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MOLECULAR PATHOGENESIS

A Novel Sialylation Site on *Neisseria gonorrhoeae* Lipooligosaccharide Links Heptose II Lactose Expression with Pathogenicity

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ABSTRACT Sialylation of lacto-*N*-neotetraose (LNnT) extending from heptose I (HepI) of gonococcal lipooligosaccharide (LOS) contributes to pathogenesis. Previously, gonococcal LOS sialyltransferase (Lst) was shown to sialylate LOS in Triton X-100 extracts of strain 15253, which expresses lactose from both HepI and HepII, the minimal structure required for monoclonal antibody (MAb) 2C7 binding. Ongoing work has shown that growth of 15253 in cytidine monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac)-containing medium enables binding to CD33/Siglec-3, a cell surface receptor that binds sialic acid, suggesting that lactose termini on LOSs of intact gonococci can be sialylated. Neu5Ac was detected on LOSs of strains 15253 and an MS11 mutant with lactose only from HepI and HepII by mass spectrometry; deleting HepII lactose rendered Neu5Ac undetectable. Resistance of HepII lactose Neu5Ac to desialylation by α2-3-specific neuraminidase suggested an α2-6 linkage. Although not associated with increased factor H binding, HepII lactose sialylation inhibited complement C3 deposition on gonococci. Strain 15253 mutants that lacked Lst or HepII lactose were significantly attenuated in mice, confirming the importance of HepII Neu5Ac in virulence. All 75 minimally passaged clinical isolates from Nanjing, China, expressed HepII lactose, evidenced by reactivity with MAb 2C7; MAb 2C7 was bactericidal against the first 62 (of 75) isolates that had been collected sequentially and were sialylated before testing. MAb 2C7 effectively attenuated 15253 vaginal colonization in mice. In conclusion, this novel sialylation site could explain the ubiquity of gonococcal HepII lactose *in vivo*. Our findings reinforce the candidacy of the 2C7 epitope as a vaccine antigen and MAb 2C7 as an immunotherapeutic antibody.

KEYWORDS *Neisseria gonorrhoeae*, complement, factor H, lipooligosaccharide, monoclonal antibodies, serum resistance, sialic acid, therapeutic

Gonorrhea affects about 78 million people annually worldwide (1); almost 470,000 of these cases are reported in the United States. Multidrug-resistant gonorrhea has been reported on every continent and is a serious public health concern (2–6). Understanding how gonococci evade host immune defenses is an important step

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toward development of urgently needed safe and effective vaccines and novel therapeutics against this infection.

Neisseria gonorrhoeae is a uniquely human-adapted pathogen (7). On a molar basis, lipooligosaccharide (LOS) is the most abundant gonococcal outer membrane molecule and plays a key role in many facets of pathogenesis (8–19). Host-like glycans expressed by lipooligosaccharide of *N. gonorrhoeae* constitute an example of molecular mimicry (8, 20).Two structures expressed by neisserial LOS from heptose I (HepI) that mimic host glycans include lacto-*N*-neotetraose (LNnT; Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1), which is identical to the terminal tetrasaccharide of paragloboside, a precursor of the major human blood group antigens (21), and globotriose (Galα1-4Galβ1-4Glcβ1), which is identical to the terminal globotriose trisaccharide of the P^K-like blood group antigen (22). The seminal work of Harry Smith and colleagues showed that *N. gonorrhoeae* scavenges cytidine monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac) from its host to sialylate its LOS (23, 24). Both, LNnT and P^K-like LOSs can be sialylated (25, 26). LOS sialylation inhibits complement activation and converts strains that are otherwise sensitive to killing by complement in serum to a serum (or complement)-resistant phenotype (16, 27–29). Several other microbes also use sialic acid expression to their advantage to subvert host immunity (30–41) by mimicking host sialic acid-based self-associated molecular patterns.

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

RESULTS

Neu5Ac caps *N. gonorrhoeae* HepII lactose. LOS glycan extensions from HepI and HepII for the strains used in this study are shown in Fig. 1. The designations 2-Hex, 3-Hex, and 4-Hex refer to mutants that express lactose (Gal-Glc), the P^K-like structure (Gal-Gal-Glc), and lacto-*N*-neotetraose (LNnT; Gal-GlcNAc-Gal-Glc) extensions, respectively, from HepI. G^+ and G^- refer to mutants in which the status of *lgtG* is fixed as on and off (or deleted), respectively. G^+ mutants express lactose from HepII, while G^- mutants lack any glycan extensions from HepII.

The ability of strains 15253 and MS11 2-Hex/G^+, which express lactose extending from HepI and HepII, and their respective isogenic mutants, 15253/G^- and MS11 2-Hex/G^-, which express lactose only from HepI, to add Neu5Ac to LOS was determined by SDS-PAGE. MS11 4-Hex/G^- (expresses LNnT LOS from HepI) was used as a positive control for sialylation. An *lst* deletion mutant of 15253 (15253 Δ*lst*) that lacks the ability to sialylate its LOS was also tested to address whether the previously described Lst enzyme is also responsible for sialylation of lactose.

Bacteria were grown in medium alone or in medium supplemented with 100 μg/ml CMP-Neu5Ac for maximal LOS sialylation. As shown in Fig. 2, the upper band in the plus lane of 2-Hex/G^+ shows slower mobility than the upper band in the corresponding minus lane. Note that even though expression of *lgtG* is fixed as on, MS11 2-Hex/G^+ expresses an LOS species with only HepI lactose (i.e., the LOS expressed by MS11 2-Hex/G^-). This is because of export of LOS to the outer membrane prior to addition of the proximal Glc on HepII, as noted previously (48). Similarly, retarded mobility of

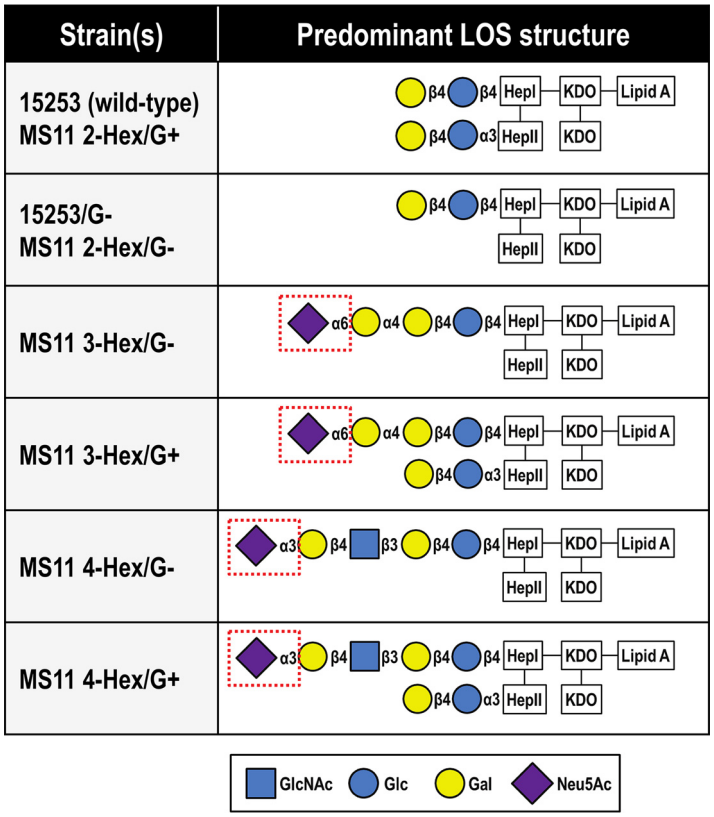

FIG 1 LOS glycan extensions from heptose I (HepI) and HepII elaborated by *N. gonorrhoeae* strains used in this study. Glycan extensions from HepI and HepII of the major LOS structures represented by the strains are shown using the symbol nomenclature for graphical representation of individual glycans (86), which represents understanding of these structures prior to this study (variable sialylation of lactose with an HepII substitution, demonstrated subsequently, is not shown). Neu5Ac is shown boxed in red because capping of *N. gonorrhoeae* LOS with Neu5Ac requires exogenous CMP-Neu5Ac as gonococci lack the ability to produce CMP-Neu5Ac.

15253 LOS was also observed when 15253 was grown in medium containing CMP-Neu5Ac. The LOS of 4-Hex/G$^-$ (positive control for sialylation) incorporated Neu5Ac and migrated more slowly. There was no appreciable alteration in LOS migration when 2-Hex/G$^-$, 15253/G$^-$, or 15253 Δ*lst* was grown in CMP-Neu5Ac, suggesting that Neu5Ac was added to the terminal Gal of HepII lactose and that Lst was the enzyme responsible for sialylation.

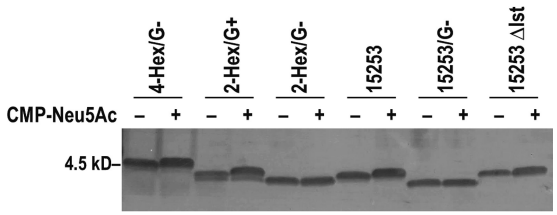

FIG 2 Evidence of sialylation of HepII lactose on *N. gonorrhoeae* 15253 and MS11 2-Hex/G$^+$. *N. gonorrhoeae* 15253 and MS11 2-Hex/G$^+$ both express lactose from HepI and HepII. Their mutants that lack HepII lactose were constructed by deleting *lgtG* (15253/G$^-$ and MS11 2-Hex/G$^-$, respectively). 15253 Δ*lst* lacks LOS sialyltransferase and cannot add Neu5Ac to LOS. MS11 4-Hex/G$^-$ expresses the sialylatable LNnT structure from HepI and served as a positive control for sialylation. All strains were grown in medium with (+) or without (−) added CMP-Neu5Ac (100 μg/ml) for 2 h at 37°C. Bacterial lysates were digested with protease K and separated on a 16% Tricine gel, and LOS was visualized by silver staining. Retardation of LOS mobility following growth in CMP-Neu5Ac-containing medium relative to that of LOS from bacteria grown in medium devoid of CMP-Neu5Ac indicates sialylation.

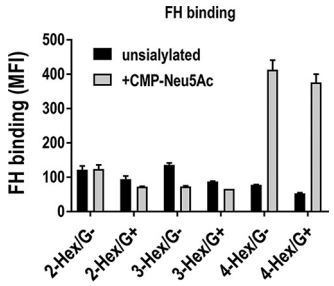

FIG 3 Enhanced FH binding upon LOS sialylation is restricted to strains that express the LNnT LOS structure. FH binding to isogenic LOS mutants of MS11 that express 2-Hex (lactose), 3-Hex (P^K-like), or 4-Hex (LNnT) structures from HepI, with (G^+) or without (G^-) lactose extensions from HepII, were grown in medium alone or medium containing CMP-Neu5Ac (25 μg/ml). Bacteria were incubated with FH (10 μg/ml), and bound FH was measured by flow cytometry. Black bars, unsialylated bacteria; gray bars, sialylated bacteria. Control reactions, where FH was excluded, showed a median fluorescence below 10. MFI, median fluorescence intensity (mean [range] of MFIs from two separate observations).

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

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

HepII lactose sialylation regulates complement activation. Neu5Ac capping of both gonococcal LNnT and P^K-like LOS inhibits complement. *N. gonorrhoeae* binds C4BP and FH in a human-specific manner (50, 51). Initial attempts at measuring human C3 fragment deposition by flow cytometry on the two unsialylated strains using normal human serum (NHS) revealed levels too low to discern the effects of sialylation on C3 deposition. Therefore, we used mouse complement, whose C4BP and FH do not bind *N. gonorrhoeae*, to study the effects of LOS sialylation on C3 deposition. Consistent with the addition of Neu5Ac to HepII lactose, C3 deposition decreased only on wild-type 15253 grown in medium containing CMP-Neu5Ac (Fig. 4 and S1). Neither 15253/G^- nor 15253 Δ*lst* inhibited C3 deposition when grown in CMP-Neu5Ac, consistent with the inability of these two mutant strains to sialylate their LOSs.

We next examined the effects of sialylation on complement activation on six MS11 LOS mutants (Fig. 5 and S2). The sialylatable 3-Hex and 4-Hex strains served as controls for Neu5Ac-mediated complement inhibition. Growth in medium containing CMP-Neu5Ac decreased C3 deposition in all tested strains. Similar to sialylation of 15253, sialylation of MS11 2-Hex/G^+ also inhibited C3 deposition. In contrast to sialylation of 15253/G^-, we noted a reproducible decrease in C3 deposition on the 2-Hex/G^- mutant, suggesting that Neu5Ac was also added to the LOS of this strain. The degree of inhibition seen with sialylation of MS11 2-Hex/G^- (a 4.1-fold decrease compared to the level of the unsialylated parent and 7.3-fold above baseline conjugate control

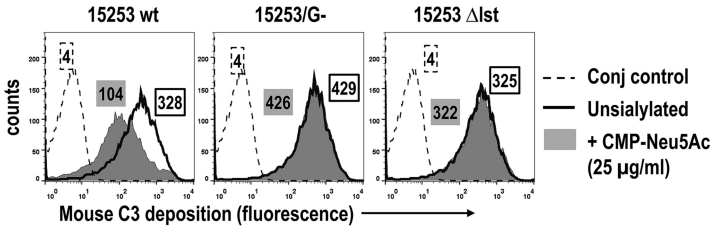

FIG 4 Sialylation of 15253 LOS inhibits complement activation. Strain 15253 (wild type [wt]) and its isogenic mutant derivatives, 15253/G$^-$ (lacks HepII glycan extensions) and 15253 Δ*lst* (lacks LOS sialyltransferase), were grown without or with CMP-Neu5Ac (25 μg/ml) and incubated in 15% normal mouse serum for 20 min at 37°C. C3 deposited on the bacterial surface was measured by flow cytometry. C3 deposited on bacteria grown in the presence or absence (unsialylated) of CMP-Neu5Ac is shown. Controls, no serum added. Numbers alongside histograms represent median fluorescence intensity (the border or shading of the text boxes that contain the numbers corresponds to that of the histograms). Fluorescence is shown on a $\log_{10}$ scale. One representative experiment of at least two reproducible repeats is shown. Aggregated data from all experiments is shown in Fig. S1 in the supplemental material. Conj, conjugate.

levels) was less than that seen with complement inhibition upon sialylation of MS11 2-Hex/G$^+$ (a 21-fold decrease compared to the level of the unsialylated 2-Hex/G$^+$ and only 3-fold greater fluorescence than that of the baseline conjugate control). This amount of LOS sialylation of MS11 2-Hex/G$^-$ LOS, although functional, was too small to be appreciated by changes in mobility on a Tricine gel or by MS analysis.

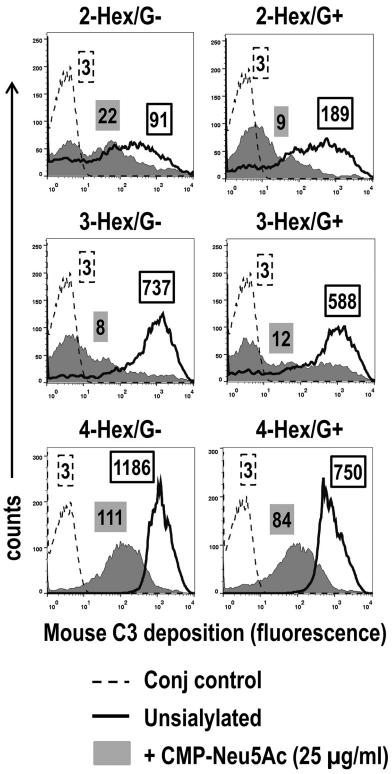

FIG 5 Complement inhibition by MS11 mutants that express lactose LOS extensions. Isogenic MS11 mutants that express predominantly lactose (2-Hex), P^K structure (3-Hex), or LNnT (4-Hex) from HepI, with (G$^+$) or without (G$^-$) lactose from HepII, were grown in the absence or presence (of CMP-Neu5Ac (25 μg/ml) and were incubated with 15% normal mouse serum for 20 min at 37°C. Mouse C3 deposited on the bacterial surface was measured by flow cytometry. C3 deposited on bacteria grown in the presence or absence of CMP-Neu5Ac is shown by the gray shaded areas and solid black lines, respectively. Results in controls (no serum added) are represented by the broken lines. Numbers alongside histograms represent median fluorescence intensity (the border or shading of the text boxes that contain the numbers corresponds to that of the histograms). Fluorescence is shown on a $\log_{10}$ scale. One representative experiment of at least two reproducible repeats is shown. Aggregated data from all experiments is shown in Fig. S2 in the supplemental material.

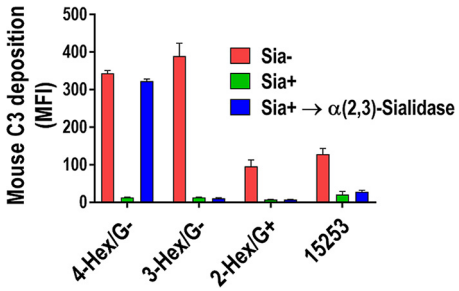

FIG 6 Neu5Ac added to HepII lactose resists removal by α2-3-sialidase. MS11 2-Hex-G$^+$ and 15253 were grown in the absence (Sia-) or presence (Sia+) of CMP-Neu5Ac (25 μg/ml). MS11 4-Hex/G$^-$, which expresses LNnT that is sialylated through an α2-3-linkage, and MS11 3-Hex/G$^-$, which expresses the P^K-like LOS that becomes sialylated through an α2-6 linkage, were used as positive and negative controls for desialylation, respectively. Bacteria were treated with recombinant α2-3-specific sialidase or with neuraminidase (sialidase) buffer alone and then incubated with 15% normal mouse serum for 20 min at 37°C. Mouse C3 deposited on bacteria (shown as median fluorescence intensity [MFI]) was measured by flow cytometry. Controls (no added serum) showed fluorescence of less than 10 units and were omitted for simplicity. Each bar represents the mean (range) of MFIs from two separate experiments.

To discern the sialic acid linkage to lactose extensions from HepII, we examined the effect of α2-3-linkage-specific neuraminidase on mouse C3 deposition (Fig. 6). Sialylated 2-Hex/G$^+$ and the corresponding wild-type strain 15253 that possessed the same pattern of HepI and HepII hexose substitutions failed to show increased C3 deposition after treatment with recombinant α2-3-specific sialidase followed by incubation with 15% normal mouse serum; resistance to α2-3-sialidase suggests an α2-6 linkage.

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

Effect of sialylation on MAb 2C7 binding and efficacy. MAb 2C7 targets a LOS epitope being developed as a gonococcal vaccine candidate. The minimal LOS structure required for MAb 2C7 binding consists of lactoses simultaneously extending from both HepI and HepII. Glycan extensions beyond lactose on HepII, for example, with GalNAc-Gal seen in the mutant strain JW31R, selected under pyocin pressure, abrogates MAb 2C7 binding (53). We therefore asked whether sialylation of HepII lactose affected MAb 2C7 binding and function. While sialylation of 15253 did not affect MAb 2C7 binding (Fig. 8A, left graph, and S3), sialylation of MS11 2-Hex/G$^+$ resulted in a reproducible ~2- to 3-fold reduction in MAb 2C7 binding (Fig. 8A, right graph, and S3). Similar levels of binding of MAb 2C7 to sialylated and unsialylated 15253 allowed us to assess the functional effects of HepII lactose sialylation when antibody binding was kept constant. As shown in Fig. 8B, increasing amounts of CMP-Neu5Ac in medium caused a dose-dependent decrease in killing by MAb 2C7.

MAb 2C7 is active against strain 15253 *in vivo*. In light of prior work that showed the importance of LOS sialylation for infection of mice (54, 55) and the observed resistance of sialylated 15253 to MAb 2C7 *in vitro* (Fig. 8), we examined the efficacy of MAb 2C7 versus 15253 in the BALB/c mouse vaginal colonization model. A passive immunization model to address the efficacy of MAb 2C7 to simulate effects of vaccine antibody was used (43). Wild-type BALB/c mice ($n = 8$) were administered 10 μg of

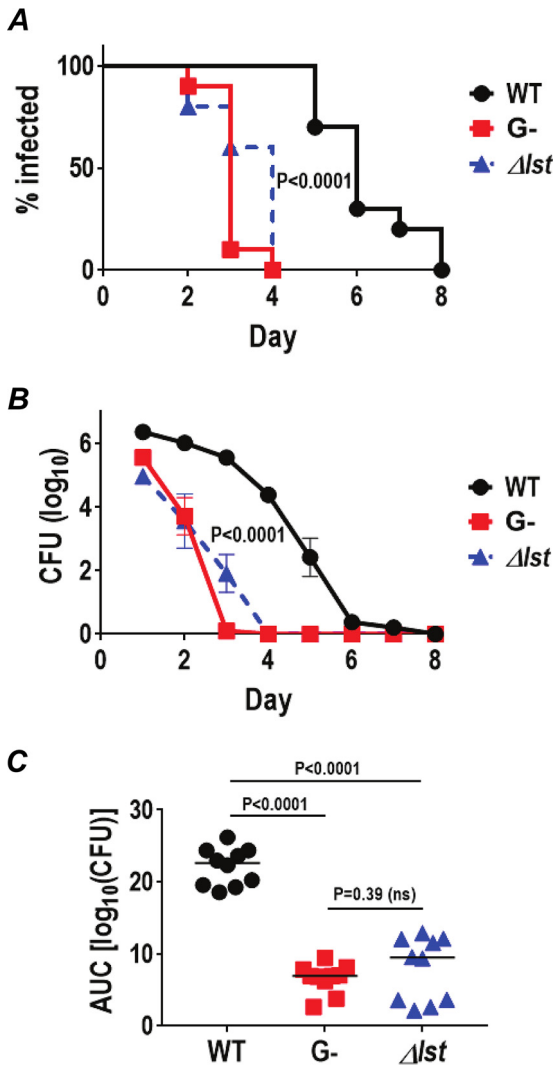

FIG 7 Sialylation of HepII lactose enhances virulence of strain 15253. *Cmah* knockout mice that express only Neu5Ac (the form of sialic acid found in humans), and not Neu5Gc (the form in wild-type mice), were infected with wild-type (WT) *N. gonorrhoeae* 15253 (5.5×10^7 CFU) and its isogenic mutants 15253/G$^-$ (lacks any HepII glycan extension; 4.3×10^7 CFU) and 15253 Δ*lst* (lacks LOS sialyltransferase; 4.9×10^7 CFU) ($n = 10$ mice per group). Vaginas were swabbed daily to enumerate *N. gonorrhoeae* CFU. (A) Kaplan-Meier curves showing time to clearance. For results with wild-type bacteria versus those with G$^-$ bacteria and with wild-type bacteria versus those with Δ*lst* bacteria, *P* is <0.0001 by Mantel-Cox log rank test. (B) CFU ($\log_{10}$) counts versus time. (C) Area under the curve (AUC) analysis for consolidated bacterial burden over time. Pairwise comparisons between results for the G$^-$ and Δ*lst* strains with the control group were made with a Mann-Whitney nonparametric *t* test. Comparisons across groups were made by one-way analysis of variance (Kruskal-Wallis nonparametric test; $P < 0.0001$).

MAb 2C7 intraperitoneally on days −2, −1, and 0, and CFU counts were monitored daily. The control group ($n = 7$) received mouse IgG3. MAb 2C7 significantly shortened the duration and burden of infection with 15253 (Fig. 9A to C).

Expression of the 2C7 epitope by contemporary clinical isolates of *N. gonorrhoeae*. Despite being under the control of a phase-variable gene, *lgtG*, the 2C7 LOS epitope (Fig. 10A) was expressed by 94% of gonococci recovered directly from cervical secretions from a cohort of women who attended a sexually transmitted disease (STD) clinic in Boston, MA (42). We examined a collection of minimally (≤3) passaged isolates cultured from the female contacts of men with gonorrhea who were referred to an STD clinic in Nanjing, China, for expression of the 2C7 LOS epitope by whole-cell enzyme-linked immunosorbent assay (ELISA). We also examined isolates for their ability to bind to MAb L8 (which recognizes an epitope defined by HepI lactose and phosphoetha-

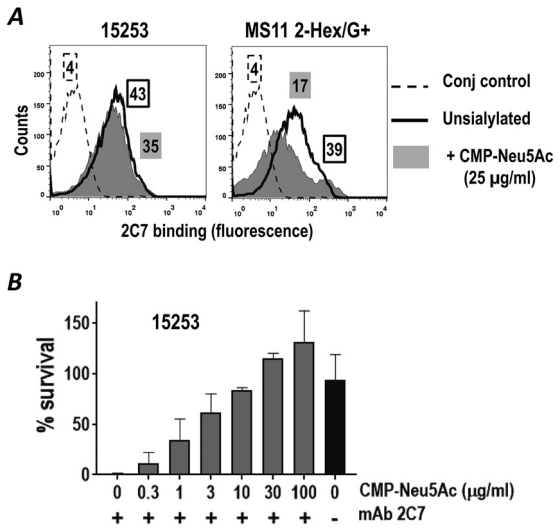

FIG 8 Effect of HepII lactose sialylation on the binding and bactericidal efficacy of MAb 2C7. (A) Sialylation decreases binding of MAb 2C7 to MS11 2-Hex/G$^+$ but not to 15253. *N. gonorrhoeae* was grown in medium alone (unsialylated) or medium supplemented with 25 μg/ml CMP-Neu5Ac and binding of MAb 2C7 (10 μg/ml) to 15253 (left graph) and MS11 2-Hex/G$^+$ was measured by flow cytometry. The control indicates bacteria incubated with anti-mouse IgG-FITC (no added MAb 2C7). One experiment of three reproducible repeats is shown. Aggregated data from all experiments is shown in Fig. S3 in the supplemental material. (B) Addition of CMP-Neu5Ac to growth medium in increasing concentrations decreases the bactericidal efficacy of MAb 2C7 against *N. gonorrhoeae* 15253. Serum bactericidal assays were performed with 20% pooled normal human serum (NHS) as the complement source. Where indicated, MAb 2C7 was added to a concentration of 10 μg/ml. Survival was determined following incubation of the reaction mixture for 30 min relative to survival at 0 min.

nolamine [PEA] substitution at the 3 position on HepII; expression of HepII lactose abrogates MAb L8 binding) and MAb 3F11 (recognizes the terminal [unsialylated] lactosamine of LNnT) (Fig. 10A). As shown in Fig. 10B, each of 75 isolates bound to MAb 2C7, albeit to various degrees, as well as to MAbs L8 and 3F11. MAb L1 barely bound to any of the tested isolates.

We next assessed the ability of MAb 2C7 to mediate complement-dependent bactericidal activity against the first 62 of 75 isolates collected from men with urethritis in a Nanjing (China) study of gonococcal transmission from men to women. Because some of the strains were sensitive to killing by 16.7% pooled normal human serum (NHS) that was used as the complement source, all isolates were grown in medium containing 2 μg/ml CMP-Neu5Ac to render them fully serum resistant (>100% survival). All (100%) of the isolates were killed at a level of >50% in the presence of 5 μg/ml of MAb 2C7 and NHS. This included two of four isolates that bound very low levels of MAb 2C7 by ELISA (optical density at 450 nm [OD_{450}] between 0.065 and 0.090). Further, serum bactericidal activity correlated with levels of MAb 2C7 binding (Fig. 10C).

DISCUSSION

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

MAb 2C7 recognized 94% of 68 gonococci examined directly from cervical secretions and 95% of 101 randomly chosen fresh (second passage) gonococcal isolates from a sexually transmitted disease clinic in Boston, MA (42). We recently surveyed 75

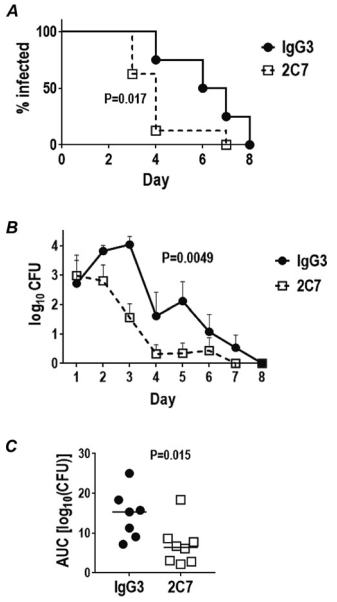

FIG 9 MAb 2C7 attenuates infection with *N. gonorrhoeae* in the murine vaginal colonization model. Wild-type BALB/c mice were treated with either MAb 2C7 (10 μg intraperitoneally twice a day on days -2, -1, and 0) or a similar dose of control mouse IgG3 and then infected with 5×10^5 CFU of 15253. Vaginas were swabbed daily to enumerate CFU. (A) Kaplan-Meier curves showing time to clearance. The two groups were compared using a Mantel-Cox log rank test. (B) CFU ($\log_{10}$) count versus time. (C) Area under the curve (AUC) analysis showing consolidated bacterial burdens over time. Pairwise comparisons between the two groups were made by a Mann-Whitney nonparametric *t* test.

minimally passaged gonococcal isolates from Nanjing, China, and noted that 100% of isolates reacted with MAb 2C7. All strains also expressed LNnT, which suggests that both sialylatable glycans are important for gonococcal pathogenesis. The importance of LNnT sialylation, in both humans and the mouse vaginal colonization model, has been established (12, 54, 55, 59). Phase variability of *lgtA* and *lgtD* controls expression of LNnT (17). Having both *lgtA* and *lgtC* off would result in expression of lactose, while the combination of *lgtA* off and *lgtC* on would result in elaboration of the P^K-like (3-Hex) structure. If *lgtA* and *lgtD* are both on, GalNAc is added to the terminal Gal of LNnT and prevents sialylation. Sialic acid likely plays a multifaceted role in neisserial pathogenesis. In addition to inhibiting complement and enhancing resistance to opsonophagocytosis and cationic antimicrobial peptides (60, 61), Neu5Ac engages sialic acid-binding immunoglobulin-type lectins (siglecs) many of which are, in turn, linked to an immunoreceptor tyrosine-based inhibition (ITIM) motif and inhibit the inflammatory response (62). Neu5Ac has also been identified in gonococcal biofilms (63). Because the gonococcus can also sialylate HepII lactose, it has the capacity to maintain LOS sialylation even when the previously described sialylatable LNnT or P^K-like structure is not expressed from HepI. While HepII lactose can be sialylated when HepI also expresses lactose, it is unclear whether HepII lactose can be sialylated when LNnT or P^K is also expressed on HepI. Gilbert and colleagues showed that meningococcal Lst could add Neu5Ac to 6-(5-fluorescein-carboxamido)-hexanoic acid succinimidyl ester (FCHASE)-aminophenyl-lactose. Lst added ~6.4-fold or ~3.2-fold more Neu5Ac onto lactosamine (LNnT is lactosamine-lactose) than lactose at substrate concentrations of 0.2 mM or 1.0 mM, respectively (64). Based on these data, we speculate that LNnT is preferentially sialylated over lactose when both glycan species are expressed.

The promiscuity of gonococcal Lst, reflected by its ability to add Neu5Ac to LNnT (through a α2-3-linkage), P^K-like LOS, and lactose (through α2-6 linkages), raises the possibility of its ability to sialylate glycans on non-LOS structures such as pilin, which can elaborate Galβ1-4Galα1-3DATDH (2,4-diacetimido-2,4,6-trideoxyhexose) (65–67). While most of the radiolabel on gonococci fed with ^{14}C-labeled CMP-Neu5Ac was

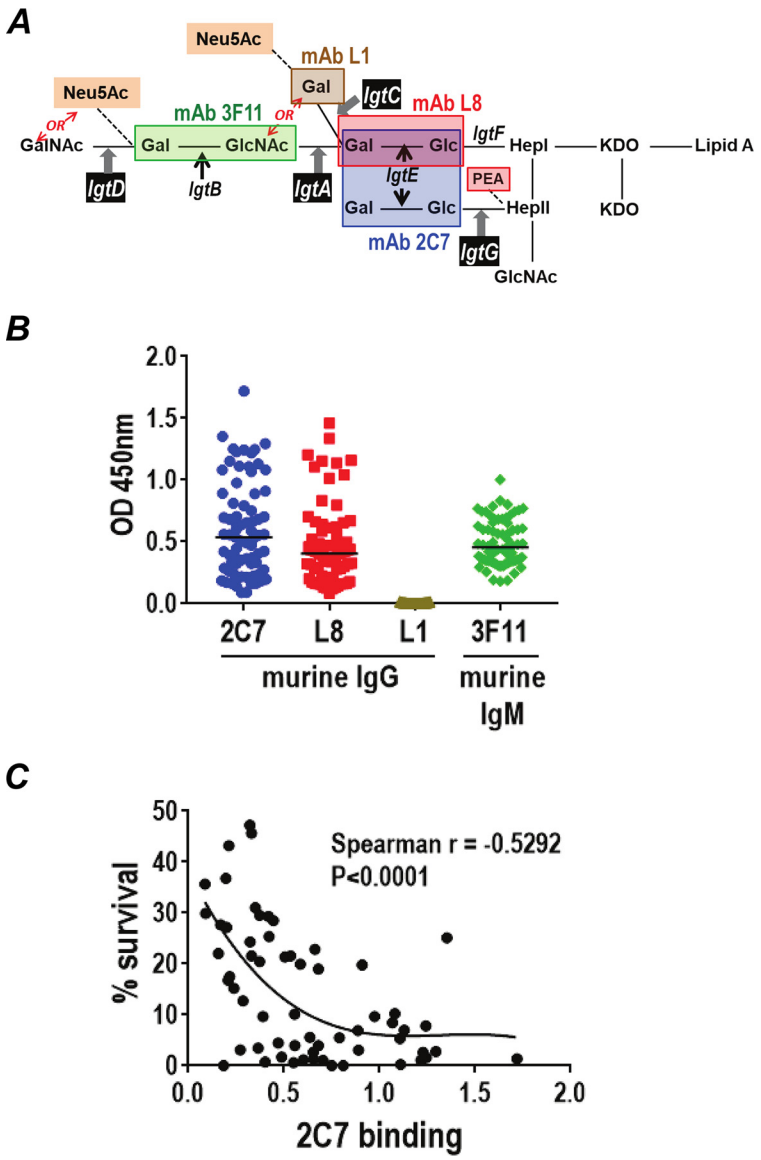

FIG 10 Expression of the 2C7 LOS epitope by clinical isolates from Nanjing, China, and bactericidal efficacy of MAb 2C7. (A) Schematic showing reactivity of anti-LOS MAbs 2C7, L1, L8, and 3F11. MAb 2C7 requires expression of lactose from HepI and HepII simultaneously (53). MAb L8 recognizes lactose from HepI in conjunction with a phosphoethanolamine (PEA) at the 3 position of HepII (81). Expression of 3-PEA from HepII requires *lgtG* to be phase off; thus binding of MAb 2C7 and L8 occurs exclusively, and they do not bind to overlapping epitopes. MAb 3F11 binds to the unsialylated terminal lactosamine of the LNnT structure; any extension beyond lactosamine—for example, with GalNAc (*lgtD* phase on) or the addition of Neu5Ac by adding CMP-Neu5Ac to growth medium—abrogates MAb 3F11 binding (80). MAb L1 binds to the P^K-like globotriose structure (Galα1-4Galβ1-4Glc). (82). Phase variation permits expression of several LOS species and binding to more than one MAb. (B) Reactivity of MAbs 2C7, L1, L8, and 3F11 to 75 minimally passaged *N. gonorrhoeae* isolates recovered from men with urethritis attending the Nanjing (China) STD clinic. Binding of MAbs was determined by whole-cell ELISA. MAbs 2C7, L1, and L8 are all mouse IgGs, while 3F11 is IgM and therefore is shown as a separate graph. (C) Complement-dependent bactericidal activity of MAb 2C7 against the first 62/75 isolates collected from men with urethritis in a Nanjing (China) study of gonococcal transmission from men to women as a function of MAb 2C7 binding (expressed as OD_{450}). Bacteria were grown in medium containing CMP-Neu5Ac (2 μg/ml) to enable them to fully resist killing (>100% survival) by 16.7% normal human serum (NHS). Survival of bacteria at 30 min following incubation with MAb 2C7 (5 μg/ml) plus NHS (16.7%) is shown as a function of MAb 2C7 binding.

located on LOS, a small amount was detected on a 28- to 30-kDa-molecular-mass protein (68). Thus, studies to examine sialylation of protein-associated gonococcal glycans by Lst may be warranted.

The importance of Neu5Ac on HepII lactose in pathogenesis was illustrated by

attenuation of 15253 Δ*lst* in the mouse vaginal colonization model. Thus, unsialylated HepII lactose does not support virulence in this model. Expression of HepII lactose by almost all clinical isolates highlights the importance of maintaining *lgtG* in the on status *in vivo*. We are not aware of any naturally occurring gonococcal isolate that lacks *lgtG*. We have shown previously that an *lgtG* deletion mutant of *N. gonorrhoeae* FA1090 was less virulent than its wild-type parent (43). Lam and Gray-Owen showed that serial passage of *N. gonorrhoeae* in mice resulted in an increased fraction of mice infected with each subpassage and in a reproducible selection of variants with *lgtG* on, providing further strong evidence of the importance of HepII lactose expression *in vivo* (69).

We noted that MS11 2-Hex/G$^-$ inhibited mouse complement when grown in CMP-Neu5Ac. The amount of Neu5Ac incorporation onto HepI lactose in this mutant was likely too small to be detected by shifts on SDS-PAGE gels or by MS analysis but was nevertheless sufficient for functional activity, limited as it was. In contrast, 15253/G$^-$, which also expresses only lactose from HepI, did not inhibit mouse C3 deposition when grown in CMP-Neu5Ac-containing medium, suggesting that the extent and influence of HepI lactose sialylation on function may differ across strains. Whether a difference in HepI lactose sialylation exists between the two strains is unclear despite differences in function but could relate to differences in Lst sequence and/or levels of Lst activity. Translation of DNA sequences of the *lst* open reading frames (ORFs) of 15253 and MS11 showed a single amino acid sequence difference; 15253 possessed a Q (seen in 16 other *N. gonorrhoeae* Lst sequences), while MS11 possessed an E (seen in >400 *N. gonorrhoeae* Lst sequences) at position 266. The −35/−10 promoter sequence, transcription start sites, and the Shine-Dalgarno sequences were identical in 15253 and MS11. Packiam et al. showed wide variation in *lst* mRNA levels across gonococcal strains, but mRNA levels often did not correlate with Lst activity as measured by sialylation of Triton X-100 bacterial extracts (70).

Linkage of Neu5Ac is a key determinant of its ability to interact with the C terminus of FH. Blaum and colleagues showed that the interaction between sialic acid and FH domain 20 is restricted only to α2-3-linked Neu5Ac; α2-6- or α2-8-linked Neu5Ac does not interact with FH (71). The Neu5Ac-lactose bond is resistant to α2-3-specific sialidase, suggesting an α2-6 linkage. In accordance with the findings of Blaum et al., sialylation of HepII lactose, presumably through an α2-6 linkage, did not increase FH binding to *N. gonorrhoeae*. We acknowledge that further structural characterization is necessary to confirm the nature of the Neu5Ac-lactose linkage. How Neu5Ac on HepII lactose regulates complement remains unclear. Similar to LNnT sialylation, Neu5Ac linked to LNnT may also inhibit the classical pathway by reducing binding of IgG directed against select surface targets to the bacterial surface.

Despite similar amounts of MAb 2C7 binding to sialylated and unsialylated 15253, the sialylated derivative was resistant (>50% survival) to MAb 2C7 plus human complement when exposed to CMP-Neu5Ac concentrations of ≥3 μg/ml. A possible explanation is that targets for C4b and C3b on LOS (72) may be obscured by the presence of Neu5Ac, thereby diminishing antibody (Ab) efficacy. However, MAb 2C7 remained effective against 15253 in the mouse vaginal colonization model, where the organism is sialylated and additional factors such as opsonophagocytosis may contribute to its bactericidal activity. Mouse FH and C4BP do not bind to gonococci (50, 51). Therefore, the barrier that MAb 2C7 must surmount to activate complement on gonococci in wild-type mice is likely to be lower than that in humans. Ongoing studies have shown efficacy of MAb 2C7 against wild-type strains MS11 and FA1090, which bind C4BP and, when sialylated, also bind FH in dual human FH and C4BP transgenic mice (S. Gulati, P. A. Rice, and S. Ram, unpublished observations), suggesting that MAb 2C7 can overcome the effects of these complement inhibitors *in vivo*.

In conclusion, this novel site of sialylation on *N. gonorrhoeae* HepII lactose can inhibit complement activation and also engage siglecs (45). These findings also explain the ubiquitous expression of HepII lactose (an integral part of the 2C7 LOS epitope) among clinical isolates of *N. gonorrhoeae* and further validate targeting the 2C7 epitope with antibody-based vaccines and immunotherapeutics.

MATERIALS AND METHODS

Bacterial strains. Strain 15253 was recovered from the pharynx (on Thayer-Martin selective medium) in an individual with disseminated gonococcal infection and has been described previously (44). 15253 was rendered resistant to streptomycin by transformation with *rpsL* from *N. gonorrhoeae* FA1090, which is naturally resistant to streptomycin, as described previously (73). Only *lgtA* and *lgtE* are intact in its *lgtA-E* locus (74). Deletion of *lgtG* in 15253 to yield 15253/G$^-$ has been described previously (75). Deletion of LOS sialyltransferase (*lst*) to yield 15253 Δ*lst* (*lst*::Kan^r) was performed as described previously (54). All of the LOS mutant derivatives of MS11 were derived from MS11 4/3/1, a variant of MS11 VD300 with an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *pilE* that controls pilus expression (76) and have been described previously (48). The LOS phenotypes of 15253, 15253/G$^-$, and the MS11 mutants used in this study are listed in Fig. 1. Seventy-five additional isolates were obtained from male subjects enrolled in a transmission study (77) of gonococcal infection from men to women in Nanjing, China. All subjects provided written informed consent in accordance with requirements by Institutional Review Boards from the University of Massachusetts Medical School, Boston University School of Medicine, and the Institute of Dermatology, Chinese Academy of Medical Sciences and Peking Union Medical College, Nanjing, China.

Normal human serum. Serum was obtained from normal healthy adult volunteers with no history of gonococcal or meningococcal infection who provided informed consent. Participation was approved by the University of Massachusetts Institutional Review Board for the protection of human subjects. Serum was obtained from whole blood that was clotted at 25°C for 30 min, followed by centrifugation at 1,500 × *g* for 20 min at 4°C. Serum from 10 donors was pooled, aliquoted, and stored at −80°C.

Mouse complement. Use of animals in this study was performed in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* endorsed by the National Institutes of Health (78). The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Massachusetts Medical School. Mouse blood obtained by terminal cardiac puncture was allowed to clot for 20 min at room temperature; it was then placed on ice for 20 min and centrifuged at 10,000 × *g* for 10 min at 4°C. Serum was harvested and stored in single-use aliquots at −80°C.

Flow cytometry. Factor H binding to *N. gonorrhoeae* was detected as described previously (73). Briefly, ~10^7 bacteria in Hanks balanced salt solution (HBSS) containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (HBSS^{++}) plus 0.1% bovine serum albumin (BSA) was incubated with 10 μg/ml purified human FH (Complement Technologies, Inc.) for 15 min at 37°C. Bacteria-bound FH was detected with goat anti-human FH (1 μg/ml; Complement Technologies, Inc.), followed by anti-goat IgG-fluorescein isothiocyanate (FITC) (Sigma) at a dilution of 1:100. Bacteria were fixed in 1% paraformaldehyde in phosphate-buffered saline (PBS), and data were acquired on a FACSCalibur flow cytometer and analyzed using FlowJo software.

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

SDS-PAGE. LOS in protease K (Calbiochem)-treated bacterial lysates prepared as described previously (48) in Tricine-SDS sample buffer (Boston Biomolecules) was visualized by electrophoresis on Criterion 16% Tris-Tricine gels (Bio-Rad) using Tris-Tricine-SDS cathode buffer (Boston Biomolecules) at 100 V and 4°C, followed by silver staining (Bio-Rad silver stain kit).

Neuraminidase treatment. Desialylation was carried out with α2-3-specific neuraminidase (catalog number P0743S; New England BioLabs). Approximately 10^7 bacteria in GlycoBuffer 1 (New England BioLabs) were treated with 16 U of neuraminidase (reaction volume, 100 μl) for 1 h at 37°C. Control reaction mixtures contained buffer alone. Bacteria were then incubated with mouse serum as described above to measure C3 deposition quantitatively.

Serum bactericidal assay. Strain 15253 was grown in gonococcal liquid medium (Morse A, Morse B, and IsoVitaleX [79]) containing CMP-Neu5Ac at concentrations ranging from 0 to 100 μg/ml in half-$\log_{10}$ increments; susceptibility to MAb 2C7 (5 μg/ml) was determined by serum bactericidal assay as described previously (79) with minor modifications. The clinical isolates from Nanjing, China, were all grown in liquid medium as described above, supplemented with 2 μg/ml of CMP-Neu5Ac. Approximately 2,000 CFU of gonococci in HBSS^{++}–0.1% BSA was incubated with 20% NHS in either the presence or absence of MAb 2C7. Final bactericidal reaction volumes were maintained at 75 μl. Aliquots of 12.5 μl were plated onto chocolate agar plates in duplicate at the beginning of the assay (time zero, t_0) and again after incubation at 37°C for 30 min (t_{30}). Survival was calculated as the number of viable colonies at t_{30} relative to that at t_0.

Mass spectroscopic analysis of LOS. O-deacylated LOS was prepared as described previously (26). Liquid chromatography-mass spectrometry (LC-MS) was performed using a Waters Premier quadrupole time of flight (Q-TOF) instrument operated in the positive-ion mode with an Agilent 1260 capillary LC system. LC separation was done on an Agilent Eclipse XDB C_8 column (particle size, 5 μm; 50 by 1 mm) operated at 55°C. The flow rate was 20 μl/min. Solvent A consisted of aqueous 0.2% formic acid–0.028% ammonia; solvent B consisted of isopropanol with 0.2% formic acid–0.028% ammonia. The following gradient was used: 0 to 2 min, 10% B; 2 to 16 min, linear gradient to 85% B; 16 to 25 min, 85%B; 25 to 30 min, equilibration at 10% B.

Anti-LOS MAbs. Anti-LOS MAbs 3F11 (80), L8 (81), L1 (82), and 2C7 (42) have been described previously. Figure 10A indicates the specificities of each of the MAbs.

Whole-cell ELISA. Whole-cell ELISA was performed as described previously (73). Briefly, U-bottom microtiter wells (Dynatech Laboratories, Inc., Chantilly, VA) were coated with 50 μl of bacterial suspensions ($\sim 10^8$ organisms/ml) in PBS for 3 h at 37°C, followed by incubation overnight at 4°C. Plates were washed with PBS containing 0.05% Tween 20. Tissue culture supernatants containing MAbs 3F11, L8, L1, and 2C7 were dispensed into wells and incubated for 1 h at 37°C, followed by washing with PBS–0.05% Tween 20. Bound 2C7, L8, and L1 were disclosed with anti-mouse IgG alkaline phosphatase (Sigma), and MAb 3F11 was detected with anti-mouse IgM alkaline phosphatase (Sigma).

***Cmah* KO mice.** Unlike mice, humans lack the ability to convert Neu5Ac to Neu5Gc because of an *Alu*-mediated deletion in a critical exon that encodes the enzyme CMP-Neu5Ac hydroxylase (CMAH) (83). Deletion of *Cmah* in mice results in expression of only Neu5Ac. *Cmah* knockout (KO) mice were generated with a human-like deletion in exon 6 of *Cmah* as described previously (84) and were subsequently back-crossed >10 generations into a BALB/c background. The genotype of *Cmah* KO mice was confirmed by PCR at Transnetyx, Inc.

Mouse infection. The mouse vaginal colonization model developed by Jerse was used (85). Briefly, female *Cmah* KO mice in the diestrus phase of the estrous cycle were started on treatment (that day) with 0.1 mg of Premarin (Pfizer) in 200 μl of water given subcutaneously on each of 3 days (−2, 0 and +2 days, indicating days before, day of, and days after inoculation, respectively) to prolong the estrus phase of the reproductive cycle and promote susceptibility to *N. gonorrhoeae* infection. Antibiotics (vancomycin and streptomycin), both ineffective against the strains of *N. gonorrhoeae* used in this experiment, were also used to reduce competitive microflora (7). Mice ($n = 10$/group) were infected on day 0 with either strain 15253, 15253/G$^-$, or 15253 Δ*lst* (inoculum specified for each experiment). Vaginas were swabbed daily, and specimens were plated on chocolate agar containing vancomycin, colistin, nystatin, trimethoprim, and streptomycin (VCNTS) to enumerate *N. gonorrhoeae* CFU. The efficacy of MAb 2C7 *in vivo* against 15253 was determined in wild-type BALB/c mice (Jackson Laboratories) as described previously. Mice were treated with MAb 2C7 or control mouse IgG3 intraperitoneally at 10 μg twice a day on days −2, −1 (prior to), and 0 (the day of infection with strain 15253), and daily vaginal CFU enumeration was carried out as described above.

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

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00285-18.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

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A Novel Sialylation Site on Neisseria gonorrhoeae Lipooligosaccharide Links Heptose II Lactose Expression with Pathogenicity

Supplemental material

- Supplemental file 1 -

  Table S1. Detected ions and proposed compositions of LPS. Fig. S1. Sialylation of strain 15253 LOS inhibits complement activation. Fig. S2. Complement inhibition by MS11 mutants that express lactose LOS extensions. Fig. S3. Effect of HepII lactose sialylation on binding of MAb 2C7.

  PDF, 836K

Supplemental Table S1. Detected ions and proposed compositions of LPS

Detected ion (m/z)	Composition	Detected mass (Da)	Calculated mass (Da)	Comments
***Ng* 15253 (no CMP-Neu5Ac)**				
1314.98	Hex4 HexNAc1 Hep2 KDO2 Lipid A (2P)	2627.94	2628.00	$[M+2H]^{2+}$
1323.50	Hex4 HexNAc1 Hep2 KDO2 Lipid A (2P)	2627.96	2628.00	$[M+H+NH_4]^{2+}$
1376.45	Hex4 HexNAc1 Hep2 KDO2 PE1 Lipid A (2P)	2750.88	2751.01	$[M+2H]^{2+}$
1385.00	Hex4 HexNAc1 Hep2 KDO2 PE1 Lipid A (2P)	2750.96	2751.01	$[M+H+NH_4]^{2+}$
***Ng* 15253 (+ CMP-NeuAc)**				
1314.99	Hex4 HexNAc1 Hep2 KDO2 Lipid A (2P)	2627.96	2628.00	$[M+2H]^{2+}$
1323.51	Hex4 HexNAc1 Hep2 KDO2 Lipid A (2P)	2627.98	2628.00	$[M+H+NH_4]^{2+}$
1376.49	Hex4 HexNAc1 Hep2 KDO2 PE1 Lipid A (2P)	2750.96	2751.01	$[M+2H]^{2+}$
1385.03	Hex4 HexNAc1 Hep2 KDO2 PE1 Lipid A (2P)	2751.02	2751.01	$[M+H+NH_4]^{2+}$
1460.54	NeuAc1 Hex4 HexNAc1 Hep2 KDO2 Lipid A (2P)	2919.06	2919.10	$[M+2H]^{2+}$
1469.07	NeuAc1 Hex4 HexNAc1 Hep2 KDO2 Lipid A (2P)	2919.10	2919.10	$[M+H+NH_4]^{2+}$
1522.04	NeuAc1 Hex4 HexNAc1 Hep2 KDO2 PE1 Lipid A (2P)	3042.06	3042.11	$[M+2H]^{2+}$
1530.54	NeuAc1 Hex4 HexNAc1 Hep2 KDO2 PE1 Lipid A (2P)	3042.04	3042.11	$[M+H+NH_4]^{2+}$
***Ng* 15253, ΔlgtG (+ CMP-NeuAc)**				
1152.95	Hex2 HexNAc1 Hep2 KDO2 Lipid A (2P)	2303.88	2303.9	$[M+2H]^{2+}$
1161.47	Hex2 HexNAc1 Hep2 KDO2 Lipid A (2P)	2303.90	2303.9	$[M+H+NH_4]^{2+}$
1214.47	Hex2 HexNAc1 Hep2 KDO2 PE1 Lipid A (2P)	2426.92	2426.91	$[M+2H]^{2+}$
1223.00	Hex2 HexNAc1 Hep2 KDO2 PE1 Lipid A (2P)	2426.96	2426.91	$[M+H+NH_4]^{2+}$
***Ng* MS11 2-Hex/G+ (+ CMP-NeuAc)**				
1152.99	Hex2 HexNAc1 Hep2 KDO2 Lipid A (2P)	2303.96	2303.9	$[M+2H]^{2+}$

1161.52	Hex2 HexNAc1 Hep2 KDO2 Lipid A (2P)	2304.00	2303.9	$[M+H+NH_4]^{2+}$
1214.51	Hex2 HexNAc1 Hep2 KDO2 PE1 Lipid A (2P)	2427.00	2426.91	$[M+2H]^{2+}$
1223.02	Hex2 HexNAc1 Hep2 KDO2 PE1 Lipid A (2P)	2427.00	2426.91	$[M+H+NH_4]^{2+}$
1315.05	Hex4 HexNAc1 Hep2 KDO2 Lipid A (2P)	2628.08	2628.00	$[M+2H]^{2+}$
1323.57	Hex4 HexNAc1 Hep2 KDO2 Lipid A (2P)	2628.10	2628.00	$[M+H+NH_4]^{2+}$
1376.54	Hex4 HexNAc1 Hep2 KDO2 PE1 Lipid A (2P)	2751.06	2751.01	$[M+2H]^{2+}$
1385.10	Hex4 HexNAc1 Hep2 KDO2 PE1 Lipid A (2P)	2751.16	2751.01	$[M+H+NH_4]^{2+}$
1460.61	NeuAc1 Hex4 HexNAc1 Hep2 KDO2 Lipid A (2P)	2919.20	2919.10	$[M+2H]^{2+}$
1469.14	NeuAc1 Hex4 HexNAc1 Hep2 KDO2 Lipid A (2P)	2919.24	2919.10	$[M+H+NH_4]^{2+}$

Ng MS11 2-Hex/G- (+ CMP-NeuAc)

1214.39	Hex2 HexNAc1 Hep2 KDO2 PE1 Lipid A (2P)	2426.76	2426.91	$[M+2H]^{2+}$
1223.06	Hex2 HexNAc1 Hep2 KDO2 PE1 Lipid A (2P)	2427.08	2426.91	$[M+H+NH_4]^{2+}$

Supplemental Material

Supplemental Fig. S1

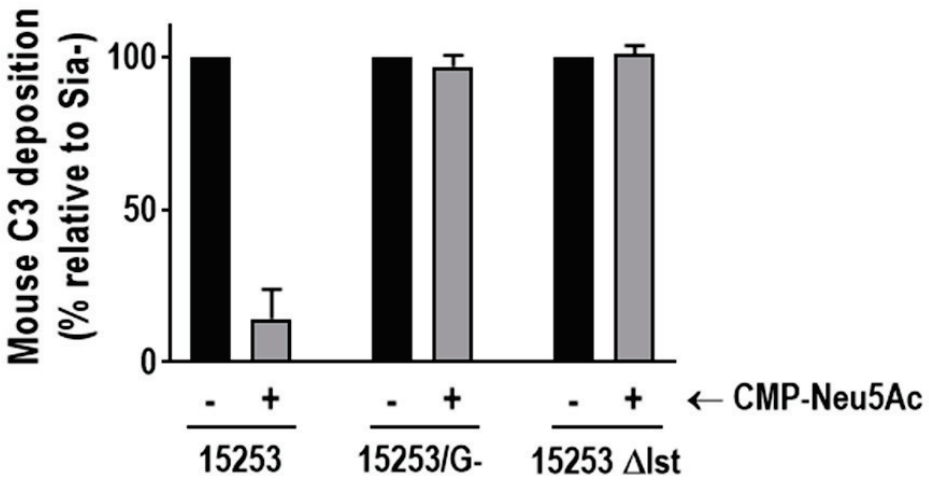

Fig. S1. Sialylation of 15253 LOS inhibits complement activation. Strains 15253 and its isogenic mutant derivatives, 15253/G- (lacks HepII glycan extensions) and 15253 Δlst (lacks LOS sialyltransferase) were grown without or with CMP-Neu5Ac (25 μg/ml), incubated in 15% normal mouse serum for 20 min at 37 °C. C3 deposited on the bacterial surface was measured by flow cytometry. C3 deposited on bacteria grown in the presence of CMP-Neu5Ac is shown by the grey bars and is represented as a percentage of C3 deposition on the corresponding unsialyated mutant (shown by solid black bars; 100% binding). Each bar represents the mean (range) of 5 separate experiments with 15253 and 2 experiments with 15253/G- and 15253 Δlst.

Supplemental Fig. S2

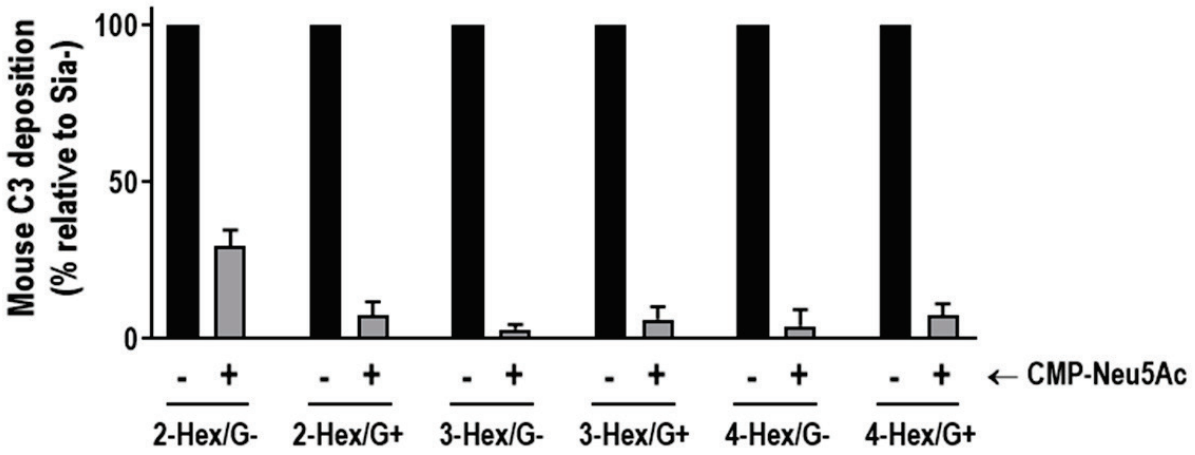

Fig. S2. Complement inhibition by MS11 mutants that express lactose LOS extensions. Isogenic MS11 mutants that express predominantly lactose (2-Hex), P^K structure (3-Hex) or LNnT (4-Hex) from HepI, with (G+) or without (G-) lactose from HepII, were grown in the absence or presence of CMP-Neu5Ac (25 µg/ml) and were incubated with 15% normal mouse serum for 20 min at 37 °C. Mouse C3 deposited on the bacterial surface was measured by flow cytometry. C3 deposited on bacteria grown in the presence of CMP-Neu5Ac is shown by the grey bars and is represented as a percentage of C3 deposition on the corresponding unsialyated mutant (shown as solid black bars; 100% binding). Each bar represents the mean (range) of 2 – 5 separate experiments.

Supplemental Fig. S3

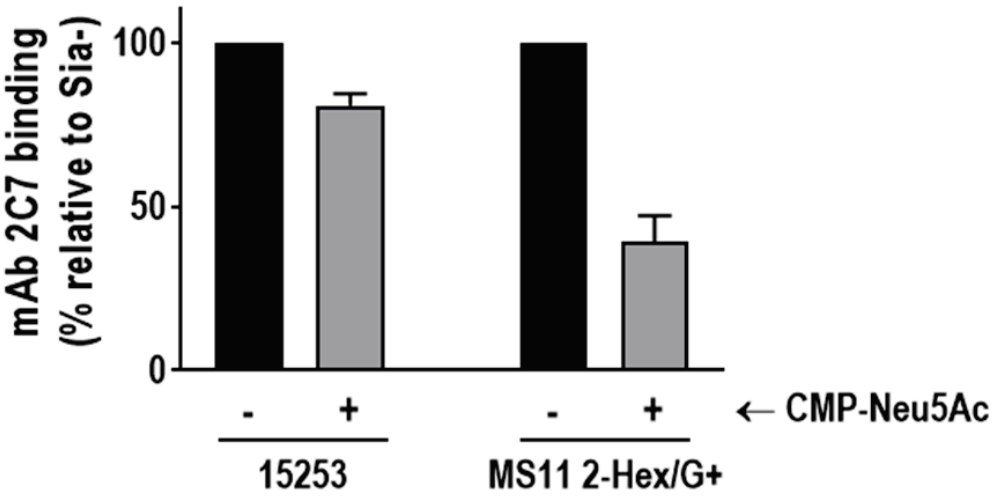

Fig. S3. Effect of HepII lactose sialylation on binding of mAb 2C7. *N. gonorrhoeae* 15253 and MS11 2-Hex/G+ were grown in media alone or media supplemented with 25 µg/ml CMP-Neu5Ac and binding of mAb 2C7 (10 µg/ml) was measured by flow cytometry. mAb 2C7 binding to the sialylated strain (grey bars) is expressed as a percentage of binding to the corresponding unsialylated strain (black bars) on the Y-axis. The mean (range) of three separate experiments is shown.