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MAJOR ARTICLE

Efficacy of Antigonococcal CMP-Nonulosonate Therapeutics Require Cathelicidins

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(See the Editorial Commentary by Shafer, on pages 1585–6.)

Novel therapies to counteract multidrug-resistant gonorrhea are urgently needed. A unique gonococcal immune evasion strategy involves capping of lipooligosaccharide (LOS) with sialic acid by gonococcal sialyltransferase (Lst), utilizing host-derived CMP-sialic acid (CMP-Neu5Ac in humans). LOS sialylation renders gonococci resistant to complement and cationic peptides, and down-regulates the inflammatory response by engaging siglecs. CMP-sialic acid analogs (CMP-nonulosonates [CMP-NulOs]) such as CMP-Leg5,7Ac$_2$ and CMP-Kdn are also utilized by Lst. Incorporation of these NulO analogs into LOS maintains gonococci susceptible to complement. Intravaginal administration of CMP-Kdn or CMP-Leg5,7Ac$_2$ attenuates gonococcal colonization of mouse vaginas. Here, we identify a key mechanism of action for the efficacy of CMP-NulOs. Surprisingly, CMP-NulOs remained effective in complement *C1q*$^{-/-}$ and *C3*$^{-/-}$ mice. LOS Neu5Ac, but not Leg5,7Ac$_2$ or Kdn, conferred resistance to the cathelicidins LL-37 (human) and mouse cathelicidin-related antimicrobial peptide in vitro. CMP-NulOs were ineffective in *Camp*$^{-/-}$ mice, revealing that cathelicidins largely mediate the efficacy of therapeutic CMP-NulOs.

Keywords. *Neisseria gonorrhoeae*; gonorrhea; complement; sialic acid; lipooligosaccharide; CMP-nonulosonate; cathelicidin; cationic antimicrobial peptide.

Gonorrhea is the second most common worldwide sexually transmitted bacterial infection (chlamydia is the most common), with 86.9 million new cases estimated to occur annually by the World Health Organization (WHO) [1]. The incidence of gonorrhea is increasing globally. In the United States, 583 405 cases were reported to the Centers for Disease Control and Prevention in 2018, which represented a 63% increase since 2014 and an 82.6% increase since the historic low in 2009 (https://www.cdc.gov/std/stats18/gonorrhea.htm). Gonorrhea commonly manifests as cervicitis, urethritis, proctitis, and conjunctivitis. If left untreated, complications including endometritis, salpingitis, tubo-ovarian abscess, bartholinitis, peritonitis, and perihepatitis in women, periurethritis and epididymitis in men, and ophthalmia neonatorum in newborns can occur. Disseminated gonococcal infection may sometimes occur; manifestations include skin lesions, tenosynovitis, septic arthritis, and rarely, endocarditis or meningitis [2].

Neisseria gonorrhoeae has become resistant to almost every antimicrobial used for treatment [3]. Strains resistant to third-generation cephalosporins and azithromycin [3, 4], the recommended first-line agents for treatment, have emerged globally. In public health efforts to stem the tide, the first-line treatment regimen was updated in 2016 to include both ceftriaxone (cephalosporin) and azithromycin—that is, combination therapy [5]. However, reports of "super-bugs" resistant to the combination therapy emerged in early 2018 [6, 7]. Three new antibiotics—solithromycin, zoliflodacin, and gepotidacin—were tested against gonorrhea in clinical trials. Solithromycin failed to meet noninferiority criteria when compared to the first-line recommended regimen of ceftriaxone plus azithromycin in a phase 3 trial [8]. Zoliflodacin and gepotidacin were effective in uncomplicated urogenital infections, but failures to eradicate oropharyngeal infection in men who have sex with men and commercial sex workers were reported [9–11]. Vaccines and new therapeutics to prevent and treat disease caused by multidrug-resistant gonorrhea are urgently needed [12].

Targeting bacterial virulence mechanisms represents an effective way to combat antimicrobial resistance, because resistance would incur a fitness cost to the organism (ie, loss of the virulence factor[s]) and the resulting attenuated "escape mutants" would likely be eliminated by host immunity. Sialic acids belong to the nonulosonate (NulO) class of monosaccharides

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(the NulO family also includes Neu5Ac, Neu5Gc, Kdn, and Leg5,7Ac$_2$); they are negatively charged 9-carbon-backbone molecules that contribute to virulence of several pathogens, including *N. gonorrhoeae* (reviewed in [13, 14]). The transfer of *N*-acetylneuraminic acid (Neu5Ac), a member of the sialic acid family prominent in humans, from host CMP-Neu5Ac onto the *N. gonorrhoeae* lipooligosaccharide (LOS), contributes to gonococcal serum resistance [15, 16], evasion of cationic antimicrobial peptides (CAMPs) [17] and biofilm formation [18]. Experimental studies in human male volunteers [19, 20] and in mice [21, 22] have demonstrated the importance of LOS sialylation in mucosal colonization and virulence. The essential role of sialylation in the gonococcal pathogenicity makes it an ideal virulence mechanism for a therapeutic to target.

We targeted gonococcal sialylation by feeding the bacteria select CMP-NulOs. These included CMP-legionaminic acid (CMP-5,7-diacetamido-3,5,7,9-tetradeoxydeoxy-D-*glycero*-D-*galacto*-nonulosonic acid; CMP-Leg5,7Ac$_2$) and CMP-ketodeoxynonulosonic acid (CMP-3-deoxy-D-*glycero*-D-*galacto*-nonulosonic acid; CMP-Kdn), which served as substrates for gonococcal LOS siayltransferase (Lst). NulO incorporation into LOS reduced the duration and burden of vaginal colonization by *N. gonorrhoeae* [23, 24]. Here, we investigate the mechanism of action for the efficacy of the therapeutic CMP-NulOs in vivo.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Neisseria gonorrhoeae strains F62 [25] and H041 (WHO Reference Strain X) [26] have been described previously. An isogenic Lst-deficient mutant of H041 (H041 Δlst) was constructed using plasmid pUC18-lst-Kan, as described previously [27]. Mouse vaginal colonization experiments required the use of streptomycin (Sm)–resistant bacteria. H041 was rendered Sm-resistant by transformation with *rpsL* derived from strain FA1090, which is naturally Sm-resistant, as described previously [24]. The Sm-resistant derivative of H041 (referred to simply as H041) was used in all experiments. LOS characterization of H041 and H041 Δlst by silver staining and Western blotting using a panel of anti-LOS monoclonal antibodies is shown in Supplementary Figure 1. A spontaneous Sm-resistant mutant of strain F62 was kindly provided by Dr Ann E. Jerse [28]. For CAMP killing assays, gonococci were grown to the mid-log phase in gonococcal liquid media (Morse A, Morse B plus Isovitalex [29]) alone, or media supplemented with CMP-NulO. Bacteria were diluted in Morse A for use in CAMP killing assays.

CMP Nonulosonates

CMP-Neu5Ac was purchased from Nacalai USA. Synthesis and characterization of CMP-Leg5,7Ac$_2$, CMP-Kdn, and CMP-Neu5,9Ac$_2$ have been described previously [23, 24].

Cationic Antimicrobial Peptides

LL-37 and mouse cathelicidin-related antimicrobial peptide (mCRAMP) were purchased from Anaspec (Eurogentec). CAMPs were dissolved in 0.01% acetic acid prior to use.

Mouse Strains

Wild-type BALB/c and C57BL/6 mice (both 6–8 weeks of age) were purchased from Jackson Laboratories. Mice were acclimatized for a week before use in challenge experiments. *C1q*$^{-/-}$ mice in a C57BL/6 background have been described previously [30]. *C3*$^{-/-}$ mice in a C57BL/6 background were from Jackson and were back-crossed 10 generations into a BALB/c background. JHD mice in a BALB/c background were provided by Dr Ann Marshak-Rothstein (University of Massachusetts Medical School). *Camp*$^{-/-}$ mice (also called *Cnlp*$^{-/-}$ mice or CRAMP knockout mice) were originally created in a C57BL/6 background [31] and back-crossed for at least 7 generations into a BALB/c background [32], and were provided by Dr Richard Gallo (University of California, San Diego). *C3*$^{-/-}$ and JHD mice were crossed with wild-type BALB/c to generate heterozygous littermates. All mice were genotyped by polymerase chain reaction to confirm deletions of the respective genes.

CAMP Killing Assay

Gonococci grown to the mid-log phase were diluted in Morse A to yield approximately 1000 colony-forming units (CFU) in 180 µL. Twenty microliters of each CAMP, diluted appropriately to yield the final concentrations specified for each experiment, was added to the bacterial suspension. Aliquots of bacteria (5 µL) were plated on chocolate agar at the start of the assay and at 90 minutes; Survival was expressed as CFU at 90 minutes relative to CFU at the start of the assay.

Murine Model of Gonococcal Vaginal Colonization

Use of animals was performed in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol (protocol number A-1717) was approved by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School. Female mice (6–8 weeks of age) in the diestrus phase of the estrous cycle were started on treatment (that day) with 0.1 mg Premarin (Pfizer) in 200 µL water given subcutaneously on each of 3 days: −2, 0, and +2 days (before, the day of, and after inoculation) to prolong the estrus phase of the cycle and promote susceptibility to *N. gonorrhoeae* infection [33]. Premarin is a mixture of sodium estrone sulfate and sodium equilin sulfate and as concomitant components, sodium sulfate conjugates of 17α-dihydroequilin, 17α-estradiol, and 17β-dihydroequilin. Mice were administered vancomycin (0.6 mg) and Sm sulfate (0.3 mg) intraperitoneally on each of 3 days: −2, −1, and 0 days (before and the day of inoculation) to reduce competitive microflora [33]. The inoculum size was specified for each experiment. Daily bacterial burdens were

measured by enumerating CFU obtained by first rinsing vaginal swabs in 100 µL of normal saline and then plating serial 10-fold dilutions onto chocolate agar plates containing Isovitalex equivalent and VCNTS (vancomycin, colistin, nystatin, and trimethoprim sulfate) supplement (Becton Dickinson) plus 100 mg of Sm sulfate (Sigma) per milliliter of media.

Statistical Analysis

Experiments that compared clearance of *N. gonorrhoeae* in independent groups of mice estimated and tested 3 characteristics of the data, as described previously [24]: time to clearance, longitudinal trends in mean $\log_{10}$ CFU, and the cumulative CFU as area under the curve (AUC). Median time to clearance was estimated using Kaplan–Meier survival curves; the times to clearance were compared between groups using a log-rank test. Mean $\log_{10}$ CFU trends over time were compared between groups using 2-way analysis of variance (ANOVA) and Dunnett multiple comparisons test. The mean AUC ($\log_{10}$ CFU vs 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 1-way ANOVA (Kruskal–Wallis test) because distributions were skewed or kurtotic; pairwise comparisons between groups were carried out using Dunn post hoc test. Killing of gonococci by CAMPs was analyzed by 2-way ANOVA and Dunnett post hoc test.

RESULTS

Key Complement Factors Are Dispensable for Efficacy of CMP-Leg5,7Ac$_2$

We previously showed that *N. gonorrhoeae* Lst can utilize several CMP-NulO substrates including CMP-Leg5,7Ac$_2$ (referred to earlier as CMP-Leg5Ac7Ac) and CMP-Kdn, which results in capping of the terminal Gal on lacto-*N*-neotetraose (LNnT) LOS with the respective NulOs. Incorporation of Leg5,7Ac$_2$ resulted in strain F62 remaining fully sensitive (100% killing or 0% survival) to human complement, even in 3.3% normal human serum (NHS), whereas Kdn and Neu5,9Ac$_2$ incorporation resulted in resistance (>100% survival) to 3.3% NHS, but >90% killing in 10% NHS [23] (Supplementary Table 1).

To determine whether complement was required for activity of the proposed therapeutic CMP-NulOs, we tested the efficacy of CMP-Leg5,7Ac$_2$ in strains of mice that lacked either complement C1q (*C1q*$^{-/-}$ mice) (engagement of C1q by antibody is the first step in classical pathway activation) or mice that lacked C3 (*C3*$^{-/-}$ mice) (C3 is the point of convergence of all 3 complement pathways). As shown in Figure 1A and 1B, CMP-Leg5,7Ac$_2$ remained active in both strains of mice. The reappearance of gonococci in 4 of 10 *C3*$^{-/-}$ mice on day 8 ($\log_{10}$ CFUs ranged from 3 to 4.6) after 2 days of negative cultures precluded significance in the Kaplan–Meier analysis. Three of these 4 mice cleared infection on day 9, while the fourth mouse remained positive on day 10 when the experiment was terminated. Nonetheless, the $\log_{10}$ CFU and AUC measures (middle and right graphs, respectively, in Figure 1B) showed efficacy of CMP-Leg5,7Ac$_2$ relative to the corresponding saline controls in *C3*$^{-/-}$ mice.

Humans possess natural antibodies against Leg5,7Ac$_2$-bearing glycans [23, 34]. While we did not detect anti-Leg5,7Ac$_2$ antibodies in naive mice, or in sera of mice infected with gonococci and treated with CMP-Leg5,7Ac$_2$ (data not shown), we tested the efficacy of CMP-Leg5,7Ac$_2$ in JHD mice that lacked the ability to produce antibodies to address the possibility that low levels of anti-Leg5,7Ac$_2$ antibodies produced locally and/or below the limit of detection of our assay were responsible for CMP-NulO efficacy, for example, by binding to the NulO and engaging Fc receptors. As shown in Figure 1C, CMP-Leg5,7Ac$_2$ remained effective in JHD mice. Collectively, these data suggest that antibodies and the classical pathway did not contribute to the efficacy of CMP-NulOs in the mouse vaginal colonization model of gonorrhea. There was only a modest decrease in efficacy of CMP-Leg5,7Ac$_2$ in *C3*$^{-/-}$ mice.

Gonococci With LOS Modified by Leg5,7Ac$_2$ and Kdn Remain Susceptible to CAMPs

Prior work has suggested that Neu5Ac on gonococcal LNnT LOS enhances resistance to cationic antimicrobial peptides (CAMPs) in vitro and in vivo [17, 35]. We hypothesized that gonococci with only Neu5Ac, but not Leg5,7Ac$_2$ or Kdn, capped LOS resisted both human and mouse cathelicidins; LL-37 and mouse CRAMP (mCRAMP), respectively. To confirm that Neu5Ac conferred resistance to CAMPs, we grew *N. gonorrhoeae* strains F62 and H041 in media alone, or in media supplemented with 25, 50, or 100 µg/mL CMP-Neu5Ac and incubated bacteria in either LL-37 or mCRAMP at concentrations ranging from 0 to 12.8 µM. A sialyltransferase-deficient (Δlst) mutant of H041 was used as a control to confirm that resistance to CAMPs was specific to incorporation of Neu5Ac into LOS. As shown in Figure 2, the addition of CMP-Neu5Ac to LOS conferred resistance to CAMPs in a dose-dependent manner (data with LL-37 and mCRAMP are shown in the top and bottom panels, respectively). In the nonsialylated state (No CMP-NulO), H041 was approximately 7-fold more resistant to killing by LL-37 than F62 (50% killing of H041 at 5.1 µM and of F62 at 0.75 µM). Both strains resisted mCRAMP better than LL-37; 50% killing of F62 occurred at approximately 10 µM mCRAMP and approximately 40% killing of H041 was observed at approximately 12.8 µM mCRAMP (the highest concentration tested). As expected, the sensitivity of H041 Δlst to either CAMP did not change with the addition of CMP-Neu5Ac to media.

Having established that maximal resistance to CAMPs was seen with the addition of 100 µg/ml of CMP-Neu5Ac in growth media (Figure 2), we proceeded to compare the ability of F62 and H041 to resist LL-37 and mCRAMP when grown in media containing CMP-Neu5Ac, CMP-Leg5,7Ac$_2$ or CMP-Kdn, each at 100 µg/mL (Figure 3). Incorporation of Leg5,7Ac$_2$ or Kdn did not enhance resistance of either strain

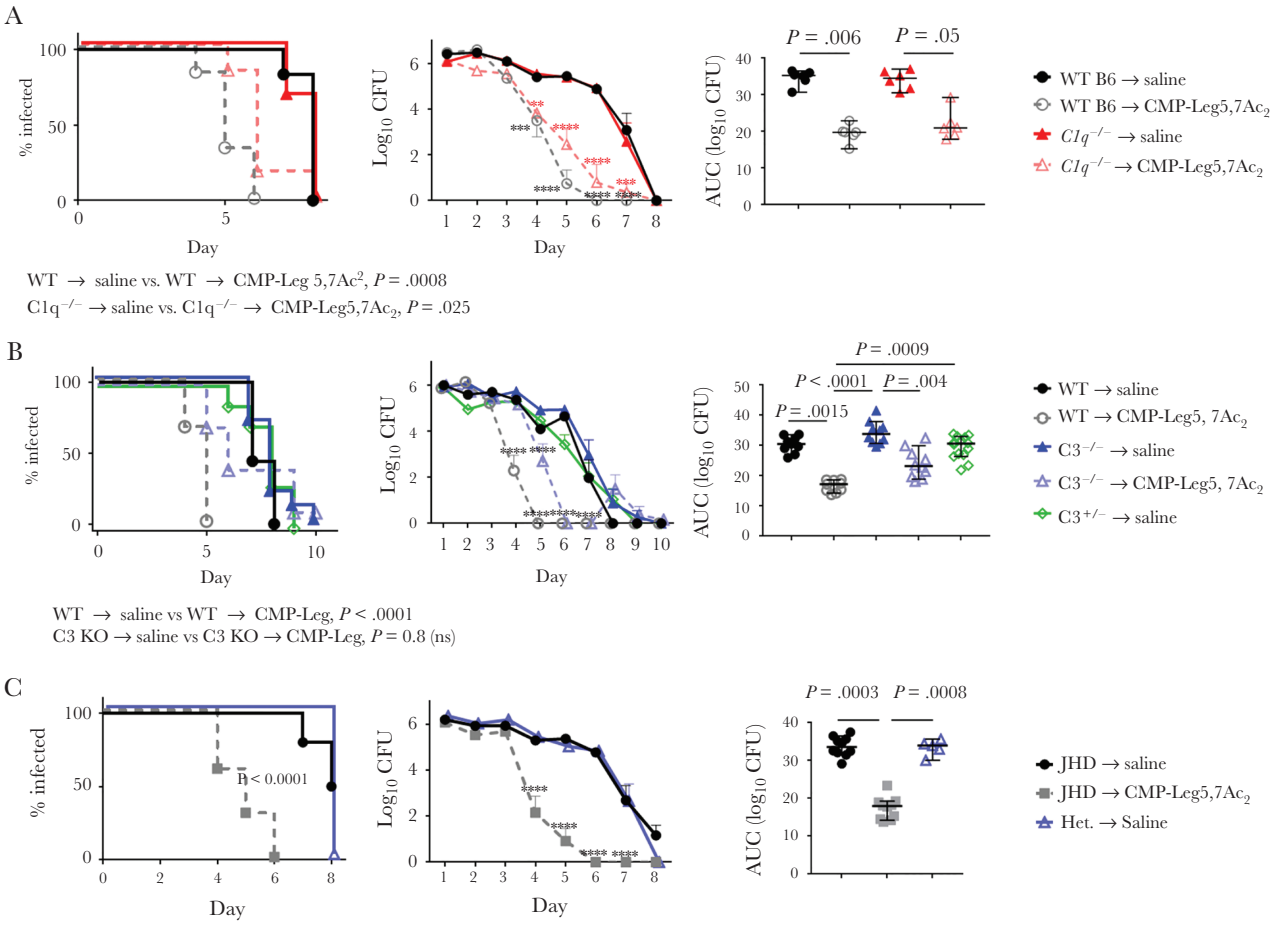

Figure 1. CMP-Leg5,7Ac$_2$ retains efficacy against vaginal *Neisseria gonorrhoeae* colonization in mice deficient in complement or antibodies. *A*, Efficacy of CMP-Leg5,7Ac$_2$ in *C1q*$^{-/-}$ mice. Premarin-treated wild-type (WT) C57BL/6 mice or *C1q*$^{-/-}$ mice (n = 6 mice per group) were infected intravaginally with 6.5×10^7 colony-forming units (CFU) of *N. gonorrhoeae* H041 and treated daily (starting 2 hours before infection) intravaginally either with saline (vehicle control) or with 10 µg CMP-Leg5,7Ac$_2$. Vaginas were swabbed daily to enumerate *N. gonorrhoeae* CFUs. The graph on the left shows Kaplan–Meier curves indicating time to clearance of infection. Groups were compared using the Mantel–Cox (log-rank) test. The middle graph shows $\log_{10}$ CFU vs time. Comparisons of the CFU over time between each treatment group and the respective saline control were made by 2-way analysis of variance (ANOVA) and Dunnett multiple comparison test. $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$. The graph on the right shows bacterial burdens consolidated over time (area under the curve [AUC] $\log_{10}$ CFU analysis). The 4 groups were compared by 1-way ANOVA using the nonparametric Kruskal–Wallis equality of populations rank test. The χ^2 with ties was 17.55 ($P = .0005$). Pairwise AUC comparisons across groups were made with Dunn multiple comparison test. *B*, Efficacy of CMP-Leg5,7Ac$_2$ *C3*$^{-/-}$ mice (n = 10/group). Wild-type BALB/c mice (n = 9/group) or *C3*$^{-/-}$ mice (n = 10/group) were infected with 6.5×10^7 CFU *N. gonorrhoeae* H041. *C3*$^{+/-}$ mice (n = 14) given saline constituted an additional control group. The infecting strain, inoculum size, procedures, and statistical analyses were as described in (*A*). Left graph: Kaplan–Meier curves. Middle graph: $\log_{10}$ CFU vs time. $^{****}P < .0001$. Right graph: AUC ($\log_{10}$ CFU) analysis). The χ^2 with ties was 33.78 ($P < .0001$). *C*, CMP-Leg5,7Ac$_2$ is efficacious against *N. gonorrhoeae* in JHD mice. Wild-type BALB/c mice or JHD mice in a BALB/c background (n = 10 mice/group) were infected intravaginally with 7.8×10^7 CFU *N. gonorrhoeae* H041 and treated with saline CMP-Leg5,7Ac$_2$ as described above. A group of 5 heterozygous (Het.) mice infected and treated with saline served as additional controls. Left graph: Kaplan–Meier curves. Middle graph: $\log_{10}$ CFU vs time. $^{****}P < .0001$ (2-way ANOVA and Dunnett posttest). Right graph: AUC analysis. The χ^2 with ties was 17.33 ($P = .0002$). Pairwise AUC comparisons across groups were made with Dunn multiple comparison test.

to both CAMPs above levels seen when no CMP-NulO was added. Again, killing of H041 Δlst by CAMPs was not influenced by any of the CMP-NulOs. These data suggest that only Neu5Ac, but not Leg5,7Ac$_2$ or Kdn, confers on gonococci, the ability to resist CAMPs.

CMP-NulOs Require mCRAMP for Efficacy in Vivo

The efficacy of CMP-Leg5,7Ac$_2$ and CMP-Kdn were next tested in mice that lacked mCRAMP (*Camp*$^{-/-}$). As shown in Figure 4, both CMP-Leg5,7Ac$_2$ (Figure 4A and Supplementary Figure 2) and CMP-Kdn (Figure 4B) lost all efficacy in *Camp*$^{-/-}$ mice, strongly suggesting that CAMPs were necessary for the therapeutic efficacy of CMP-NulOs against *N. gonorrhoeae* in the mouse vaginal colonization model.

Dissociation Between Complement Sensitivity In Vitro and Efficacy in Mice Revealed by CMP-Neu5,9Ac$_2$

Incorporation of Kdn or Neu5,9Ac$_2$ into gonococcal LOS result in similar complement resistance profiles [23] (Supplementary Table 1). However, unlike CMP-Kdn, CMP-Neu5,9Ac$_2$ had no activity against *N. gonorrhoeae* in the mouse vaginal colonization model [23]. We hypothesized that Neu5,9Ac$_2$ incorporation

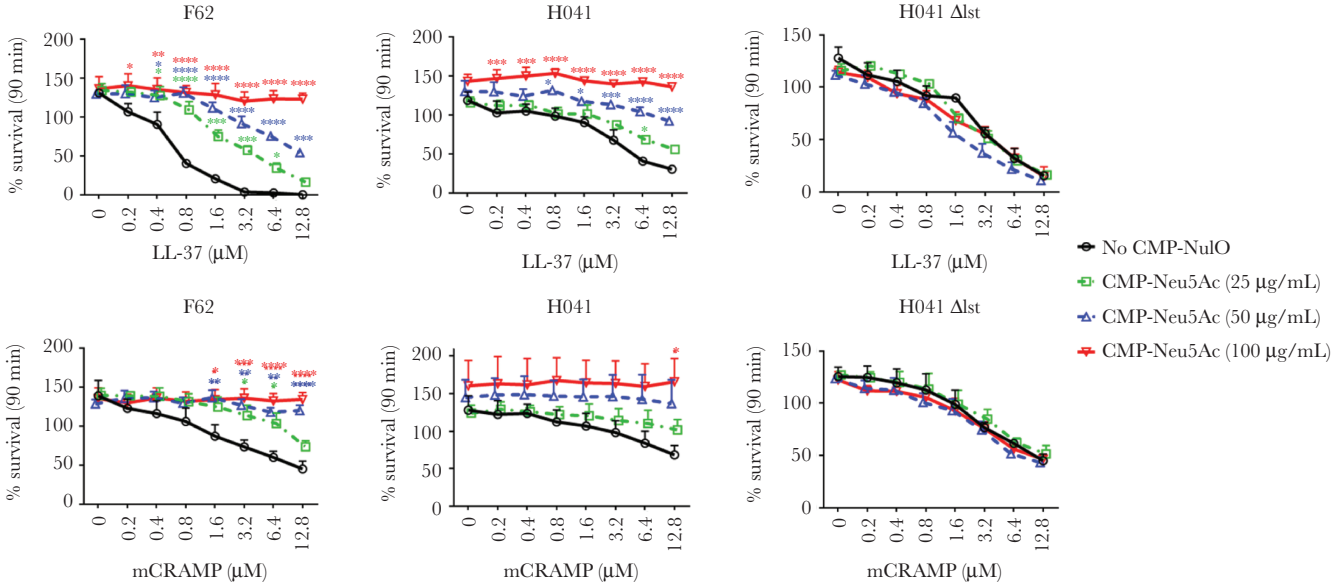

Figure 2. Gonococcal lipooligosaccharide (LOS) sialylation confers resistance to cationic antimicrobial peptides in a dose-dependent manner. Gonococcal strains F62 (graphs on left), H041 (graphs in middle), and LOS sialyltransferase-deficient mutant H041 Δlst (graphs on right; negative control) that were grown in media alone ("no CMP-NulO") or media supplemented with 25, 50, or 100 μg/mL CMP-Neu5Ac were incubated with either LL-37 (top row) or mouse cathelicidin-related antimicrobial peptide (mCRAMP; bottom row) at concentrations ranging from 0 to 12.8 μM (*x*-axis). Survival at 90 minutes relative to colony-forming units at 0 minute is shown on the y-axis (mean [standard error of the mean] of 3 experiments). Groups were compared using 2-way analysis of variance, and pairwise comparisons between each of the CMP-Neu5Ac groups and the "No CMP-NulO" group were made with Dunnett test. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.

into LOS would confer resistance against CAMPs. As shown in Figure 5, growth of bacteria in CMP-Neu5,9Ac$_2$ conferred partial protection against LL-37 (more so in H041 than F62) and a similar level of protection against mCRAMP (similar in both strains) as bacteria grown in CMP-Neu5Ac. Supplementary Figure 3 lists the half-maximal inhibitory concentration of LL-37 and mCRAMP against strains F62 and H041 when grown in media alone, or media supplemented with CMP-Leg5,7Ac$_2$, CMP-Kdn, or CMP-Neu5,9Ac$_2$. Although restricted to CMP-Neu5,9Ac$_2$, these data suggest that sensitivity to CAMPs, but not sensitivity to complement, may better predict activity of CMP-NulO therapeutic candidates against *N. gonorrhoeae* in vivo.

DISCUSSION

We have identified CMP-NulOs that show promise against *N. gonorrhoeae* in preclinical studies using a mouse model. Previously, we had shown that *N. gonorrhoeae* grown in media that contained the CMP-NulOs, CMP-Leg5,7Ac$_2$ and CMP-Kdn, prevented resistance to complement-mediated killing of gonococci that results from Neu5Ac incorporation into LOS, putatively facilitating bacterial clearance. We surmised that, in mice, susceptibility to complement killing was required for activity of CMP-NulOs against *N. gonorrhoeae*. Importantly, we have shown here that efficacy of certain antigonococcal CMP-nonulosonates require cathelicidins and are less dependent on complement.

Previous work has shown that incorporation of Neu5Ac into gonococcal LOS contributes to resistance to CAMPs [17, 35]. Sialylated gonococci, although more resistant to CAMPs than their nonsialylated counterparts, nonetheless, bound higher amounts of LL-37 and mCRAMP [35]. Neu5Ac retains CAMPs on bacterial surfaces and prevents intercalation of CAMPs into membranes that disrupts them and kills the bacteria, offering a possible explanation for resistance to CAMPs by sialylated bacteria. Our data demonstrate variability among NulOs in conferring resistance to CAMPs. While Neu5Ac fully protected gonococci against LL-37 and mCRAMP, Neu5,9Ac$_2$ protected gonococci only partially against LL-37 but fully against mCRAMP. Of note, Neu5,9Ac$_2$ can be 9-*O*-deacetylated by esterases [36, 37] converting it to Neu5Ac, which may explain its observed protection against CAMPs and the absence of activity of CMP-Neu5,9Ac$_2$ in vivo [23]. Leg5,7Ac$_2$ and Kdn on LOS provided no protection against either CAMP, while maintaining their mode of action to prevent resistance to complement-mediated killing. Although the molecular basis for Neu5Ac-mediated resistance to CAMPs remains unclear, our studies suggest that the negative charge of a NulO alone (Neu5Ac, Kdn, and Leg5,7Ac$_2$ are all similarly negatively charged) is insufficient to mediate resistance to CAMPs.

Cathelicidins—named because they contain a cathelin domain—are produced by cervical epithelial cells, neutrophils, and T and B lymphocytes (reviewed in [38]). Humans, rhesus macaques, mice, rats, and guinea pigs all possess a single cathelicidin species. The inactive precursor of LL-37 is an 18 kDa molecule called hCAP-18. Cleavage of the cathelin

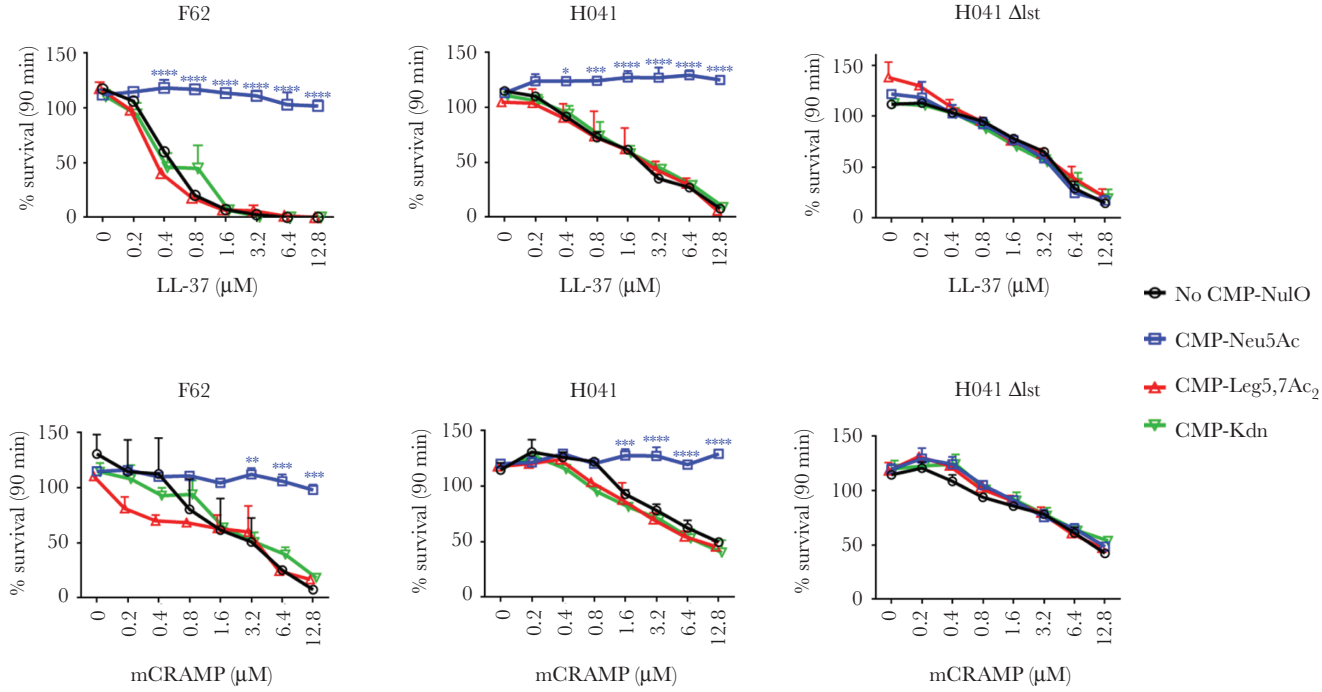

Figure 3. Incorporation of Leg5,7Ac$_2$ or Kdn into LOS does not confer resistance to cationic antimicrobial peptides (CAMPs). *Neisseria gonorrhoeae* strains F62 (graphs on left), H041 (graphs in middle), and LOS sialyltransferase-deficient mutant H041 Δlst (graphs on right; negative control) that were grown in media alone ("No CMP-NulO") or media supplemented with 100 μg/mL CMP-Neu5Ac (control for maximal resistance to CAMPs on wild-type gonococci), CMP-Leg5,7Ac$_2$ or CMP-Kdn were incubated with either LL-37 (top row) or mouse cathelicidin-related antimicrobial peptide (mCRAMP; bottom row) at concentrations ranging from 0 to 12.8 μM (x-axis). Survival at 90 minutes relative to CFU at 0 minute is shown on the y-axis (mean [range] of 2 separate experiments). Groups were compared using 2-way analysis of variance and pairwise comparisons between each of the CMP-NulO-treated groups and the "No CMP-NulO" group was made with Dunnett test. *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$.

domain of hCAP-18 releases the active 37 amino acid antimicrobial LL-37 peptide. Localization of mCRAMP to specific cells in the mouse genital tract, responsible for clearance of NulO-coated gonococci, has not been determined. Sialylated vs nonsialylated gonococci were shown to survive killing by mouse neutrophils; however, there was no difference in survival when neutrophils from *Camp*$^{-/-}$ mice were used [35], highlighting the role of LOS sialylation in the defense against CAMPs. The MtrC-MtrD-MtrE efflux pump, which serves to expel CAMPs from gonococci is, at least in part, responsible for gonococcal defense against CAMPs [17, 39, 40]. Upon deletion of the *mtrE* gene Neu5Ac sialylation of LOS was unable to protect gonococcal strains MS11 and F62 from killing by LL-37 or mCRAMP when tested in vitro. MtrE deletion mutants were even more attenuated than the Lst mutants in wild-type mice using vaginal inocula that contained wild-type organisms and deletion mutants in equal amounts [17]. Interestingly, the loss of mCRAMP (*Camp*$^{-/-}$ mice) restored virulence of only the Lst mutants, but not the MtrE deletion mutants [17]. Of note, gonococci down-regulate expression of LL-37 by ME-180 cells [41] and macrophages [42], suggesting that gonococci may have evolved mechanisms to suppress this arm of host immunity. The extent to which gonococci suppress LL-37 production in humans remains to be elucidated. Cathelicidins have been increasingly recognized as immunomodulators. They regulate neutrophil and monocyte chemotaxis, promote phagocytosis, skew polarization of macrophages to a proinflammatory phenotype, induce expression of chemokines and regulate intra- and extracellular Toll-like receptor activation [43]. Whether cathelicidins eliminate gonococci in vivo by direct bacterial killing and/or by recruiting additional immune effector mechanisms remains to be determined.

Phosphoethanolamine (PEtn), a lipid A substituted moiety, encoded by *lptA*, also plays a key role in defending the bacterium against CAMPs. Gonococcal *lptA* deletion mutants are hypersusceptible to polymyxin B, a CAMP [44–46], and are less virulent than their wild-type counterparts in female mice and in the human male urethral challenge model [47]. Deleting *lptA* from another pathogenic *Neisseria* species, represented by *Neisseria meningitidis* strain NMB, resulted in an 8-fold reduction of LL-37 minimum inhibitory concentrations (from 15.6 μg/mL to 1.95 μg/mL [48]), suggesting the likelihood that lipid A PEtn also contributes to gonococcal resistance to LL-37. By contrast, loss of PEtn from *Haemophilus ducreyi* lipid A did not alter resistance to LL-37 [49], suggesting that the same moiety in different species may serve different functions. Of note, gonococcal *lptA* mutants are also more susceptible to killing by complement [45, 50]. Whether attenuation of gonococcal *lptA* mutants in vivo is interrelated with

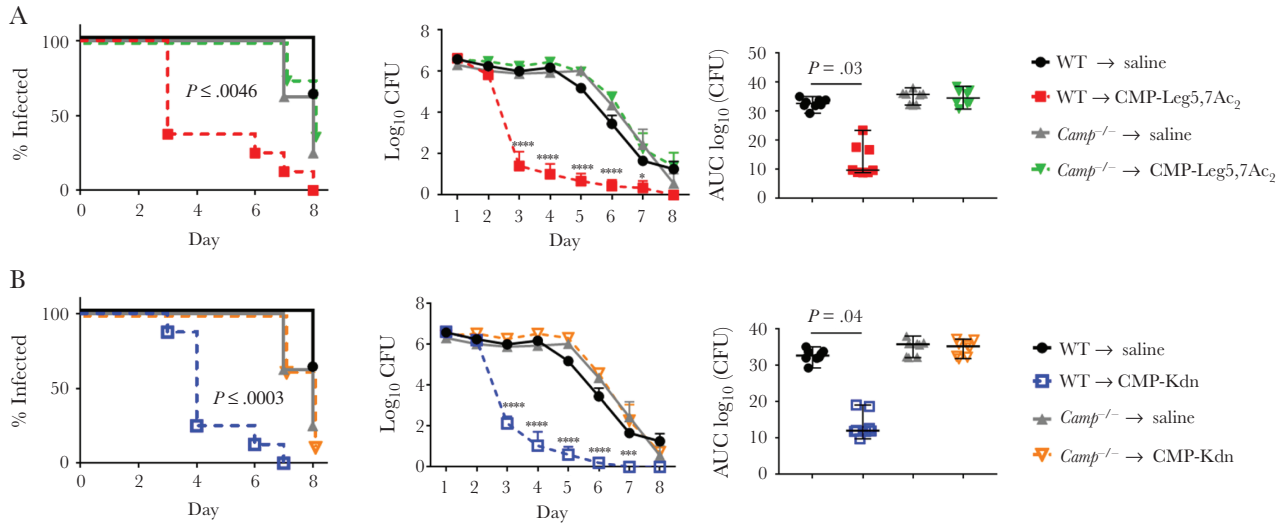

Figure 4. Efficacy of topically administered CMP-Leg5,7Ac$_2$ (*A*) and CMP-Kdn (*B*) against *Neisseria gonorrhoeae* was abrogated in *Camp*$^{-/-}$ mice. Premarin-treated wild-type (WT) BALB/c mice or *Camp*$^{-/-}$ mice in a BALB/c background (n = 8 in each group) were infected with 2.7×10^7 colony-forming units (CFU) of *N. gonorrhoeae* H041 and treated daily (starting 2 hours before infection) intravaginally either with saline (vehicle control) or with 10 µg CMP-NulO. The graphs on the left indicate time to clearance (Mantel–Cox analysis). WT mice given CMP-NulO were compared with each of the other groups and the highest *P* value is indicated. The graphs in the middle show $\log_{10}$ CFU vs time. Comparisons of the CFU over time between each treatment group and the respective saline control were made by 2-way analysis of variance (ANOVA) and Dunnett multiple comparison test. **P* < .05; ****P* < .001; *****P* < .0001. Graphs on the right show area under the curve ($\log_{10}$ CFU). The 4 groups were compared by 1-way ANOVA using the nonparametric Kruskal–Wallis equality of populations rank test. The χ^2 with ties was 19.65 (*P* = .0002) for (*A*) and 20.13 (*P* = .0002) for (*B*). Pairwise area under the curve (AUC) comparisons across groups were made with Dunn multiple comparison test, and significant values are indicated.

loss of resistance to complement, CAMPs, or both remains to be elucidated.

In conclusion, our data show that resistance to cathelicidins mediated by NulOs varies with NulO structure. The efficacy of candidate therapeutic CMP-NulOs against multidrug-resistant *N. gonorrhoeae* in vivo is attributable to cathelicidins. Elucidating the mechanism of action of therapeutic CMP-NulOs will facilitate further preclinical development of lead

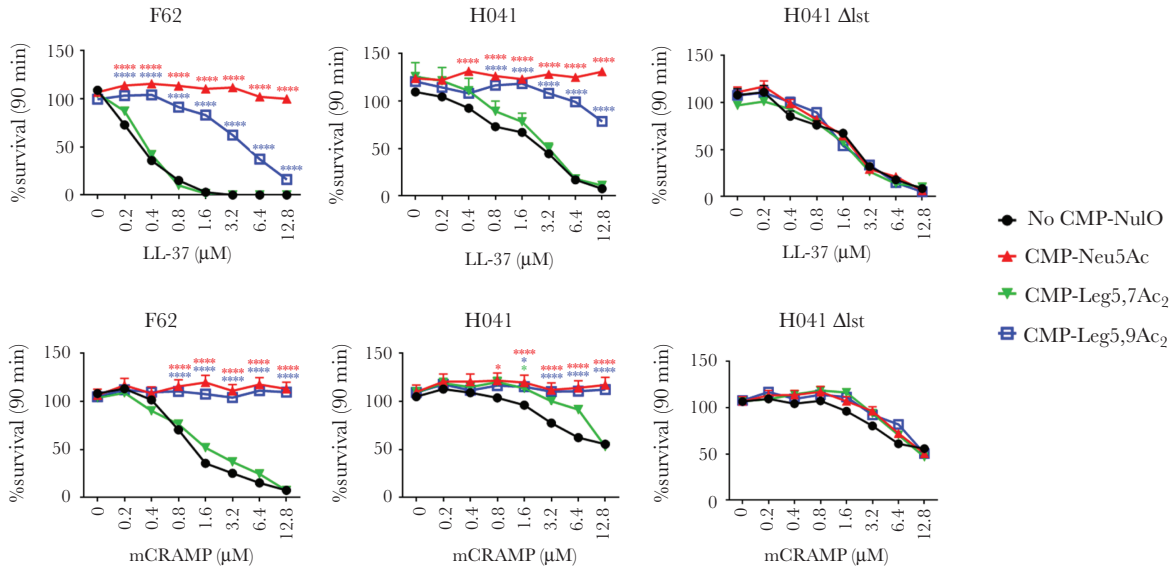

Figure 5. Neu5,9Ac$_2$-capped gonococcal lipooligosaccharide (LOS) confers resistance to cationic antimicrobial peptides. *Neisseria gonorrhoeae* strains F62 (graphs on left), H041 (graphs in middle), and LOS sialyltransferase-deficient mutant H041 Δlst (graphs on right; negative control) that were grown in media alone ("No CMP-NulO") or media supplemented with 100 µg/mL CMP-Neu5Ac (control for maximal resistance) or CMP-Neu5,9Ac$_2$ were incubated with either LL-37 (top row) or mouse cathelicidin-related antimicrobial peptide (mCRAMP; bottom row) at concentrations ranging from 0 to 12.8 µM (x-axis). Survival at 90 minutes relative to colony-forming units at 0 minute is shown on the y-axis (mean [range] of 2 separate experiments). Groups were compared using 2-way analysis of variance, and pairwise comparisons between each of the CMP-NulO-treated groups and the "No CMP-NulO" group was made with Dunnett test. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001.

compounds, as well as enable identification of additional NulO-based therapeutics in the fight against antimicrobial-resistant *N. gonorrhoeae.*

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. I. C. S. and S. R. are listed as inventors on patents related to the use of CMP-nonulosonates for prevention and treatment of gonococcal infections (assignees: National Research Council of Canada and University of Massachusetts Medical School). All other authors report no potential conflicts of interest.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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