

1 **A novel sialylation site on *Neisseria gonorrhoeae* lipooligosaccharide links**
2 **heptose II lactose expression with pathogenicity**

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17 **Running title:** HepII lactose sialylation in gonococcal pathogenicity

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22 **Abstract**

23 Sialylation of lacto-*N*-neotetraose (LNnT) extending from heptose I (HepI) of
24 gonococcal lipooligosaccharide (LOS) contributes to pathogenesis. Previously,
25 gonococcal LOS sialyltransferase (Lst) was shown to sialylate LOS in Triton X-100
26 extracts of strain 15253, which expresses lactose from both HepI and HepII, the minimal
27 structure required for mAb 2C7 binding. Ongoing work has shown that growth of 15253
28 in cytidine monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac)-containing media
29 enables binding to CD33/Siglec-3, a cell surface receptor that binds sialic acid,
30 suggesting that lactose termini on LOS of intact gonococci can be sialylated. Neu5Ac
31 was detected on LOSs of strains 15253 and a MS11 mutant with only lactose from HepI
32 and HepII by mass spectrometry; deleting HepII lactose rendered Neu5Ac undetectable.
33 Resistance of HepII lactose Neu5Ac to desialylation by α 2-3-specific neuraminidase
34 suggested an α 2-6-linkage. Although not associated with increased factor H binding,
35 HepII lactose sialylation inhibited complement C3 deposition on gonococci. 15253
36 mutants that lacked Lst or HepII lactose were significantly attenuated in mice,
37 confirming the importance of HepII Neu5Ac in virulence. All 75 minimally passaged
38 clinical isolates from Nanjing, China, expressed HepII lactose, evidenced by reactivity
39 with mAb 2C7; mAb 2C7 was bactericidal against the first 62 (of 75) isolates that had
40 been collected sequentially and were sialylated before testing. mAb 2C7 effectively
41 attenuated 15253 vaginal colonization in mice. In conclusion, this novel sialylation site
42 could explain the ubiquity of gonococcal HepII lactose *in vivo*. Our findings reiterate the
43 candidacy of the 2C7 epitope as a vaccine antigen and mAb 2C7 as an
44 immunotherapeutic antibody.

45 **Introduction**

46 Gonorrhea affects about 78 million people annually worldwide (1); almost
47 470,000 of these are reported in the U.S. Multidrug-resistant gonorrhea has been
48 reported on every continent and is a serious public health concern (2-6). Understanding
49 how gonococci evade host immune defenses is an important step toward development
50 of urgently needed safe and effective vaccines and novel therapeutics against this
51 infection.

52 *N. gonorrhoeae* is a uniquely human-adapted pathogen (7). On a molar basis,
53 lipooligosaccharide (LOS) is the most abundant gonococcal outer membrane molecule
54 and plays a key role in many facets of pathogenesis (8-19). Host-like glycans
55 expressed by lipooligosaccharide (LOS) of *N. gonorrhoeae* constitutes an example of
56 molecular mimicry (8, 20). Two structures expressed by Neisserial LOS from heptose
57 (Hep) I that mimic host glycans include lacto-*N*-neotetraose (LNnT; Gal β 1-4GlcNAc β 1-
58 3Gal β 1-4Glc β 1-), identical to the terminal tetrasaccharide of paragloboside, a precursor
59 of the major human blood group antigens (21), and globotriose (Gal α 1-4Gal β 1-4Glc β 1-)
60 that is identical to terminal globotriose trisaccharide of the P^K-like blood group antigen
61 (22). The seminal work of Harry Smith and colleagues showed that *N. gonorrhoeae*
62 scavenge cytidine monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac) from its host
63 to sialylate its LOS (23, 24). Both, LNnT and P^K-like LOSs can be sialylated (25, 26).
64 LOS sialylation inhibits complement activation and converts strains that are otherwise
65 sensitive to killing by complement in serum to a 'serum (or complement)-resistant'
66 phenotype (16, 27-29). Several other microbes also use sialic acid expression to their

67 advantage to subvert host immunity (30-41), by mimicking host sialic acid-based “self-
68 associated molecular patterns”

69 Among members of the species *Neisseria*, the gonococcus uniquely expresses
70 lactose extending from HepII. A monoclonal antibody (mAb) called 2C7 binds an
71 epitope on LOS that requires expression of HepI and HepII lactose. Despite being under
72 control of a phase variable LOS glycosyltransferase (*lgt*) gene called *lgtG*, HepII lactose
73 is expressed by ~95% of clinical isolates of *N. gonorrhoeae* (42), which suggests a key
74 role in virulence. Isogenic mutant strains that lack *lgtG* show decreased virulence in the
75 mouse vaginal colonization model of gonorrhea (43). Why HepII lactose promotes
76 gonococcal virulence remains unclear. In recent work, we noted that growth of a
77 gonococcal strain called 15253 that expresses lactose simultaneously from HepI and
78 HepII (44) was capable of binding to Siglec-3 when grown in CMP-Neu5Ac-containing
79 media (45). Siglec-3 binds exclusively to sialyoglycans (46, 47). These data suggested
80 that lactose expressed by *N. gonorrhoeae* LOS could also be sialylated. This study
81 describes sialylation of gonococcal LOS lactose termini and elucidates its function in
82 complement evasion and virulence.

83

84

85 Results

86 **Neu5Ac 'caps' *N. gonorrhoeae* HepII lactose.** LOS glycan extensions from
87 HepI and HepII for the strains used in this study are shown in Fig. 1. The ability of
88 strains 15253 and MS11 2-Hex/G+, which express lactose extending from HepI and
89 HepII, and their respective isogenic mutants, 15253/G- and MS11 2-Hex/G-, which
90 express only lactose only from HepI, to add Neu5Ac to LOS was determined by SDS-
91 PAGE. MS11 4-Hex/G- (expresses LNnT LOS from HepI) was used as a positive
92 control for sialylation. An *lst* deletion mutant of 15253 (15253 Δ /*lst*) that lacks the ability
93 to sialylate its LOS was also tested to address whether the previously described Lst
94 enzyme is also responsible for sialylation of lactose.

95 Bacteria were grown in media alone, or media supplemented with 100 μ g/ml
96 CMP-Neu5Ac for maximal LOS sialylation. As shown in Fig. 2, the upper band in the '+'
97 lane of 2-Hex/G+ shows slower mobility compared to the upper band in the
98 corresponding '-' lane. Note that despite fixing *igtG* 'on', MS11 2-Hex/G+ expresses an
99 LOS species with only HepI lactose (i.e., the LOS expressed by MS11 2-Hex/G-). This
100 is because of export of LOS to the outer membrane prior to addition of the proximal Glc
101 on HepII, as noted previously (48). Similarly, retarded mobility of 15253 LOS was also
102 observed when grown in media containing CMP-Neu5Ac. The LOS of 4-Hex/G-
103 (positive control for sialylation) incorporated Neu5Ac and migrated slower. There was
104 no appreciable alteration in LOS migration when 2-Hex/G-, 15253/G- or 15253 Δ /*lst*
105 were grown in CMP-Neu5Ac, suggesting that Neu5Ac was added to the terminal Gal of
106 HepII lactose and that Lst was the enzyme responsible for sialylation.

107 Mass spectroscopic analysis of LOS purified from strains 15253, 15253/G-,
108 MS11 2-Hex/G+ and MS11 2-Hex/G- grown in CMP-Neu5Ac and unsialylated 15253
109 (negative control) is shown in Supplemental Table S1. The data confirm the presence of
110 sialic acid on 15253 and MS11 2-Hex/G+, but not on their isogenic mutants lacking
111 HepII lactose. Collectively, the data strongly suggest that Neu5Ac is added to HepII
112 lactose.

113

114 **Sialylation of HepII lactose does not enhance FH binding.** Previously, we
115 showed that sialylation of *N. gonorrhoeae* LNnT LOS, but not P^K-like LOS, enhances
116 human FH binding (26). We used strain MS11 to determine whether sialylation of HepII
117 lactose enhances FH binding because this strain expresses PorB.1B and binds FH
118 relatively weakly in the unsialylated state (49), which would more readily reveal
119 increased FH binding with sialylation, if it were to occur. By contrast, strain 15253
120 (PorB.1A) binds high levels of FH even when unsialylated (49), which would limit the
121 ability to detect an increase in FH binding with sialylation. FH binding to MS11 LOS
122 mutants that expressed 2, 3 or 4 hexoses from HepI, each with (G+) or without (G-)
123 HepII lactose was examined. The 3-Hex (P^K-like LOS) and 4-Hex (LNnT) mutants
124 served as negative and positive controls for FH binding with sialylation, respectively. As
125 shown in Fig. 3, growth of the 2-Hex mutants in CMP-Neu5Ac did not enhance FH
126 binding. Thus, enhanced FH binding to *N. gonorrhoeae* is restricted to LNnT LOS.

127

128 **HepII lactose sialylation regulate complement activation.** Neu5Ac capping of
129 gonococcal LNnT and P^K-like LOS both inhibit complement. *N. gonorrhoeae* bind C4BP

130 and FH in a human-specific manner (50, 51). Initial attempts at measuring human C3
131 fragment deposition by flow cytometry on the two unsialylated strains using normal
132 human serum revealed levels too low to discern the effects of sialylation on C3
133 deposition. Therefore, we used mouse complement, whose C4BP and FH do not bind
134 *N. gonorrhoeae*, to study the effects of LOS sialylation on C3 deposition. Consistent
135 with the addition of Neu5Ac to HepII lactose, C3 deposition decreased only on wild-type
136 15253 grown in media containing CMP-Neu5Ac (Fig. 4). Neither 15253/G- nor 15253
137 Δ/st inhibited C3 deposition when grown in CMP-Neu5Ac, consistent with the inability of
138 these two mutant strains to sialylate their LOSs.

139 We next examined the effects of sialylation on complement activation on six
140 MS11 LOS mutants (Fig. 5). The sialylatable 3-Hex and 4-Hex strains served as
141 controls for Neu5Ac-mediated complement inhibition. Growth in media containing CMP-
142 Neu5Ac decreased C3 deposition all tested strains. Similar to 15253, sialylation of
143 MS11 2-Hex/G+ also inhibited C3 deposition. In contrast to 15253/G-, we noted a
144 reproducible decrease in C3 deposition on the 2-Hex/G- mutant, suggesting that
145 Neu5Ac was also added to the LOS of this strain. The degree of inhibition seen with
146 sialylation of MS11 2-Hex/G- (a 4.1-fold decrease compared to the unsialylated parent,
147 and 7.3-fold above baseline conjugate control levels) was less than that seen with
148 complement inhibition upon sialylation of MS11 2-Hex/G+ (a 21-fold decrease
149 compared to unsialylated 2-Hex/G+, and only 3-fold greater fluorescence compared to
150 the baseline conjugate control). This amount of LOS sialylation of MS11 2-Hex/G- LOS,
151 although functional, was too small to be appreciated by changes in mobility on a Tricine
152 gel or by MS analysis.

153 To discern the sialic acid linkage to HepII substituted lactose we examined the
154 effect of α 2-3-linkage-specific neuraminidase on mouse C3 deposition (Fig. 6).
155 Sialylated 2-Hex/G+ and the corresponding wild-type strain 15253 that possessed the
156 same pattern of Hep I and Hep II hexose substitutions, failed to show increased C3
157 deposition after treatment with recombinant α 2-3-specific sialidase followed by
158 incubation with 15% normal mouse serum; resistance to α 2-3-sialidase suggests an α 2-
159 6 linkage.

160

161 **Loss of sialic acid on HepII lactose impairs *N. gonorrhoeae* vaginal**
162 **colonization in mice.** The ability of 15253 (wild-type), 15253/G- and 15253 Δ lst to
163 colonize the genital tract of *Cmah* knockout (KO) mice was compared. *Cmah* KO mice
164 lack the enzyme CMP-*N*-acetylneuraminic acid hydroxylase (CMAH) and akin to
165 humans, cannot convert Neu5Ac to Neu5Gc. Thus, these mice provide a 'human-like'
166 sialic acid milieu to study the effects of LOS sialylation on virulence. *Cmah* KO mice
167 support *N. gonorrhoeae* colonization slightly better than control wild-type BALB/c mice
168 (52). Loss of either HepII lactose or Lst significantly attenuated the duration and burden
169 of bacterial colonization (Fig. 7A-C). These data provide strong evidence for the
170 importance of sialylation of HepII lactose in gonococcal virulence. We were unable to
171 evaluate the effects of LOS sialylation in MS11 2-Hex/G+ because this strain colonized
172 mice for only 3 days (data not shown).

173

174 **Effect of sialylation on mAb 2C7 binding and efficacy.** mAb 2C7 targets a
175 LOS epitope being developed as a gonococcal vaccine candidate. The minimal LOS

176 structure required for mAb 2C7 binding are lactoses simultaneously extending from both
177 HepI and HepII. Glycan extensions beyond lactose on HepII, for example with GalNAc-
178 Gal seen in a mutant strain selected under pyocin pressure called JW31R, abrogates
179 mAb 2C7 binding (53). We therefore asked whether sialylation of HepII lactose affected
180 mAb 2C7 binding and function. While sialylation of 15253 did not affect mAb 2C7
181 binding (Fig. 8A, left graph), sialylation of MS11 2-Hex/G+ resulted in a reproducible ~2-
182 to 3-fold reduction in mAb 2C7 binding (Fig. 8A, right graph). Similar binding of mAb
183 2C7 to sialylated and unsialylated 15253 allowed us to assess the functional effects of
184 HepII lactose sialylation when antibody binding was kept constant. As shown in Fig. 8B,
185 increasing amounts of CMP-Neu5Ac in media caused a dose-dependent decrease in
186 killing by mAb 2C7.

187

188 **mAb 2C7 is active against strain 15253 in vivo.** In light of prior work that
189 showed the importance of LOS sialylation for infection of mice (54, 55) and the
190 observed resistance of sialylated 15253 to mAb 2C7 *in vitro* (Fig. 8), we examined the
191 efficacy of mAb 2C7 versus 15253 in the BALB/c mouse vaginal colonization model. A
192 'passive immunization model' to address the efficacy of mAb 2C7 to simulate effects of
193 vaccine antibody was used (43). Wild-type BALB/c mice (n=8) were administered mAb
194 2C7 10 µg intraperitoneally on Days -2, -1 and 0, and CFUs were monitored daily. The
195 control group (n=7) received mouse IgG3. mAb 2C7 significantly shortened the duration
196 and burden of infection with 15253 (Fig. 9A-C).

197

198 **Expression of the 2C7 epitope by contemporary clinical isolates of *N.***
199 ***gonorrhoeae***. Despite being under control of a phase variable gene, *lgtG*, the 2C7 LOS
200 epitope (Fig. 10A) was expressed by 94% of gonococci recovered directly from cervical
201 secretions from a cohort of women who attended a sexually transmitted disease (STD)
202 clinic in Boston (42). We examined a collection of minimally (≤ 3) passaged isolates
203 cultured from the female contacts of men with gonorrhea who were referred to a STD
204 clinic in Nanjing, China, for expression of the 2C7 LOS epitope by whole cell ELISA. We
205 also examined isolates for their ability to bind to mAb L8 (recognizes an epitope defined
206 by HepI lactose and phosphoethanolamine [PEA] substitution at the 3-position on HepII;
207 expression of HepII lactose abrogates mAb L8 binding) and mAb 3F11 (recognizes
208 terminal [unsialylated] lactosamine of LNnT) (Fig. 10A). As shown in Fig. 10B, each of
209 75 isolates bound to mAb 2C7, albeit to varying degrees, as did mAb L8 and 3F11. mAb
210 L1 barely bound to any of the tested isolates.

211 We next assessed the ability of mAb 2C7 to mediate complement-dependent
212 bactericidal activity against the first 62 of 75 isolates collected from men with urethritis in
213 a Nanjing (China) study of gonococcal transmission from men to women. Because
214 some of the strains were sensitive to killing by 16.7% pooled normal human serum
215 (NHS) that was used as the complement source, all isolates were grown in media
216 containing 2 $\mu\text{g/ml}$ CMP-Neu5Ac to render them fully serum resistant ($>100\%$ survival).
217 All (100%) of isolates were killed $>50\%$ in the presence of 5 $\mu\text{g/ml}$ of mAb 2C7 and
218 NHS. This included two of four isolates that bound very low levels of mAb 2C7 by ELISA
219 ($\text{OD}_{450\text{nm}}$ between 0.065 and 0.090). Further, serum bactericidal activity correlated with
220 levels of mAb 2C7 binding (Fig. 10C).

221 Discussion

222 The novel finding in this report is the presence of Neu5Ac on *N. gonorrhoeae*
223 HepII lactose. To our knowledge, *N. gonorrhoeae* is the only member of the genus
224 Neisseria that expresses lactose extending from HepII. Certain *N. meningitidis* strains
225 possess *IgtG* and can substitute Glc at the 3 position of HepII (seen in LOS
226 immunotypes L2 and L4 (56, 57)), but extensions beyond the proximal Glc in
227 meningococci have not been described. Prior work by Mandrell et al provided evidence
228 for the ability of 15253 Lst to sialylate lactose, although not in context of intact bacteria;
229 LOS in Triton X-100 extracts of strain 15253 (which also contains Lst) could incorporate
230 radiolabeled Neu5Ac when supplied with exogenous CMP-[¹⁴C]-Neu5Ac (58).

231 mAb 2C7 recognized 94% of 68 gonococci examined directly from cervical
232 secretions express and 95% of 101 randomly chosen fresh (second passage)
233 gonococcal isolates from a sexually transmitted disease clinic in Boston (42). We
234 recently surveyed 75 minimally passaged gonococcal isolates from Nanjing, China and
235 noted that 100% of isolates reacted with mAb 2C7. All strains also expressed LNnT,
236 which suggests that both sialylatable glycans are important for gonococcal
237 pathogenesis. The importance of LNnT sialylation, both in humans and in the mouse
238 vaginal colonization model has been established (12, 54, 55, 59). Phase variability of
239 *IgtA* and *IgtD* control expression of LNnT (17). *IgtA* and *IgtC* both 'off' would result in
240 expression of lactose, while the combination of *IgtA* off and *IgtC* on would result in
241 elaboration of the P^K-like (3-Hex) structure. If *IgtA* and *IgtD* are both 'on', GalNAc is
242 added to the terminal Gal of LNnT and prevents sialylation. Sialic acid likely plays a
243 multifaceted role in Neisserial pathogenesis. In addition to inhibiting complement,

244 enhancing resistance to opsonophagocytosis and cationic antimicrobial peptides (60,
245 61), Neu5Ac engages sialic acid-binding immunoglobulin-type lectins (Siglecs) many of
246 which are, in turn, linked to an immunoreceptor tyrosine-based inhibition (ITIM) motif
247 and inhibit the inflammatory response (62). Neu5Ac has also been identified in
248 gonococcal biofilms (63). Because it can also sialylate HepII lactose, the gonococcus
249 has the capacity to maintain LOS sialylation even when the previously described
250 sialylatable LNnT or P^K-like structures is not expressed from HepI. While HepII lactose
251 can be sialylated when HepI also expresses lactose, it is unclear whether HepII lactose
252 can be sialylated when LNnT or P^K is also expressed on HepI. Gilbert and colleagues
253 showed that meningococcal Lst could add Neu5Ac to 6(5-fluorescein-carboxamido)-
254 hexanoic acid succinimidyl ester (FCHASE)-aminophenyl-lactose. Lst added ~6.4-fold or
255 ~3.2-fold more Neu5Ac onto lactosamine (LNnT is lactosamine-lactose) compared to
256 lactose at substrate concentrations of 0.2 mM or 1.0 mM, respectively (64). Based on
257 these data, we speculate that LNnT is preferentially sialylated over lactose when both
258 glycan species are expressed.

259 The importance of Neu5Ac on HepII lactose in pathogenesis was illustrated by
260 attenuation of 15253 Δ lst in the mouse vaginal colonization model. Thus, unsialylated
261 HepII lactose does not support virulence in this model. Expression of HepII lactose by
262 almost all clinical isolates highlights the importance of maintaining *IgtG* 'on' *in vivo*. We
263 are not aware of any naturally occurring gonococcal isolate that lacks *IgtG*. We have
264 shown previously that a *IgtG* deletion mutant of *N. gonorrhoeae* FA1090 was less
265 virulent than its wild-type parent (43). Lam and Gray-Owen showed that serial passage
266 of *N. gonorrhoeae* in mice resulted an increased fraction of mice infected with each

267 subpassage and in a reproducible selection of variants with IgtG 'on', providing further
268 strong evidence of the importance of HepII lactose expression in vivo (65).

269 We noted that MS11 2-Hex/G- inhibited mouse complement when grown in
270 CMP-Neu5Ac. The amount of Neu5Ac incorporation onto HepI lactose in this mutant
271 was likely too small to be detected by shifts on SDS-PAGE or by MS analysis, but was
272 nevertheless sufficient for functional activity, limited as it was. In contrast, 15253/G-,
273 which also expresses only lactose from HepI, did not inhibit mouse C3 deposition when
274 grown in CMP-Neu5Ac-containing media, suggesting that the extent and influence of
275 HepI lactose sialylation on function may differ across strains. Whether a difference in
276 HepI lactose sialylation exists between the two strains is unclear despite differences in
277 function, but could relate to differences in Lst sequence and/or levels of Lst activity.
278 Translation of DNA sequences of the *lst* ORF of 15253 and MS11 showed a single
279 amino acid sequence difference; 15253 possessed a Q (seen in 16 other *N.*
280 *gonorrhoeae* Lst sequences), while MS11 possessed an E (seen in >400 *N.*
281 *gonorrhoeae* Lst sequences) at position 266. The -35/-10 promoter sequence,
282 transcription start sites and the Shine-Dalgarno sequence were identical in 15253 and
283 MS11. Packiam et al showed wide variation in *lst* mRNA levels across gonococcal
284 strains, but mRNA levels often did not correlate with Lst activity as measured by
285 sialylation of Triton X-100 bacterial extracts (66).

286 Linkage of Neu5Ac is a key determinant of its ability to interact with the C-
287 terminus of FH. Blaum and colleagues showed that the interaction between sialic acid
288 and FH domain 20 is restricted only to α 2-3-linked Neu5Ac; α 2-6- or α 2-8-linked
289 Neu5Ac do not interact with FH (67). The Neu5Ac-lactose bond is resistant to α 2-3

290 specific sialidase, suggesting an α 2-6 linkage. In accordance with the findings of Blaum
291 et al, sialylation of HepII lactose – presumably through an α 2-6 linkage – did not
292 increase FH binding to *N. gonorrhoeae*. We acknowledge that further structural
293 characterization is necessary to confirm the nature of the Neu5Ac-lactose linkage. How
294 Neu5Ac on HepII lactose regulates complement remains unclear. Similar to LNnT
295 sialylation, Neu5Ac linked to LNnT may also inhibit the classical pathway by reducing
296 binding of IgG directed against select surface targets to the bacterial surface.

297 Despite similar amounts of mAb 2C7 binding to sialylated compared to
298 unsialylated 15253, the sialylated derivative was resistant (>50% survival) to mAb 2C7
299 plus human complement when exposed to CMP-Neu5Ac concentrations ≥ 3 μ g/ml. A
300 possible explanation is that targets for C4b and C3b on LOS (68), may be obscured by
301 the presence of Neu5Ac, thereby diminishing Ab efficacy. However, mAb 2C7 remained
302 effective against 15253 in the mouse vaginal colonization model, where the organism is
303 sialylated and additional factors such as opsonophagocytosis may contribute to its
304 bactericidal activity. Mouse FH and C4BP do not bind to gonococci (50, 51). Therefore,
305 the barrier that mAb 2C7 must surmount to activate complement on gonococci in wild-
306 type mice is likely to be lower than in humans. Ongoing studies have shown efficacy of
307 mAb 2C7 against wild-type strains MS11 and FA1090, which both bind C4BP and when
308 sialylated, also bind FH, in ‘dual’ human FH and C4BP transgenic mice (S.G, P.A.R and
309 S.R, unpublished observations) suggesting that mAb 2C7 can overcome the effects of
310 these complement inhibitors in vivo.

311 In conclusion, this novel site of sialylation on *N. gonorrhoeae* HepII lactose can
312 inhibit complement activation and also engage Siglecs (45). These findings also explain

313 the ubiquitous expression of HepII lactose (an integral part of the '2C7 LOS epitope')
314 among clinical isolates of *N. gonorrhoeae* and further validate targeting the 2C7 epitope
315 with antibody-based vaccines and immunotherapeutics.

316 **Materials and Methods**

317

318 **Bacterial strains.** Strain 15253 was recovered from an individual with
319 disseminated gonococcal infection and has been described previously (44). Only *IgtA*
320 and *IgtE* are intact in its *IgtA-E* locus (69). Deletion of *IgtG* in 15253 to yield 15253/G-
321 has been described previously (70). Deletion of LOS sialyltransferase (*Ist*) to yield
322 15253 Δ *Ist* (*Ist::kan^R*) was performed as described previously (54). All the LOS mutant
323 derivatives of MS11 have been described previously (48). The LOS phenotypes of
324 15253, 15253/G- and the MS11 mutants used in this study are listed in Figure 1.
325 Seventy five additional isolates were obtained from subjects enrolled in a transmission
326 study (Ref.) of gonococcal infection from men to women in Nanjing, China. All subjects
327 provided written informed consent in accordance with requirements by Institutional
328 Review Boards from: the University of Massachusetts Medical School; Boston
329 Univeristy School of Medicine and the Institute of Dermatology, Chinese Academy of
330 Medical Sciences & Peking Union Medical College, Nanjing, China.

331

332 **Normal human serum.** Serum was obtained from normal healthy adult
333 volunteers with no history of gonococcal or meningococcal infection who provided
334 informed consent. Participation was approved by the University of Massachusetts
335 Institutional Review Board for the protection of human subjects. Serum was obtained
336 from whole blood that was clotted at 25 °C for 30 min followed by centrifugation at 1500
337 g for 20 min at 4 °C. Serum from 10 donors was pooled, aliquoted and stored at -80 °C.

338

339 **Mouse complement.** Use of animals in this study was performed in strict
340 accordance with the recommendations in the Guide for the Care and Use of Laboratory
341 Animals of the National Institutes of Health. The protocol was approved by the
342 Institutional Animal Care and Use Committee (IACUC) at the University of
343 Massachusetts Medical School. Mouse blood obtained by terminal cardiac puncture
344 was allowed to clot for 20 min at room temperature, then placed on ice for 20 min and
345 centrifuged at 10,000 g for 10 min at 4 °C. Serum was harvested and stored in single-
346 use aliquots at -80 °C.

347

348 **Flow cytometry.** Factor H binding to *N. gonorrhoeae* was detected as described
349 previously (71). Briefly, $\sim 10^7$ bacteria in HBSS containing 1 mM CaCl₂ and 1 mM MgCl₂
350 (HBSS⁺⁺) containing 0.1% BSA was incubated with 10 µg/ml purified human FH
351 (Complement Technologies, Inc.) for 15 min at 37 °C. Bacteria-bound FH was detected
352 with goat anti-human FH (1 µg/ml) (Complement Technologies, Inc.), followed by anti-
353 goat IgG FITC (Sigma) at a dilution of 1:100. Bacteria were fixed in 1%
354 paraformaldehyde in PBS and Data were acquired on a FACSCalibur flow cytometer
355 and analyzed using FlowJo software.

356 Mouse C3 deposition on bacteria was measured by incubating 10^7 CFU of
357 bacteria in HBSS⁺⁺/BSA with mouse complement (concentration stated for each
358 experiment) for 20 min at 37 °C. Mouse C3 fragments deposited on bacteria were
359 detected using anti-mouse IgG FITC (MP Biomedicals) at a dilution of 1:100, and flow
360 cytometry was performed as described above.

361

362 **SDS-PAGE.** LOS in Protease K (Calbiochem)-treated bacterial lysates prepared
363 as described previously (48) in Tricine-SDS Sample Buffer (Boston Biomolecules) was
364 visualized by electrophoresis on Criterion™ 16% Tris-Tricine gels (Bio-Rad) using Tris-
365 Tricine-SDS Cathode buffer (Boston Biomolecules) at 100 V at 4 °C followed by silver
366 staining (Bio-Rad Silver Stain kit).

367
368 **Neuraminidase treatment.** Desialylation was carried out with α 2-3-specific
369 neuraminidase (New England Biolabs; Cat. No. P0743S). Approximately 10^7 bacteria in
370 GlycoBuffer 1 (New England Biolabs) were treated with 16 U neuraminidase (reaction
371 volume 100 μ l) for 1 h at 37 °C. Control reactions contained buffer alone. Bacteria were
372 then incubated with mouse serum as described above to measure quantitatively, C3
373 deposition.

374
375 **Serum bactericidal assay.** Strain 15253 was grown in gonococcal liquid media
376 (Morse A, Morse B and IsoVitaleX™ (72)) containing CMP-Neu5Ac at concentrations
377 ranging from 0 to 100 μ g/ml in half- \log_{10} increments; susceptibility to mAb 2C7 (5 μ g/ml)
378 was determined by serum bactericidal assay as described previously (72) with minor
379 modifications. The clinical isolates from Nanjing, China were all grown in liquid media as
380 described above, supplemented with 2 μ g/ml of CMP-Neu5Ac. Approximately 2000
381 CFU gonococci in HBSS⁺⁺/0.1% BSA were incubated with 20% NHS either in the
382 presence or absence of mAb 2C7. Final bactericidal reaction volumes were maintained
383 at 75 μ l. Aliquots of 12.5 μ l were plated onto chocolate agar plates in duplicate at the

384 beginning of the assay (t_0) and again after incubation at 37°C for 30 min (t_{30}). Survival
385 was calculated as the number of viable colonies at t_{30} relative to t_0 .

386

387 **Mass spectroscopic analysis of LOS.** O-deacylated LOS was prepared as
388 described previously (26). LC-MS was performed using a Waters Premier Q-TOF
389 operated in the positive-ion mode with an Agilent 1260 capillary LC system. LC
390 separation was done on an Agilent Eclipse XDB C8 column (5 μ m, 50 x 1mm) operated
391 at 55 °C. The flow rate was 20 μ L/min. Solvent A: aqueous 0.2 % formic acid/0.028 %
392 ammonia; solvent B: Isopropanol with 0.2% formic acid/0.028% ammonia. The following
393 gradient was used: 0-2 min. 10 % B, 2-16 min linear gradient to 85 % B, 16-25 min. 85
394 %B, 25-30 min. equilibration at 10% B.

395

396 **Anti-LOS mAbs.** Anti-LOS mAbs 3F11 (73), L8 (74), L1 (75) and 2C7 (42) have
397 been described previously. Fig. 10A indicates the specificities of each of the mAbs

398

399 **Whole cell ELISA.** Whole cell ELISA was performed as described previously
400 (71). Briefly, U-bottomed microtiter wells (Dynatech Laboratories, Inc., Chantilly, VA)
401 were coated with 50 μ l of bacterial suspensions ($\sim 10^8$ organisms/ml) in PBS for 3 h at
402 37 °C, followed by incubation overnight at 4 °C. Plates were washed with PBS
403 containing 0.05% Tween 20. Tissue culture supernatants containing mAbs 3F11, L8, L1
404 and 2C7 were dispensed into wells and incubated for 1 h at 37 °C, followed by washing
405 with PBS/0.05% Tween 20. Bound 2C7, L8 and L1 were disclosed with anti-mouse IgG

406 alkaline phosphatase (Sigma), and mAb 3F11 was detected with anti-mouse IgM
407 alkaline phosphatase (Sigma).

408

409 ***Cmah KO mice.*** Unlike mice, humans lack the ability to convert Neu5Ac to
410 Neu5Gc, because of an *Alu*-mediated deletion in a critical exon that encodes the
411 enzyme, CMP-Neu5Ac hydroxylase (CMAH) (76). Deletion of *Cmah* in mice results in
412 expression of only Neu5Ac. *Cmah* knockout (KO) mice were generated with a human-
413 like deletion in exon 6 of *Cmah* as described previously (77) and were subsequently
414 back-crossed >10 generations into a BALB/c background.

415

416 ***Mouse infection.*** The mouse vaginal colonization model developed by Jerse
417 was used (78). Briefly, female *Cmah* KO mice in the diestrus phase of the estrous cycle
418 were started on treatment (that day) with 0.1 mg Premarin[®] (Pfizer) in 200 μ l of water
419 given subcutaneously on each of three days; -2, 0 and +2 days (before, the day of and
420 after inoculation) to prolong the estrus phase of the reproductive cycle and promote
421 susceptibility to *Ng* infection. Antibiotics (vancomycin, colistin, neomycin, trimethoprim
422 and streptomycin (VCNTS)) ineffective against *N. gonorrhoeae* were also used to
423 reduce competitive microflora (7). Mice (n=10/group) were infected on Day 0 with either
424 strain 15253, 15253/G- or 15253 Δ *lst* (inoculum specified for each experiment).
425 Vaginas were swabbed daily and plated on chocolate agar containing VCNTS to
426 enumerate *N. gonorrhoeae* CFUs. The efficacy of mAb 2C7 in vivo against 15253 was
427 performed in wild-type BALB/c mice (Jackson Laboratories) as described previously.
428 Mice were treated with mAb 2C7 or control mouse IgG3 intraperitoneally, 10 μ g twice a

429 day on days -2, -1 (prior to) and 0 (the day of infection with strain 15253 and daily
430 vaginal CFU enumeration was carried out as described above.

431

432 **Statistical analyses.** Experiments that compared clearance of *N. gonorrhoeae* in
433 independent groups of mice estimated and tested three characteristics of the data (43):
434 Time to clearance, longitudinal trends in mean \log_{10} CFU and the cumulative CFU as
435 area under the curve (AUC). Statistical analyses were performed using mice that initially
436 yielded bacterial colonies on Days 1 and/or 2. Median time to clearance was estimated
437 using Kaplan-Meier survival curves; times to clearance were compared between groups
438 using the Mantel-Cox log-rank test. Mean \log_{10} CFU trends over time were compared
439 between groups using a linear mixed model with mouse as the random effect using both
440 a random intercept and a random slope. A cubic function in time was determined to
441 provide the best fit; random slopes were linear in time. A likelihood ratio test was used
442 to compare nested models (with and without the interaction term of group and time) to
443 test whether the trend differed over time between the two groups. The mean AUC (\log_{10}
444 CFU versus time) was computed for each mouse to estimate the bacterial burden over
445 time (cumulative infection); the means under the curves were compared between
446 groups using the nonparametric two-sample Wilcoxon rank-sum (Mann-Whitney) test
447 because distributions were skewed or kurtotic. The Kruskal-Wallis equality-of-
448 populations rank test was also applied to compare more than two groups in an
449 experiment. Correlation between survival in serum bactericidal assays and mAb 2C7
450 binding was performed by Spearman's non-parametric test. A cubic equation as used to
451 generate the best-fit curve.

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733

734 **Figure Legends**

735 **Fig. 1.** LOS glycan extensions from heptose (Hep) I and HepII elaborated by *N.*
736 *gonorrhoeae* strains used in this study. Glycan extensions from HepI and HepII of the
737 major LOS structure represented by the strains is shown using the symbol
738 nomenclature for graphical representation of individual glycans (79). *N. gonorrhoeae*
739 lack the ability to produce CMP-Neu5Ac, therefore capping of LOS with Neu5Ac
740 requires the addition of CMP-Neu5Ac to growth media.

741
742 **Fig. 2.** Evidence of sialylation of HepII lactose on *N. gonorrhoeae* 15253 and MS11 2-
743 Hex/G+. *N. gonorrhoeae* 15253 and MS11 2-Hex/G+ both express lactose from HepI
744 and HepII. Their mutants that lack HepII lactose were constructed by deleting *IgtG*
745 (15253/G- and MS11 2-Hex/G-). 15253 Δ Ist lacks LOS sialyltransferase and cannot add
746 Neu5Ac to LOS. MS11 4-Hex/G- expresses the sialylatable LNnT structure from HepI
747 and served as a positive control for sialylation. All strains were in media with (+) or
748 without (-) added CMP-Neu5Ac (100 μ g/ml) for 2 h at 37 °C. Bacterial lysates were
749 digested with protease K, separated on a 16% Tricine gel and LOS was visualized by
750 silver staining. Retardation of LOS mobility following growth in CMP-Neu5Ac-containing
751 media relative to LOS from bacteria grown in media devoid of CMP-Neu5Ac indicates
752 sialylation.

753
754 **Fig. 3.** Enhanced FH binding upon LOS sialylation is restricted to strains that express
755 the LNnT LOS structure. FH binding to isogenic LOS mutants of MS11 that express 2-
756 Hex (lactose), 3-Hex (PK-like) or 4-Hex (LNnT) structures from HepI, with (G+) or

757 without (G-) lactose extensions from HepII were grown in media alone or media
758 containing CMP-Neu5Ac (25 µg/ml). Bacteria were incubated with FH (10 µg/ml) and
759 bound FH measured by flow cytometry. Black bars, unsialylated bacteria; grey bars,
760 sialylated bacteria. Control reactions, where FH was excluded, showed a median
761 fluorescence below 10. Y-axis, median fluorescence (mean [range] of 2 separate
762 observations).

763

764 **Fig. 4.** Sialylation of 15253 LOS inhibits complement activation. Strains 15253 and its
765 isogenic mutant derivatives, 15253/G- (lacks HepII glycan extensions) and 15253 Δ lst
766 (lacks LOS sialyltransferase) were grown without or with CMP-Neu5Ac (25 µg/ml),
767 incubated in 15% normal mouse serum for 20 min at 37 °C. C3 deposited on the
768 bacterial surface was measured by flow cytometry. C3 deposited on bacteria grown in
769 the presence or absence of CMP-Neu5Ac is shown by the grey shaded and solid black
770 line histograms, respectively. Controls (no serum added) are shown by the broken lines.
771 Numbers alongside histograms represent median fluorescence intensity (the border or
772 shading of the text boxes that contain the numbers) correspond to that of the
773 histograms). X-axis, fluorescence (\log_{10} scale); Y-axis, counts. One representative
774 experiment of at least two reproducible repeats is shown.

775

776 **Fig. 5.** Complement inhibition by MS11 mutants that express lactose LOS extensions.
777 Isogenic MS11 mutants that express predominantly lactose (2-Hex), PK structure (3-
778 Hex) or LNnT (4-Hex) from HepI, with (G+) or without (G-) lactose from HepII, were
779 grown in the absence or presence of CMP-Neu5Ac (25 µg/ml) and were incubated with

780 15% normal mouse serum for 20 min at 37 °C. Mouse C3 deposited on the bacterial
781 surface was measured by flow cytometry. C3 deposited on bacteria grown in the
782 presence or absence of CMP-Neu5Ac is shown by the grey shaded areas and solid
783 black lines, respectively. Controls (no serum added) are shown by the broken lines.
784 Numbers alongside histograms represent median fluorescence intensity (the border or
785 shading of the text boxes that contain the numbers) correspond to that of the
786 histograms). X-axis, fluorescence (\log_{10} scale); Y-axis, counts. One representative
787 experiment of at least two reproducible repeats is shown.

788

789

790 **Fig. 6.** Neu5Ac added to HepII lactose resists removal by α 2-3-sialidase. MS11 2-Hex-
791 G+ and 15253 were grown in the absence or presence of CMP-Neu5Ac (25 μ g/ml).
792 MS11 4-Hex/G-, which expresses LNnT that is sialylated through an α 2-3-linkage and
793 MS11 3-Hex/G-, which expresses the P^K-like LOS that becomes sialylated through an
794 α 2-6-linkage, were used as positive and negative controls for desialylation, respectively.
795 Bacteria were treated with recombinant α 2-3-specific sialidase or with neuraminidase
796 (sialidase) buffer alone, then incubated with 15% normal mouse serum for 20 min at 37
797 °C. Mouse C3 deposited on bacteria (shown as median fluorescence intensity (MFI) on
798 the Y-axis) was measured by flow cytometry. Controls (no added serum) showed
799 fluorescence less than 10 units and have been omitted for simplicity. Each bar
800 represents the mean (range) of two separate experiments.

801

802 **Fig. 7.** Sialylation of HepII lactose enhances virulence of strain 15253. Cmah knockout
803 mice that express only Neu5Ac (the form of sialic acid found in humans), but not
804 Neu5Gc (the form in wild-type mice), were infected with wild-type (WT) *N. gonorrhoeae*
805 15253 (5.5×10^7 CFU) and its isogenic mutants 15253/G- (lacks any HepII glycan
806 extension; 4.3×10^7 CFU) and 15253 Δ/st (lacks LOS sialyltransferase; 4.9×10^7 CFU)
807 ($n=10$ mice per group). Vaginas were swabbed daily to enumerate *N. gonorrhoeae*
808 CFUs. **A.** Kaplan Meier curves showing time to clearance. WT bacteria versus G- and
809 WT versus Δ/st , $P<0.0001$ by Mantel-Cox log-rank test. **B.** CFU versus time. X-axis,
810 day; Y-axis, CFU (\log_{10}). **C.** Area Under Curve (AUC) analysis for consolidated bacterial
811 burden over time. Pairwise comparisons between G- and Δ/st with the control group
812 were made by Mann-Whitney's non-parametric t test. Comparisons across groups were
813 made by one-way ANOVA (Kruskal-Wallis non-parametric test; $P<0.0001$).

814

815 **Fig. 8.** Effect of HepII lactose sialylation on the binding and bactericidal efficacy of mAb
816 2C7. **A.** Sialylation decreases binding of mAb 2C7 to MS11 2-Hex/G+, but not to 15253.
817 *N. gonorrhoeae* were grown in media alone or media supplemented with 25 $\mu\text{g/ml}$ CMP-
818 Neu5Ac and binding of mAb 2C7 (10 $\mu\text{g/ml}$) to 15253 (left graph) and MS11 2-Hex/G+
819 was measured by flow cytometry. The solid black line shows mAb 2C7 binding to
820 unsialylated bacteria; the grey shaded histogram, mAb 2C7 binding to sialylated
821 bacteria. The control indicates bacteria incubated with anti-mouse IgG-FITC (no added
822 mAb 2C7). One experiment of two reproducible repeats is shown. **B.** Addition of CMP-
823 Neu5Ac to growth media in increasing concentrations decreases the bactericidal
824 efficacy of mAb 2C7 against *N. gonorrhoeae* 15253. Serum bactericidal assays were

825 performed with 20% pooled normal human serum (NHS) as the complement source.
826 Where indicated, mAb 2C7 was added to a concentration of 10 µg/ml. Y-axis, percent
827 survival following incubation of the reaction for 30 min relative to survival at 0 min.

828

829 **Fig. 9.** mAb 2C7 is attenuates infection with *N. gonorrhoeae* in the murine vaginal
830 colonization model. Wild-type BALB/c mice were treated with either mAb 2C7 (10 µg
831 intraperitoneally twice a day on days -2, -1 and 0) or a similar dose of control mouse
832 IgG3 and then infected with 5×10^5 CFU of 15253. Vaginas were swabbed daily to
833 enumerate CFUs. **A.** Kaplan Meier curves showing time to clearance. The two groups
834 were compared using the Mantel-Cox log-rank test. **B.** CFU versus time. X-axis, day; Y-
835 axis, CFU (\log_{10}). **C.** Area Under Curve (AUC) analysis showing consolidated bacterial
836 burdens over time. Pairwise comparisons between the two groups were made by Mann-
837 Whitney's non-parametric t test.

838

839 **Fig. 10.** Expression of the 2C7 LOS epitope by clinical isolates from Nanjing, China and
840 bactericidal efficacy of mAb 2C7. **A.** Schematic showing reactivity of anti-LOS mAbs
841 2C7, L1, L8 and 3F11. mAb 2C7 requires expression of lactose from HepI and HepII
842 simultaneously (53). mAb L8 recognizes lactose from HepI in conjunction with a
843 phosphoethanolamine (PEA) at the 3-position of HepII (74). Expression of 3-PEA from
844 HepII requires *IgtG* to be phase-varied 'OFF', thus binding of mAb 2C7 and L8 occur
845 exclusively and do not bind to overlapping epitopes. mAb 3F11 binds to unsialylated
846 terminal lactosamine of the LNnT structure; any extension beyond lactosamine – for
847 example with GalNAc [*IgtD* phase-varied 'ON'] or the addition of Neu5Ac by adding

848 CMP-Neu5Ac to growth media – abrogates mAb 3F11 binding (73). mAb L1 binds to the
849 P^K-like globotriose structure (Gal α (1,4)-Gal β (1,4)-Glc) (75). **B.** Reactivity of mAbs 2C7,
850 L1, L8 and 3F11 to 75 minimally passaged *N. gonorrhoeae* isolates recovered from men
851 with urethritis attending the Nanjing (China) STD clinic. Binding of mAbs was
852 determined by whole-cell ELISA. mAbs 2C7, L1 and L8 are all mouse IgG, while 3F11 is
853 IgM, therefore shown as a separate graph. **C.** Complement-dependent bactericidal
854 activity of mAb 2C7 against the first 62/75 isolates collected from men with urethritis in a
855 Nanjing (China) study of gonococcal transmission from men to women as a function of
856 mAb 2C7 binding. Bacteria were grown in media containing CMP-Neu5Ac (2 μ g/ml) to
857 enable them to fully resist killing (>100% survival) by 16.7% normal human serum
858 (NHS). Survival of bacteria at 30 min following incubation with mAb 2C7 (5 μ g/ml) plus
859 NHS (16.7%) is shown as a function of mAb 2C7 binding (X-axis).

Fig. 1

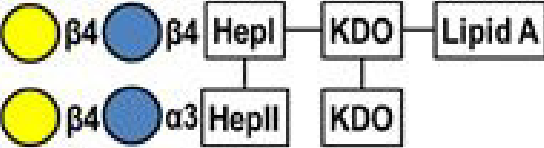
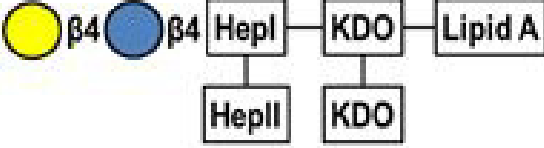



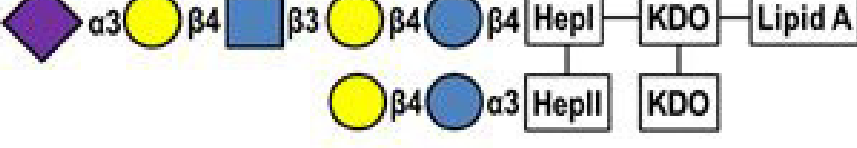
Strain(s)	Predominant LOS structure
15253 (wild-type) MS11 2-Hex/G+	
15253/G- MS11 2-Hex/G-	
MS11 3-Hex/G-	
MS11 3-Hex/G+	
MS11 4-Hex/G-	
MS11 4-Hex/G+	



Fig. 2

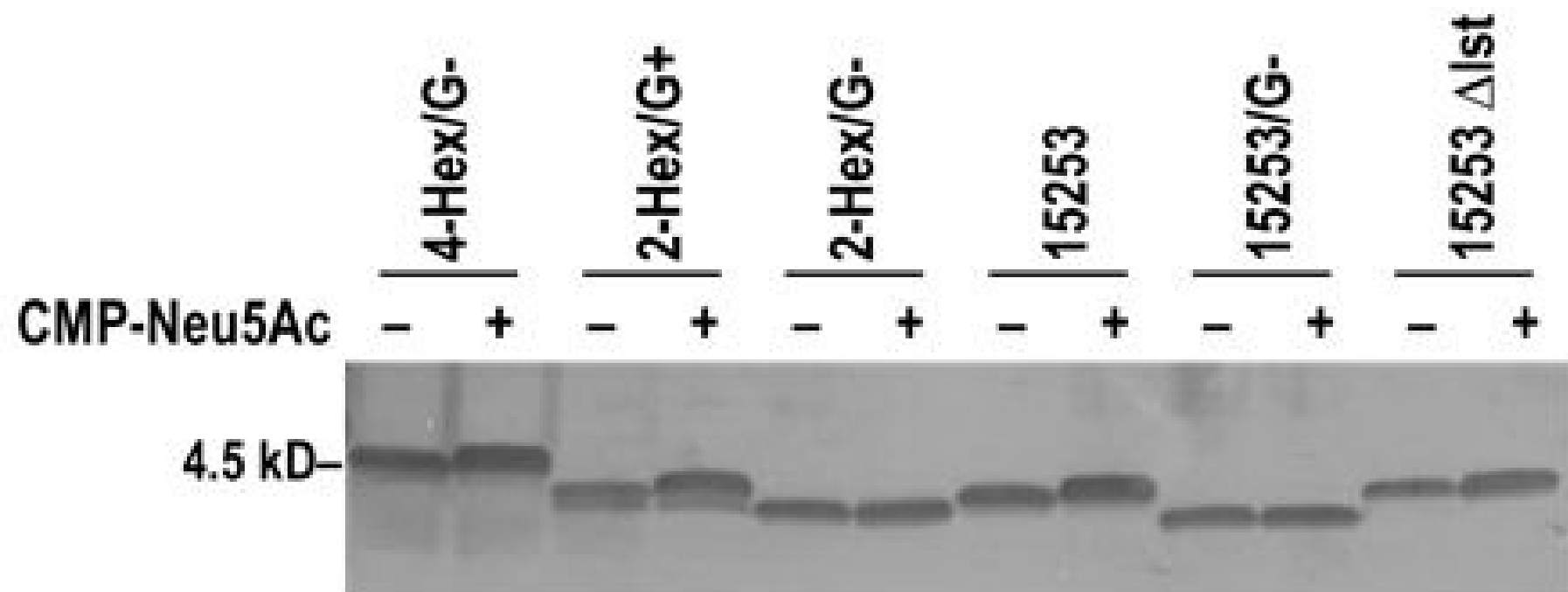


Fig. 3

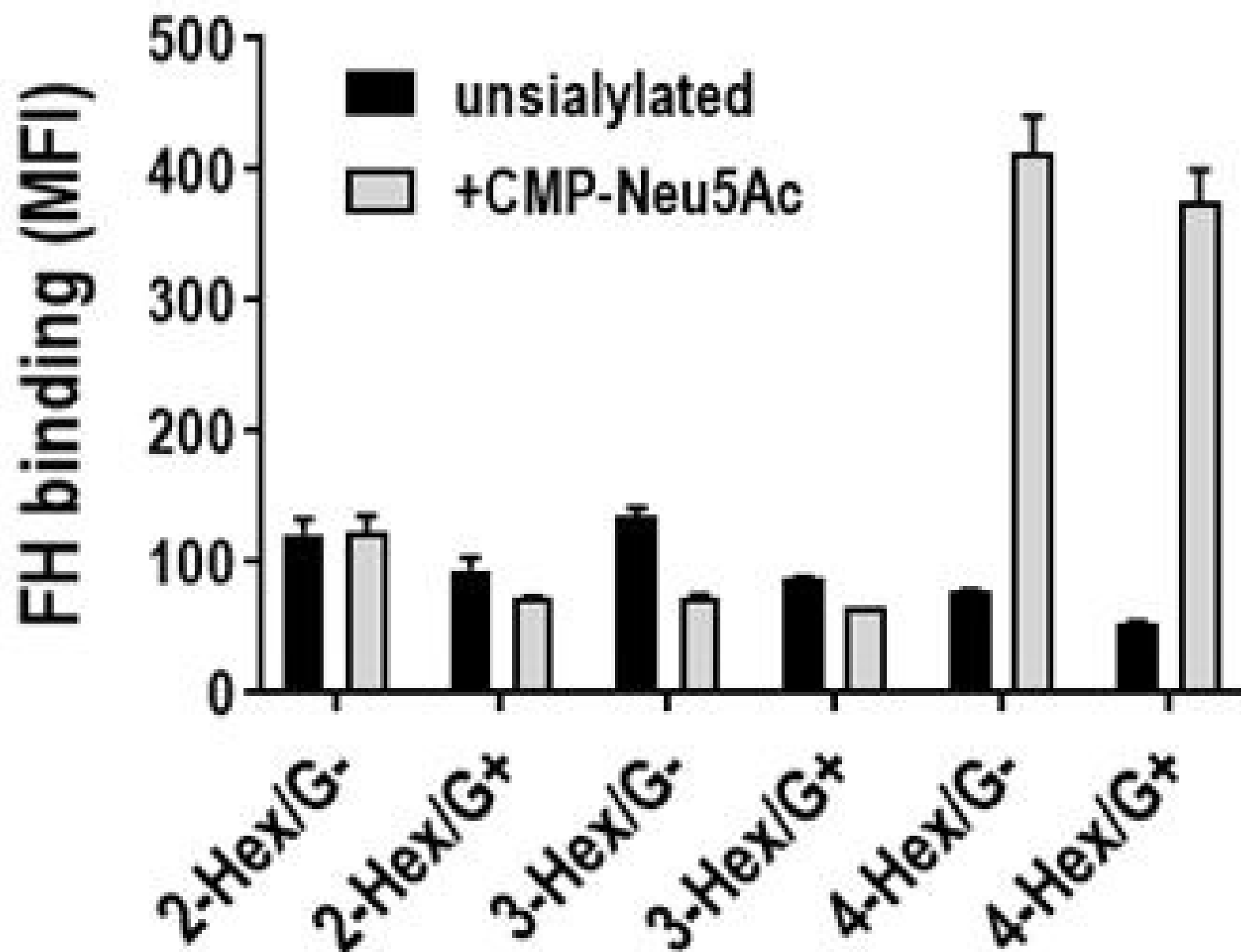
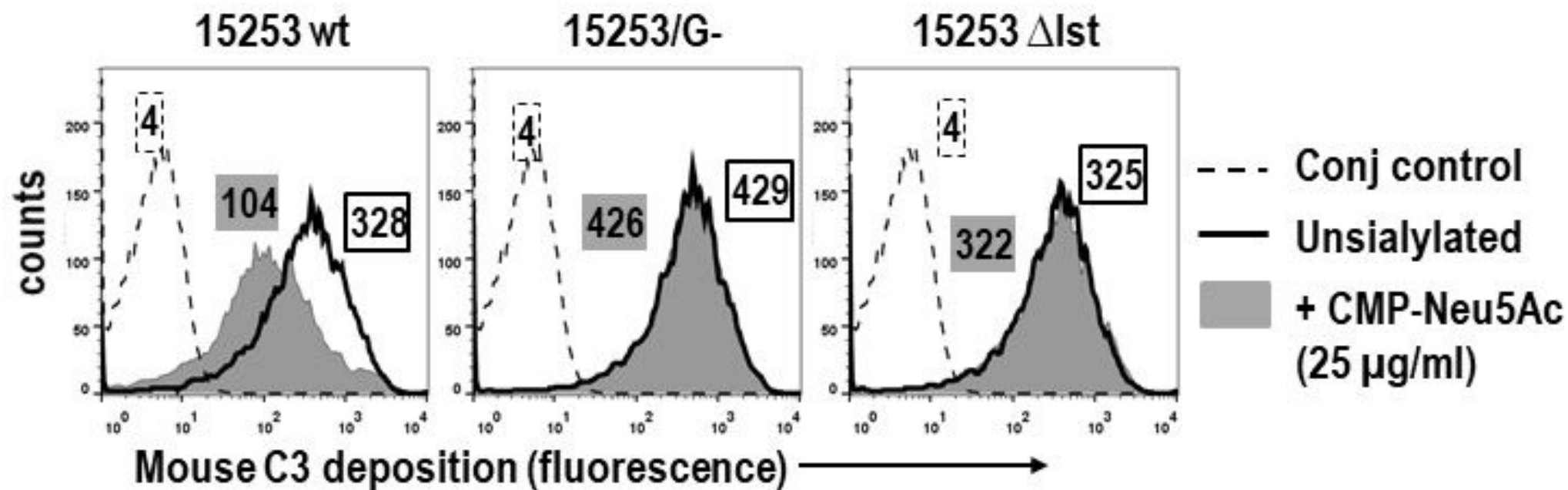
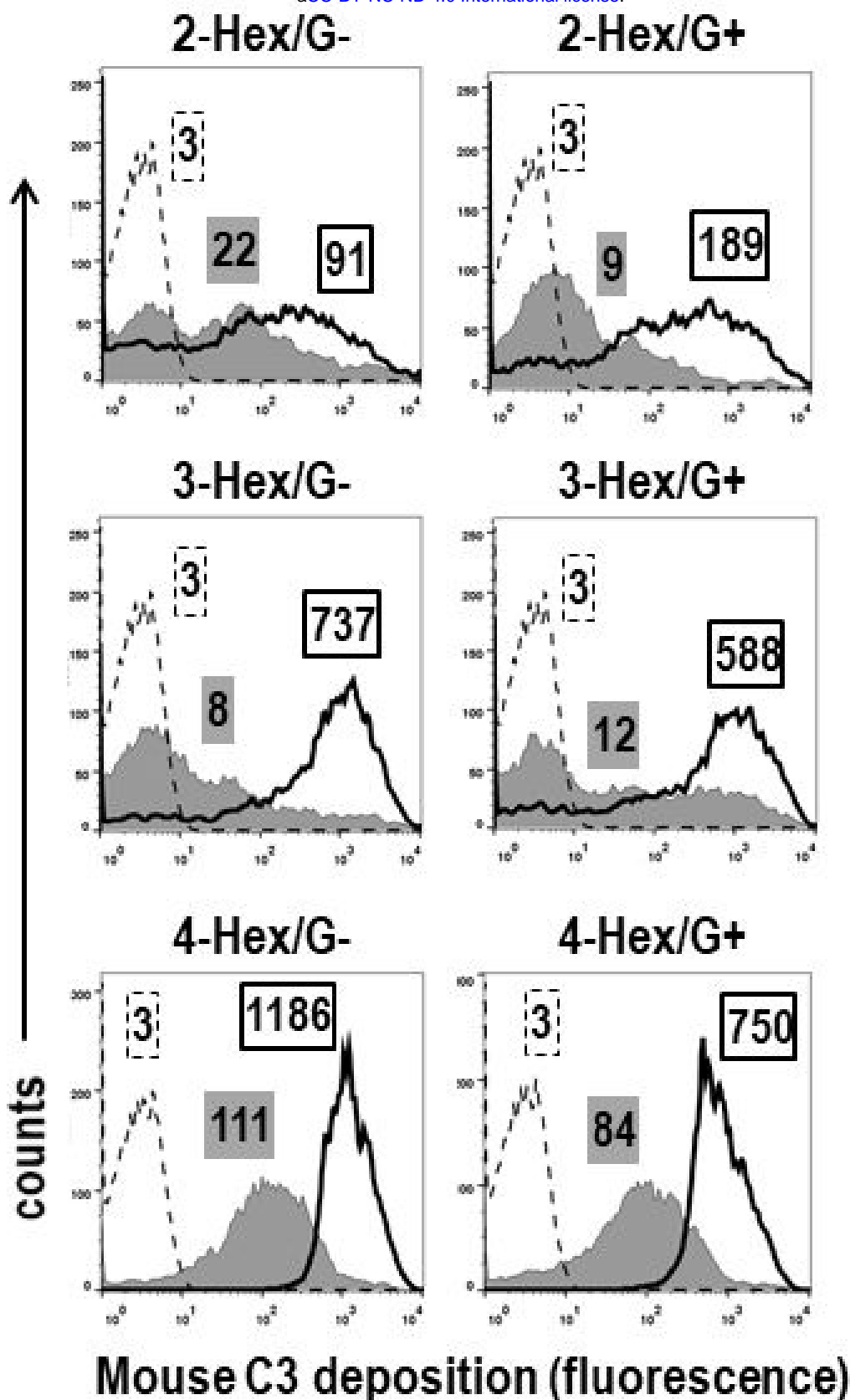


Fig. 4

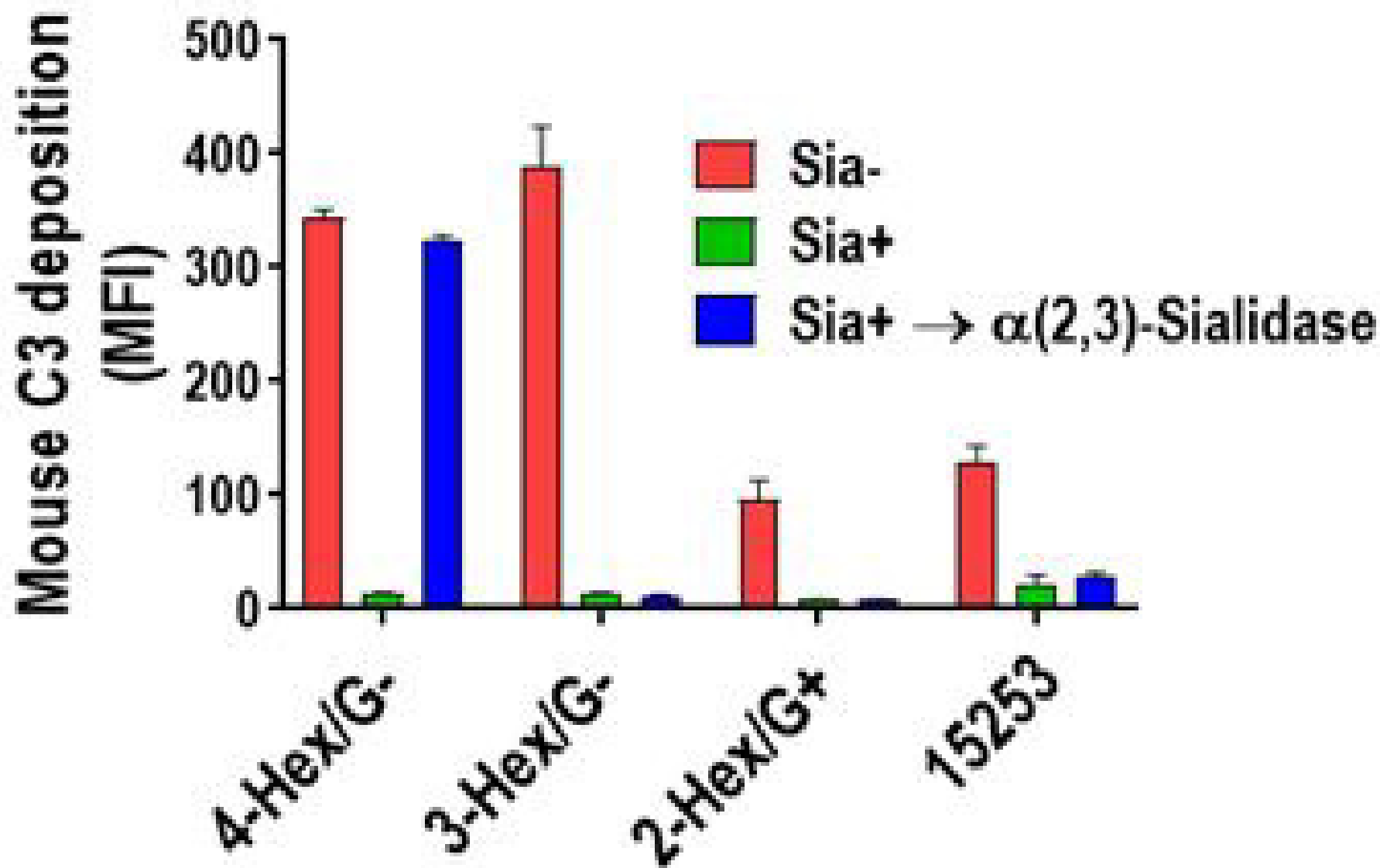


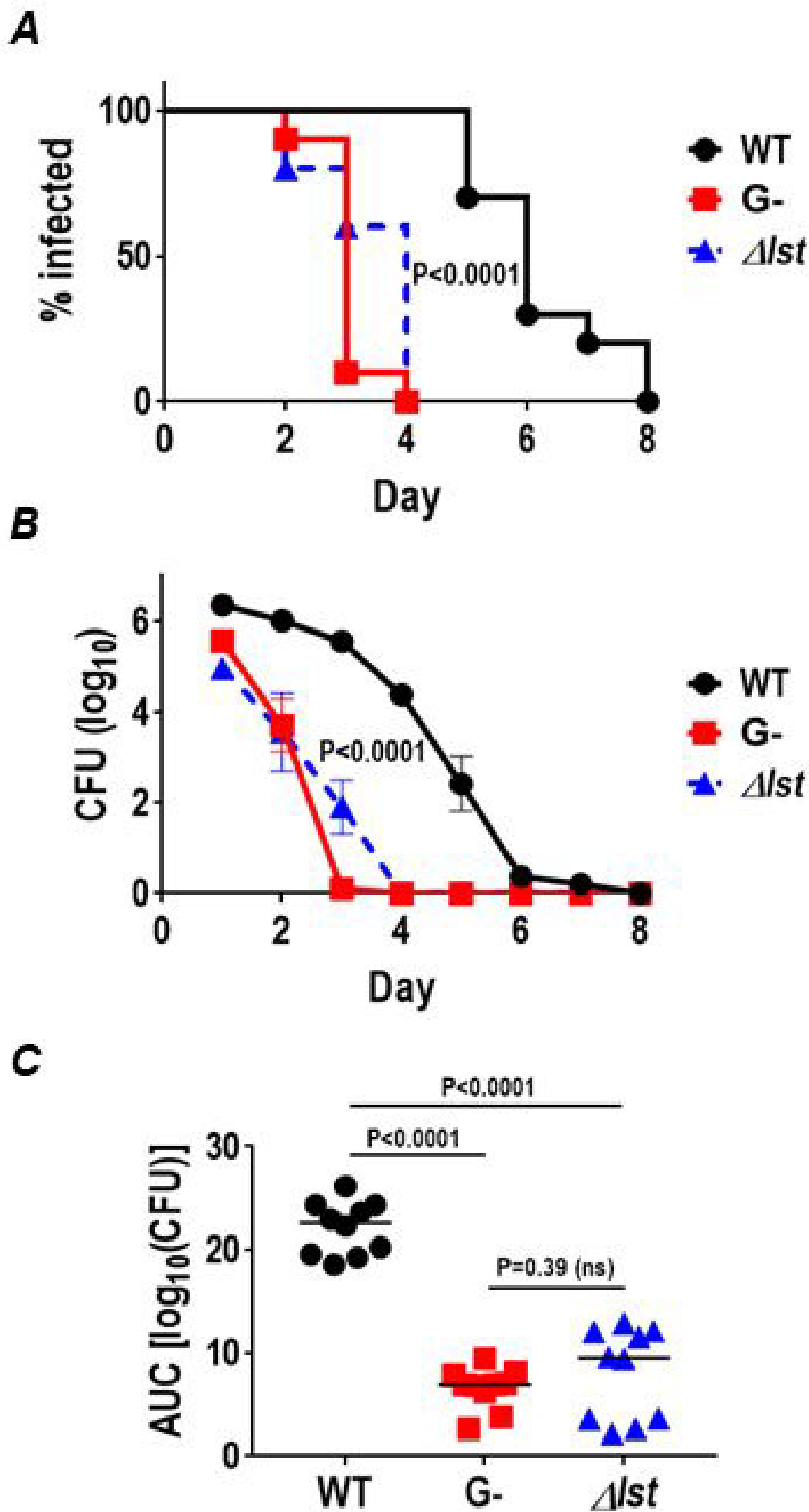
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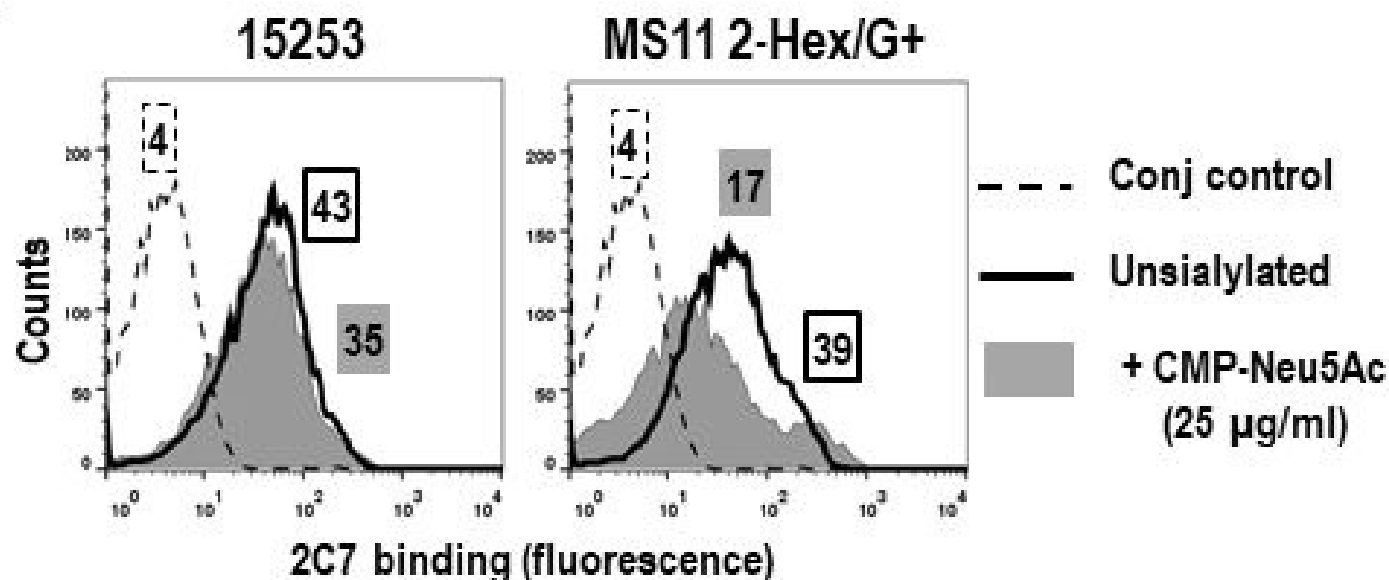
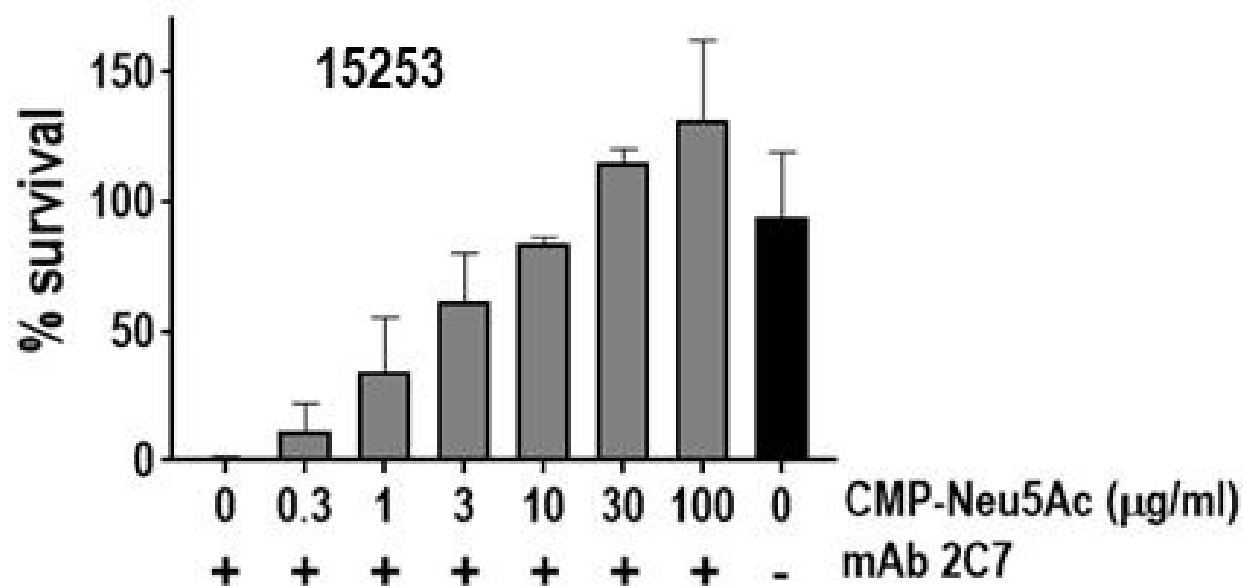
— Unsialylated

■ + CMP-Neu5Ac (25 µg/ml)

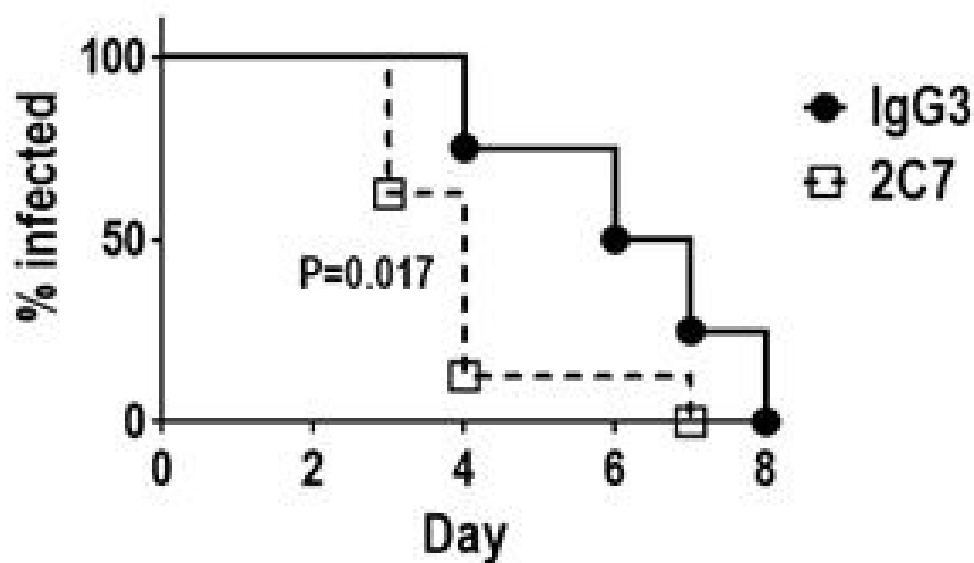
Fig. 6



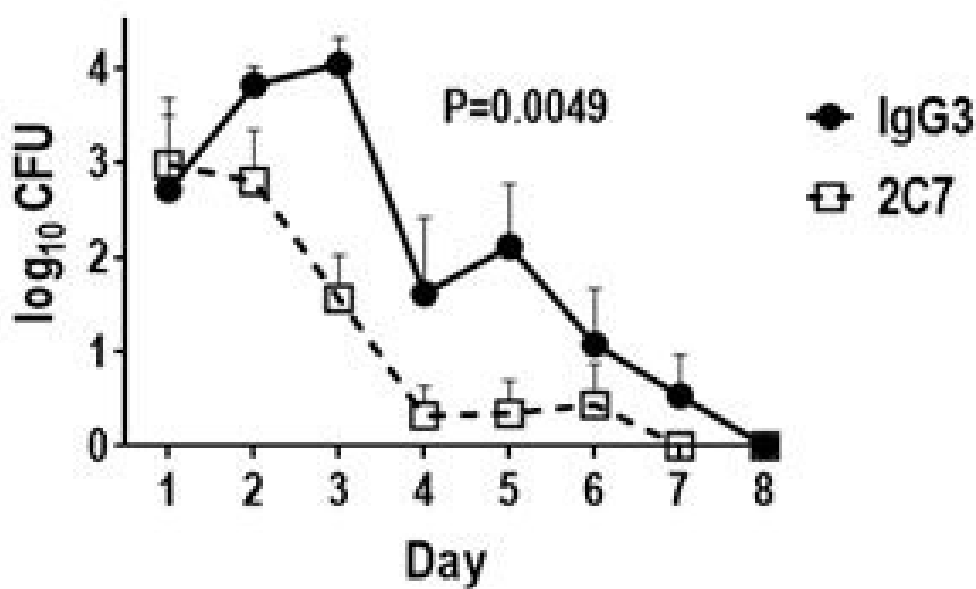


A**B**

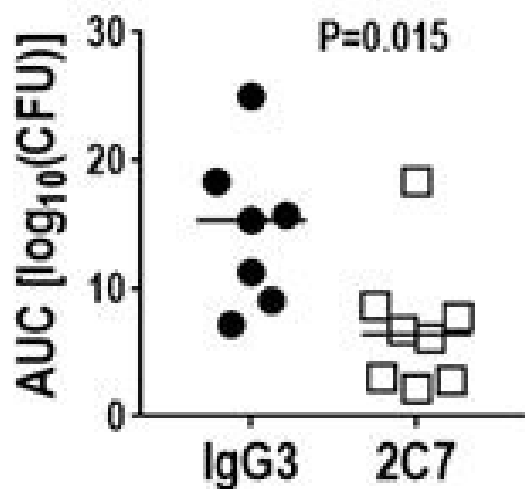
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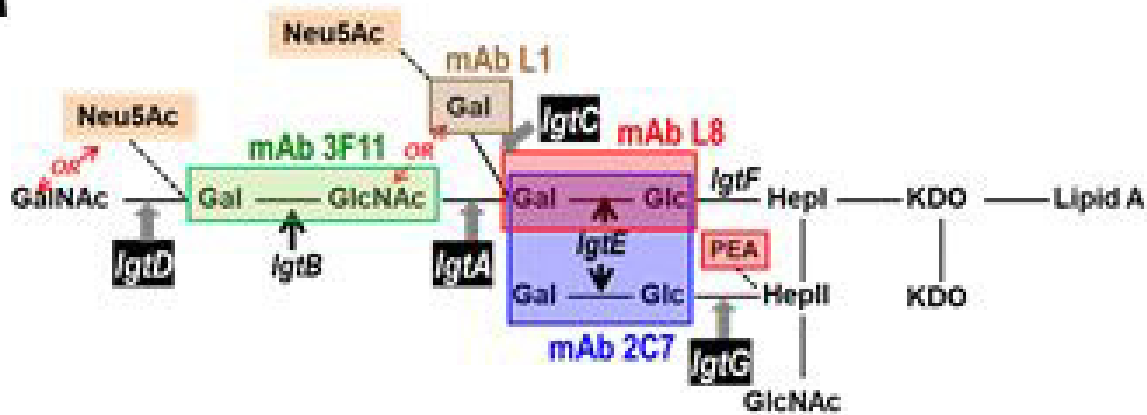
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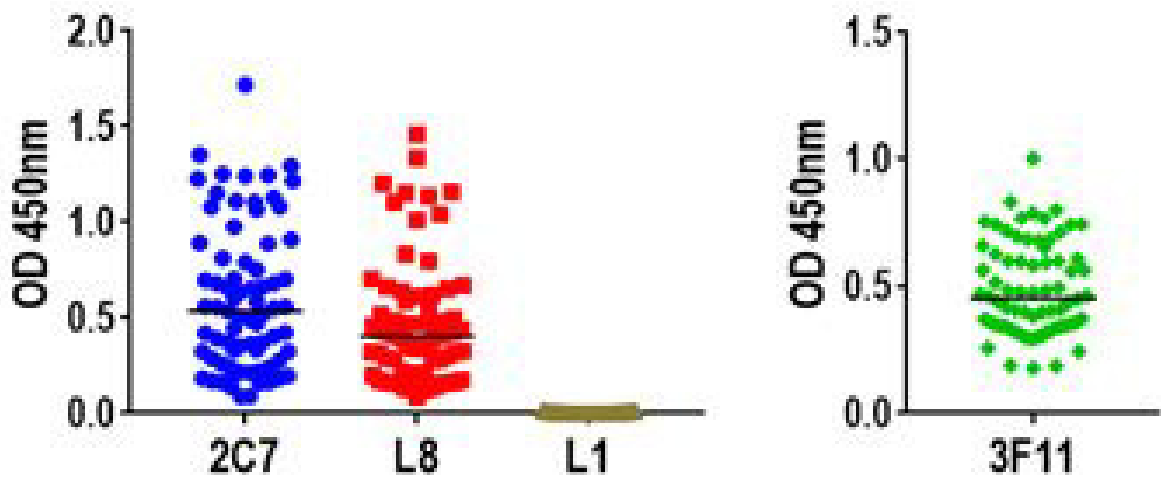
C



A



B



C

