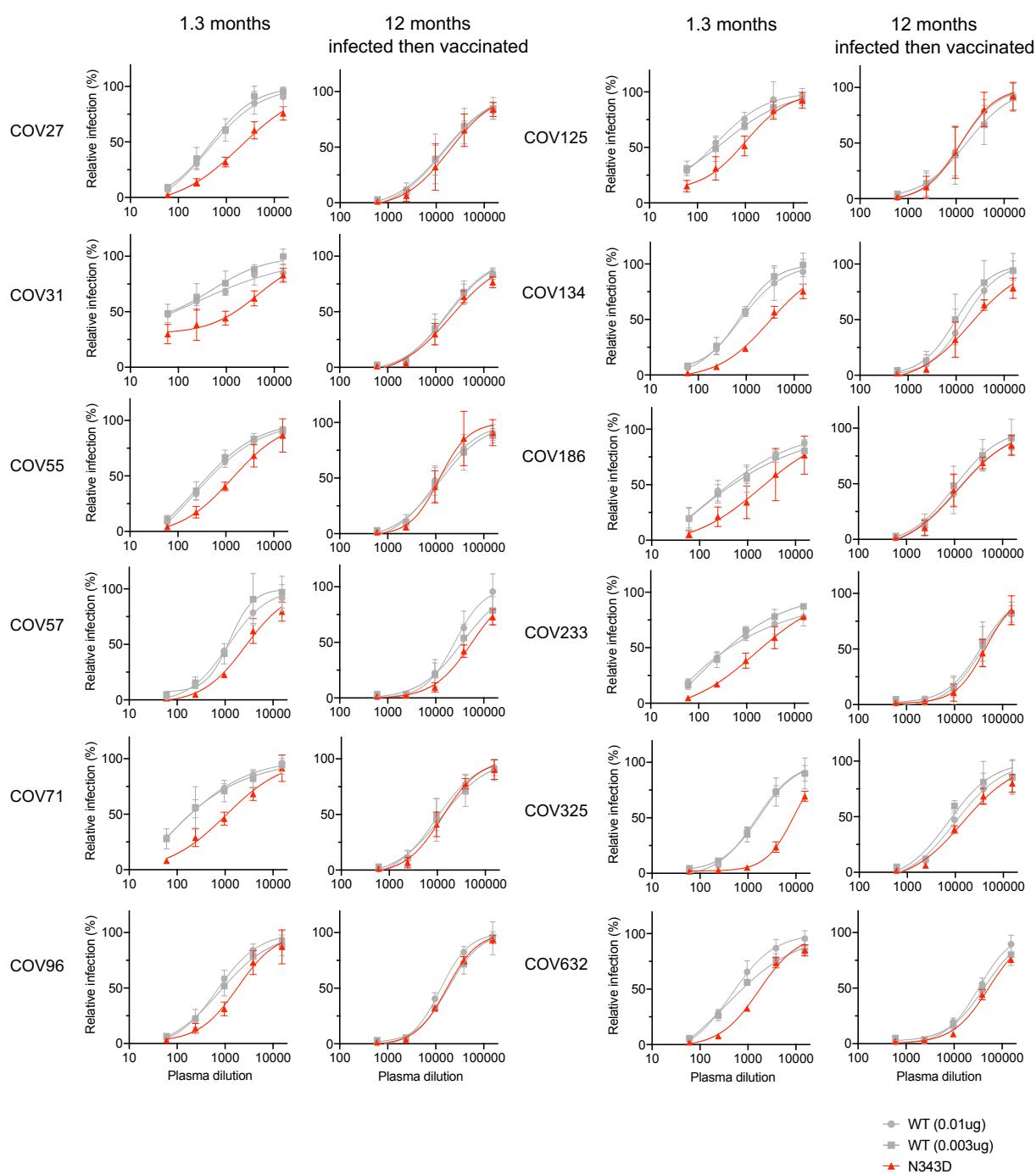
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FIG S6 Effect of glycosylation at N282 on neutralization sensitivity

622 Neutralization of glycosylation site mutant N282D pseudotyped virus infection in the
623 presence of the indicated concentrations of a panel of monoclonal antibodies, including
624 class 1 (C098, C099, and C936), class 2 (C121 and C144), class 3 (C032, C080, C135,
625 C581, and C952), and class 4 (C022 and C118). As controls, glycosylation intact spike
626 (WT in the furin uncleavable R683G background) was transfected at two doses, 10 ng
627 or 3 ng, and the resulting viruses were assessed for neutralization sensitivity in parallel.
628 The mean and range of two technical replicates are shown.
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FIG S7 Neutralization sensitivity of N343D mutant to convalescent plasma

633 Additional examples of plasma neutralization of N343D or glycosylation site intact spike
 634 (in the furin uncleavable R683G background, same as FIG 5) pseudotyped virus using
 635 293T/ACE2.cl22 target cells. The mean and range of two technical replicates are
 636 shown.
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