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Phosphoethanolamine Decoration of Neisseria gonorrhoeae Lipid A Plays a Dual Immunostimulatory and Protective Role during Experimental Tract Infection


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The induction of an intense inflammatory response by Neisseria gonorrhoeae and the persistence of this pathogen in the presence of innate effectors is a fascinating aspect of gonorrhea. Phosphoethanolamine (PEA) decoration of lipid A increases gonococcal resistance to complement-mediated bacteriolysis and cationic antimicrobial peptides (CAMPs), and recently we reported that wild-type N. gonorrhoeae strain FA1090 has a survival advantage relative to a PEA transferase A (lptA) mutant in the human urethral-challenge and murine genital tract infection models. Here we tested the immunostimulatory role of this lipid A modification. Purified lipooligosaccharide (LOS) containing lipid A devoid of the PEA modification and an lptA mutant of strain FA19 induced significantly lower levels of NF-κB in human embryonic kidney Toll-like receptor 4 (TLR4) cells and murine embryonic fibroblasts than wild-type LOS of the parent strain. Moreover, vaginal proinflammatory cytokines and chemokines were not elevated in female mice infected with the isogenic lptA mutant, in contrast to mice infected with the wild-type and complemented lptA mutant bacteria. We also demonstrated that lptA mutant bacteria were more susceptible to human and murine cathelicids due to increased binding by these peptides and that the differential induction of NF-κB by wild-type and unmodified lipid A was more pronounced in the presence of CAMPs. This work demonstrates that PEA decoration of lipid A plays both protective and immunostimulatory roles and that host-derived CAMPs may further reduce the capacity of PEA-deficient lipid A to interact with TLR4 during infection.

Neisseria gonorrhoeae causes an estimated 106 million infections annually worldwide (1). The majority of N. gonorrhoeae infections are uncomplicated mucosal infections of the urogenital tract. Pharyngeal and rectal infections are also common, and upper reproductive tract infections can occur in both sexes. Ascending infection occurs in 10 to 20% of women with gonococcal cervicitis, and the major morbidity and mortality associated with gonorrhea is due to gonococcal pelvic inflammatory disease (PID) and the associated complications of involuntary infertility, chronic pelvic pain, and ectopic pregnancy (2, 3). Neonates of infected mothers are at risk of a purulent conjunctivitis that can lead to corneal scarring and blindness, and gonorrhea during pregnancy is associated with low birth weight and septic abortions (4). Gonorrhea is also a known cofactor in the spread of human immunodeficiency virus (5, 6). There is no immunity to repeat infections, and the rapid emergence of antibiotic resistance in N. gonorrhoeae seriously threatens control measures (1, 7).

N. gonorrhoeae urogenital tract infections range from inapparent to symptomatic, the latter of which are characterized by a purulent exudate that contains numerous polymorphonuclear leukocytes (PMNs) with intracellular gonococci. Recently, the inflammatory response to N. gonorrhoeae was shown to occur through the Th17 pathway in the female mouse model of gonococcal infection (8). This finding is consistent with the detection of Th17 responses in humans with gonorrhea (9) and the central role that the Th17 pathway plays in infections by other pyogenic cocci (10). Differentiation and proliferation of Th17 cells occur under the influence of the interleukin 6 (IL-6), transforming growth factor β (TGF-β), IL-1β, and IL-23 produced by antigen-presenting cells in response to microbial stimulation. The subsequent production of IL-17 and its binding to IL-17 receptor A (IL-17RA) on endothelial and stromal cells induces granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), and the CXC chemokines KC, LIX, and MIP-2α, which are potent recruiters of PMNs. Lipid A-mediated signaling through Toll-like receptor 4 (TLR4) is critical for the induction of Th17 responses by N. gonorrhoeae (8, 11), and IL-17 and IL-22 produced by the Th17 pathway lead to the production of cationic antimicrobial peptides (CAMPs) by epithelial cells (12, 13), which are also carried within PMN granules. Variations in surface structures can influence interactions between microbes and the host, and in the pathogenic neisseriae, phosphoethanolamine (PEA) substitution at the 4′ position of lipid A increases resistance to polymyxin B (PMB), a bacterium-derived CAMP (14). PEA decoration of heptose II of the oligosaccharide side chain of lipid A also increases resistance to bactericidal activity of human serum and human cationic peptides (15, 16).
charide core of the lipooligosaccharide (LOS) β-chain, in contrast, reduces resistance to PMB in Neisseria meningitidis but has no effect in N. gonorrhoeae. The presence of a lipid A PEA moiety also increases N. gonorrhoeae resistance to complement-mediated killing (15, 16) but does not affect the serum resistance of N. meningitidis. We recently demonstrated that an lptA mutant of N. gonorrhoeae strain FA1090 was attenuated during experimental urethral infection of male subjects and cervicovaginal infection of female mice (17), which strongly supports the protective role of this lipid A modification during infection.

There is also evidence that PEA decoration of neisserial lipid A impacts interactions with TLR4 and innate effectors. Jarvis and colleagues reported that the 4’ lipid A PEA modification and the PEA transferase A (lptA) gene, which catalyzes this modification, are found only in N. meningitidis, N. gonorrhoeae, and one of seven commensal Neisseria species that are generally considered non-pathogenic (18). These investigators also showed that the PEA lipid A modification had a major impact on the induction of tumor necrosis factor alpha (TNF-α) in a human monocytic cell line (THP-1 cells) (19) and enhanced both MyD88- and TRIF-dependent TLR4 signaling pathways (20), from which they hypothesized that the absence of this modification in commensal neisseriae allows these bacteria to reside in the host without inducing potentially protective host responses (18).

Evidence from these collective studies suggests that PEA decoration of lipid A in N. gonorrhoeae plays the dual role of inducing inflammation and protecting the pathogen from complement and CAMPs, which are increased during the inflammatory response. In our recent experiments with human subjects and female mice, competitive infections were performed between mutant bacteria and the wild-type parent strain (17), and therefore, the effect of the PEA lipid A decoration on host responses could not be measured. Here we examined the impact of lipid A PEA decoration on induction of a proinflammatory response through TLR4 using human and murine tissue culture cell lines and on the host inflammatory response to infection as assessed by noncompetitive murine infection. We also examined the bactericidal and immunomodulatory activities of cationic lipids against gonococci with wild-type or PEA-deficient lipid A, which are a class of host-derived CAMPS that protect against other bacterial infections (21, 22). The outcome of these studies is the demonstration that PEA decoration of gonococcal lipid A is an important adaptation of N. gonorrhoeae that plays both immunomodulatory and protective roles, thereby providing a balance between inducing and surviving host innate defenses.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** Wild-type N. gonorrhoeae strain FA19, an isogenic lptA::spc mutant, and a complemented lptA (C′ lptA) mutant containing the wild-type lptA gene from strain FA19 were the primary test strains and have been described previously (14). The lptA mutant and C′ lptA mutant are spectinomycin (Spc) resistant due to the inactivation of the lptA gene with a spectinomycin resistance (spc) gene (14). Streptomycin (Sm)-resistant derivatives of these strains were constructed as described previously (23). Bacteria were cultured on gonococcal agar (GC agar) supplemented with Kellogg’s supplement I and ferric nitrate as described previously (24). For mouse infection experiments, GC agar supplemented with Sm (100 μg/ml) or Sm plus Spc (50 μg/ml) was used to isolate N. gonorrhoeae from vaginal swab suspensions. All cultures were incubated at 35 to 37°C in 7% CO₂.

**NF-kB reporter assay.** Human embryonic kidney (HEK) cells expressing the TLR4-MD2-CD14 receptor complex and mouse embryonic fibroblasts (MEFs) from C3H/HeN (wild-type TLR4) mice carrying the NF-kB-inducing secreted embryonic alkaline phosphatase (SEAP) reporter gene (InvivoGen, CA) were used to assess the NF-kB-stimulatory property of purified LOS and whole bacteria according to the manufacturer’s instructions. Briefly, LOS from wild-type or lptA mutant gonococci was purified as previously described (17) and added to 5 x 10⁵ HEK TLR4 cells or MEFs at concentrations of 0.1, 1, 10, or 100 ng/ml in 96-well plates and incubated for 20 h at 37°C (total reaction volume, 200 μl/well). For whole bacteria, a multiplicity of infection (MOI) of 1 was used. For SEAP detection, 20 μl of supernatant from LOS-treated or infected cells was added to 180 μl of the QUANTI-blue substrate (InvivoGen) in 96-well microtiter plates. Supernatants from untreated or uninfected cells were used as negative controls. The reaction mixture was incubated at 37°C for 30 min, and SEAP activity was assessed by reading the optical density at 650 nm (OD₆₅₀). For LOS neutralization assays, whole bacteria or LOS from wild-type or lptA mutant gonococci were preincubated alone or with 10 μg of CAMPS for 30 min at 37°C before the NF-kB reporter cells were added to the 96-well plates, and SEAP measurement was performed as described above.

**Bacterial viability assay and LDH assay.** MEFs containing the NF-kB-inducing SEAP reporter gene were incubated with 10⁸ CFU of strain FA19S⁶, the lptA mutant, or the C′ lptA mutant at an MOI of 1 in 96-well plates. Wells with media alone were used as controls. Survival of the bacteria was measured after 24 h of incubation by quantitatively culturing 20 μl of the culture supernatant on GC agar. The viability of MEFs was measured by a lactate dehydrogenase (LDH) assay in which 20 μl of the supernatant was added to twice the volume of the LDH assay mix (Sigma tox7 kit). After 30 min of incubation, the reaction was stopped by adding 1/10 the volume of 1 N HCl, and the amount of color produced was measured at 490 nm. Growth medium alone with no MEFs and growth medium with lysed MEFs were used as negative and positive controls, respectively.

**Experimental murine infection.** Female BALB/c mice (6 to 8 weeks old; National Cancer Institute) were treated with water-soluble 17β-estradiol and antibiotics to increase their susceptibility to N. gonorrhoeae as described previously (25). For noncompetitive infections, groups of estradiol-treated mice were inoculated vaginally with 10⁶ CFU of wild-type FA19S⁶, the lptA mutant, or the C′ lptA mutant bacteria or inoculated vaginally with phosphate-buffered saline (PBS) alone (uninfected control group). Vaginal swabs were collected the following day and every other day for 7 days and suspended in 100 μl of GC broth. Samples were serially diluted and quantitatively cultured on GC agar with Sm. A small portion of sample was also cultured on heart infusion agar to monitor the presence of potentially inhibitory facultatively anaerobic commensal flora. Vaginal washes were also collected an hour after the cultures were taken on day 5 postinoculation with bacteria by pipetting 50 μl of PBS in and out of the vaginas of control and test mice 20 times. The lavage fluid was then centrifuged at 13,000 x g for 3 min. The supernatant was frozen immediately and stored at −70°C for subsequent cytokine/chemokine analysis using the Bio-Plex system (Bio-Rad, Hercules, CA). For competitive-infection experiments, groups of mice were inoculated with 10⁶ CFU of wild-type FA19S⁶ bacteria combined with similar numbers of lptA mutant or C′ lptA bacteria (total dose, 10⁶ CFU) (n = 3 to 8 mice/group). Vaginal swabs were collected every other day for 6 days starting with day 2 and suspended in 100 μl of GC broth. Vaginal swab suspensions and the inocula were cultured quantitatively on GC agar with Sm (total CFU) and GC agar with Sm plus Spc (lptA mutant or C′ lptA mutant CFU), and the number of wild-type CFU was calculated by subtracting the number of Spc-resistant CFU from the total number of CFU. Results were expressed as the competitive index (CI) for individual mice using the following equation: the number of mutant CFU over the number of wild-type CFU (output) divided by the number of mutant CFU over the number of wild-
type CFU (input). Competitive infections were performed three times and the data combined (a total of 17 to 18 mice per mixture were tested).

**Multiplex cytokine/chemokine analysis.** Cytokine concentrations (pg/ml) were measured in cell-free vaginal lavage supernatants by multiplex assay using the 20-plex mouse inflammatory cytokine and chemokine panel (Invitrogen Life Sciences).

**Susceptibility to CAMPs.** Synthetic CRAMP-38, LL-37, and the scrambled LL-37 peptide RSLEGTDRIFPVRKLNSKLEFKDIKIGKRQFKVLKIL were obtained from Jan Pohl (Centers for Disease Control). Polymyxin B (PMB) was purchased from Sigma. Bacteria were cultured in GC broth to the mid-logarithmic phase, and 10^6 CFU were suspended in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Quality Biologicals) containing LL-37, CRAMP-38, or scrambled LL-37 peptide (final peptide concentration, 1 μg/ml). Suspensions were incubated in 96-well polystyrene microtiter plates (total assay volume, 200 μl) at 37°C in 7% CO2. After overnight incubation at 37°C, 10 μl of the cultures were spotted onto GC agar plates, incubated overnight, and scored for growth the following day. We also assessed the bactericidal capacity of LL-37 and the scrambled peptide (16 μg/ml) against test strains using a colony-forming reduction assay described previously (26).

**Flow cytometry assay.** Deposition of LL-37 or CRAMP-38 on the surfaces of whole bacteria was assayed by flow cytometry. Bacteria were washed twice with Hanks’ balanced buffer solution (HBSS) and passed through 1.2-μm filters to obtain single-cell suspensions. To minimize lysis of the bacteria by the peptides, an excess of bacteria and a short incubation time were used. Filtered suspensions containing 10^6 CFU were incubated with CRAMP-38 or LL-37 (10 μg/ml) at 37°C for 15 min. The bacteria were washed twice in HBSS, and surface-bound CAMPs were detected by incubation with rabbit anti-LL-37 (product number ab64892) or anti-CRAMP (product number ab74868) antibodies (Abcam) for 30 min and then with Alexa 488-conjugated secondary antibody (Invitrogen) for 15 min. After the plates were centrifuged and washed twice with HBSS, the bacteria were resuspended in 1 ml of HBSS and applied to a Becton, Dickinson LSRII flow cytometer to count at least 30,000 events. Bacteria incubated without CAMPs were used as the negative control for background fluorescence. Each strain was also incubated with CAMPs but without secondary antibodies in each assay, and we observed no difference from samples to which no CAMPs were added.

**LAL assay.** LOS from wild-type strain FA19 or the lptA mutant (10 ng) was preincubated alone or with increasing concentrations of CAMPs (final concentrations, 1, 10, and 100 μg/ml of PMB, 1 and 10 μg/ml of CRAMP and LL-37, and 10 μg/ml of lysozyme) for 30 min at 37°C in 96-well microtiter plates (total reaction volume, 50 μl). Fifty microliters of Limulus amebocyte lysate (LAL) factor resuspended in endotoxin-free water according to the manufacturer’s guidelines was added to each reaction well and incubated for 10 min at 37°C. The prewarmed chromogenic substrate (100 μl) was then added, and after 4 to 6 min, the reaction was stopped by the addition of 50 μl of stop solution (25% [vol/vol] acetic acid). Color development was measured spectrophotometrically, and the amount of free LOS that bound to LAL factor was reported as the OD405.

**Statistical analyses.** The numbers of bacteria recovered from mice over time in noncompetitive infections were compared by a repeated-measures analysis of variance. Levels of vaginal proinflammatory cytokines and chemokines were compared using a nonparametric Kruskal-Wallis test for comparison of multiple groups followed by Dunn’s multiple-comparison posttest. Differences in the levels of SEAP activity and LAL were measured by an unpaired Student’s t test (GraphPad).

**Animal use assurances.** All animal experiments were conducted at the Uniformed Services University of the Health Sciences, a facility fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, under a protocol that was approved by the University’s Institutional Animal Care and Use Committee.

**RESULTS**

Wild-type lipid A stimulates the human TLR4-MD2-CD14 complex to a greater extent than lipid A devoid of the PEA moiety. To examine the impact of the PEA lipid A modification on the capacity of *N. gonorrhoeae* strain FA19 to induce a proinflammatory response through TLR4 signaling, we incubated increasing amounts of purified LOS from wild-type and lptA mutant bacteria with human embryonic kidney (HEK) cells that express the human TLR4-MD2-CD14 receptor complex and carry an NF-κB-inducing secreted alkaline phosphatase (SEAP) reporter gene. A dose-dependent increase in SEAP activity occurred for both LOS species, but wild-type LOS induced a significantly greater increase in NF-κB expression than the LOS containing unmodified lipid A when concentrations of 1 and 10 ng of LOS were used (Fig. 1A). Results with murine embryonic fibroblasts (MEFs) with the NF-κB SEAP reporter gene from C3H/HeN (lps^−^) mice were similar, with wild-type LOS inducing higher levels of NF-κB expression than LOS lacking the lipid A PEA modification when 0.1, 1, and 10 ng/ml were tested (Fig. 1B). These results confirm an earlier report that the lipid A PEA modification in *N. gonorrhoeae* is important in inducing TNF-α in human THP1 monocytic cells (19).

Interactions between surface molecules other than lipid A and their cognate innate receptors can also influence innate responses to *N. gonorrhoeae*. We therefore also tested the inflammatory po-
potential of whole bacteria using the MEF NF-κB reporter system that utilizes fibroblasts from C3H/HeN mice, which express a functional TLR4 molecule as well as several other innate immunity receptors, including TLR2. At a multiplicity of infection of 1, similar levels of SEAP activity were detected in supernatants from cells incubated with wild-type, lptA mutant, and C’lptA mutant bacteria, suggesting that other bacterial ligands may induce NF-κB expression in this system (Fig. 2A). However, we also observed that greater than a log fewer lptA mutant gonococci were recovered after 24 h of incubation with the MEFs than when the wild-type and complemented strains were used (Fig. 2B). We saw no evidence of differential killing of the MEFs by these three strains when levels of lactate dehydrogenase activity were used as a marker of fibroblast viability (Fig. 2C). This result suggests that the reduced survival of the mutant during incubation with MEFs is due to soluble factors produced by the MEFs (the identity of which are not yet known but are under investigation) and not due to the differential death of the MEFs.

Wild-type but not lptA mutant gonococci induce a proinflammatory response during infection. Experimental gonococcal infection of BALB/c mice is characterized by a significant increase in proinflammatory vaginal cytokines and chemokines and an influx of vaginal PMNs on day 5 postinoculation (27). We therefore used this model to assess whether wild-type and lptA mutant gonococci differ in their inflammatory potentials in vivo. Groups of mice were inoculated with wild-type, lptA mutant, or C’lptA mutant bacteria (10⁵ CFU/group). Colonization load was measured by culturing vaginal swabs for N. gonorrhoeae on days 1, 3, 5, and 7 postinoculation, and vaginal washes were collected for multiplex cytokine and chemokine analysis on day 5. In three different experiments, animals in each group were colonized for an average of 4.7 to 5.4 days (range, 0 to 7 days), with no statistically significant difference in the durations of colonization observed among groups. There was also no significant difference in the numbers of bacteria recovered from each group (Fig. 3). However, levels of vaginal proinflammatory cytokines were higher in mice infected with the wild-type or C’lptA mutant strains than in the untreated control group. Using the Kruskal-Wallis test, levels of TNF-α, IL-1α, and MIP-1α showed a significant difference among groups, with the levels of TNF-α induced by wild-type gonococci and the C’lptA mutant being significantly higher than levels in the mock-infected control mice; levels induced by the wild-type strain were also significantly higher than those produced by the lptA mutant (P<0.05, Dunn’s multiple-comparison test) (Fig. 4). Levels of IL-1β appeared to be lower in mice infected with the lptA mutant than in mice infected with wild-type and C’lptA mutant gonococci and similar to that of uninfected mice, although the results were not statistically significant. In contrast, mice inoculated with the lptA mutant showed no elevation in proinflammatory cytokines compared to uninfected control mice. The levels of IL-10 were similar in all groups, from which we conclude that the lower levels of proinflammatory cytokines in mice infected with the lptA mutant were not due to increased suppression by this anti-inflammatory cytokine. Accordingly, we
conclude that PEA decoration of lipid A, which is found in wild-type N. gonorrhoeae, plays a critical role in inducing an inflammatory response to gonococcal infection.

**PEA decoration of lipid A confers a survival advantage to N. gonorrhoeae during competitive genital tract infection.** The host innate response to N. gonorrhoeae infection in female BALB/c mice is well characterized (8, 11, 27, 28), and this model has been used to study other mechanisms by which N. gonorrhoeae evades innate effectors, including CAMPs, during infection (23, 29–31). When tested by competitive infection with the wild-type strain, the FA19 lptA mutant was attenuated in vivo, similar to our recent results with an lptA mutant of strain FA1090 (17). Groups of female BALB/c mice were inoculated with mixed bacterial suspensions containing similar numbers of wild-type bacteria and either lptA mutant or C’lptA mutant gonococci. The number of bacteria of each strain recovered from vaginal swab suspensions was determined over a period of 6 days and the ratio was normalized to the ratio of the numbers of each strain in the inoculum. In three experiments, we observed a 10- to 100-fold decrease in the recovery of the lptA mutant relative to the wild-type parent strain FA19SmR (geometric mean CI, 0.12 and 0.04 on days 4 and day 6 of infection, respectively) (Fig. 5A). Importantly, no mutant bacteria were recovered from 6 of 13 infected mice on day 6 postinoculation. In contrast, wild-type gonococci were recovered from these mice at levels of >10³ to 10⁷ CFU/100 μl vaginal swab suspension at this time point (Fig. 5A, open diamonds). Genetic complementation restored recovery of the mutant and in fact increased fitness >1,000-fold relative to that of the wild-type strain (Fig. 5B). Neither the lptA nor the C’lptA mutant showed a difference in fitness from that of the wild-type strain when cocultured in vitro through stationary phase (data not shown). The increased fitness of the C’lptA mutant relative to the wild-type strain may be due to higher expression of the lptA gene in the C’lptA mutant. In this strain, the complementing lptA gene is under the control of the lac promoter (14), and increased expression of isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible genes has been demonstrated previously during murine infection (17, 31). These results suggest that the PEA moiety on wild-type lipid A is beneficial to N. gonorrhoeae during infection and provides a fitness advantage over the fitness of gonococci devoid of PEA-decorated lipid A.

**PEA-decorated lipid A protects gonococci against cathelicidins by reducing surface binding.** CAMPs are carried by phagocytes and inducibly produced by epithelial cells, including genital tract epithelial cells (22), in response to infection. Like PMB, a bacterial CAMP, cathelicidins kill bacteria by binding to and permeabilizing and/or depolarizing negatively charged bacterial membranes (32). To test the effect of PEA lipid A decoration on gonococcal resistance to the cathelicidins, we compared the susceptibilities of wild-type N. gonorrhoeae strain FA19, isogenic lptA mutant, and complemented mutant (C’lptA) bacteria to the human and mouse cathelicidins LL-37 and CRAMP-38. Bacteria

![FIG 3](image-url) **FIG 3** Wild-type and lptA mutant gonococci colonize mice equally well during noncompetitive infections. Groups of BALB/c mice were inoculated with 10⁷ CFU of wild-type, lptA mutant, or C’lptA mutant bacteria, and the number of bacteria recovered from vaginal swabs was determined by quantitative culture (n = 11 to 16 mice per strain).

![FIG 4](image-url) **FIG 4** Gonococci devoid of the PEA lipid A moiety do not induce a proinflammatory response. Vaginal cytokines and chemokines were measured on day 5 postinoculation in the noncompetitive experiments whose results are shown in Fig. 3. Horizontal bars indicate the geometric mean. Kw*, Kruskal-Wallis test. $P < 0.05$, Dunn’s multiple comparison.
were incubated with or without CAMPs, and the numbers of viable bacteria remaining were determined by semiquantitative culture. Similar numbers of wild-type and \( C^{\prime}/lptA \) mutant bacteria were recovered from wells containing no peptide, LL-37, CRAMP-38, or a scrambled LL-37 peptide. In contrast, no \( lptA \) mutant bacteria were recovered following incubation with cathelicidin, while high numbers were recovered from wells containing scrambled peptide and no peptide (Fig. 6). In order to confirm that PEA decoration of lipid A increases gonococcal resistance to CAMPs, we also assessed the antigonococcal activities of LL-37 and a scrambled peptide variant against our test strains using a more quantitative assay. In two separate experiments, we found (data not presented) that, on average, the \( lptA::spc \) mutant exhibited increased susceptibility to 16 \( \mu \)g/ml of LL-37 (5% survival) compared to the susceptibilities of the wild-type and \( C^{\prime}/lptA \) mutant strains (25% and 35% survival, respectively); the scrambled peptide did not exhibit bactericidal action against any of the test strains. This finding is consistent with earlier results that showed that \( lptA \) null mutants are more susceptible than wild-type gonococci to PMB (14, 17).

We next determined whether the relative susceptibilities of the strains to the cathelicidins correlated with their surface binding. Bacteria were incubated with 10 \( \mu \)g/ml of cathelicidin, and bound CAMPs were detected by flow cytometry using CAMP-specific antibodies. Higher levels of CRAMP-38 and LL-37 were detected in \( lptA \) mutant bacteria than in wild-type or \( C^{\prime}/lptA \) bacteria (Fig. 7). A population of bacteria that did not bind the CAMPs was detected for all strains, which we hypothesize is due to the design of the assay conditions, which required a short incubation period and an excess of bacteria to detect binding without subsequent lysis of the bacteria. The outcome of these experiments suggests that cathelicidins have high binding affinity for bacteria devoid of PEA-decorated lipid A, which leads to efficient killing of the bacteria.

FIG 5 The lipid A PEA modification provides a competitive advantage during experimental genital tract infection. Female BALB/c mice were inoculated vaginally with similar numbers of bacteria of wild-type FA19SmR and the \( lptA \) mutant (A) or wild-type FA19SmR and the \( C^{\prime}/lptA \) mutant (B), and the relative recovery of each strain was determined over time. The competitive index (CI) for each mouse at each time point is shown, with the geometric mean indicated by a horizontal bar. A CI of 1.0 indicates equal levels of fitness, a CI of <1.0 indicates reduced fitness of the mutants, and a CI of >1.0 indicates greater fitness of the mutants. The limit of detection (4 CFU) was used for the number of mutant or wild-type gonococci when these strains were not recovered. Open diamonds correspond to mice in which only wild-type bacteria (A) or \( C^{\prime}/lptA \) mutant bacteria (B) were recovered. The open circle in panel A corresponds to a time point from which only mutant bacteria were recovered from one mouse. The decrease in the number of data points over time is due to some mice clearing infection or being culture negative at that time point. Results are the combined data from three different experiments.

FIG 6 PEA-decorated lipid A protects \( N. \) gonorrhoeae from cathelicidins. A semiquantitative assay was used to assess bacterial susceptibility to human and murine cathelicidins. A scrambled LL-37 peptide was used as a negative control. hLL-37, human LL-37.
Differential induction of proinflammatory cytokines by wild-type and \( lptA \) mutant LOS is enhanced by CAMPs. The LOS used in these studies showed no evidence of contaminating protein when analyzed by mass spectrophotometry (data not shown). However, as a second measure of contaminating proteins, we performed LOS neutralization assays with PMB. Consistent with lipid A being the sole inducer of NF-\( \kappa \)B expression, 125 \( \mu \)g/ml of PMB neutralized >95% of NF-\( \kappa \)B activity (data not shown). However, preincubation with low concentrations (1 and 10 \( \mu \)g/ml) of PMB neutralized unmodified LOS to greater extents than LOS with the PEA moiety (Fig. 8A). A similar observation was reported by Tzeng et al. (16) in studies with an \( lptA \) mutant of \( N. \) meningitidis, and based on the capacity of PMB to differentially neutralize modified and unmodified lipid A, we hypothesized that host-derived CAMPs may contribute to the differences in the inflammatory potentials of wild-type and \( lptA \) mutant gonococci observed in

FIG 7 Cathelicidins preferentially bind to gonococci with lipid A devoid of the 4'-PEA moiety. Flow cytometry was used to measure surface binding of CRAMP-38 and LL-37 wild-type, \( lptA \), and \( C' lptA \) mutant bacteria. The open and shaded areas represent the presence and absence of CAMPS, respectively. The assay was performed three times, and the results were similar.

FIG 8 Preincubation with CAMPs differentially neutralizes the inflammatory potential of unmodified LOS. LOS from wild-type (filled bars) or \( lptA \) mutant (open bars) gonococci was incubated with PMB (10 \( \mu \)g/ml) or 10 \( \mu \)g of CRAMP-38, human LL-37, or lysozyme and then tested for its capacity to induce NF-\( \kappa \)B expression in MEFs or bind to the LAL reagent. (A) SEAP activity produced by MEFs; (B) LAL activity. Results using 1 \( \mu \)g/ml and 100 \( \mu \)g/ml of PMB were similar (data not shown). Single, double, and triple asterisks correspond to \( P \) values that are \( \leq 0.05 \), \( \leq 0.01 \), and \( \leq 0.001 \), respectively.
chicken lysozyme has LPS neutralization properties (data not shown). These results support the hypothesis that the increased binding of cathelicidins to PEA-unmodified lipid A alters the inflammatory potential of unmodified lipid A.

To further assess this hypothesis, we used the LAL assay to measure the amount of unbound LOS after preincubation with various CAMPS. In the absence of preincubation with CAMPS, LAL factor bound equal amounts of lipid A from PEA-modified and unmodified LOS (Fig. 8B). Higher levels of lipid A were detected in the LAL assay when PEA-modified LOS was preincubated with 1, 10, and 100 μg/ml of PMA than when unmodified LOS was used. Similar results were obtained for CRAMP-38 and CRAMP-38 when a concentration of 10 μg/ml was used. Preincubation with 10 μg/ml of chicken lysozyme did not affect the degree of LAL binding. This result differs from an earlier report that chicken lysozyme has LPS neutralization properties (33). We conclude that CAMPS more efficiently neutralize unmodified lipid A due to increased binding to the PEA-deficient lipid A molecule and that CAMPS encountered during infection may contribute to the absence of proinflammatory responses in mice infected with the lptA mutant.

DISCUSSION

Sexually transmitted bacteria, such as N. gonorrhoeae, encounter a variety of innate effectors in the genital tract that are constitutively present or increased during periods of inflammation. Cervical and endometrial cells produce cathelicidins, defensins, secretory leukocyte protease inhibitor (SLPI), and bactericidal/permeability-increasing protein (BPI) (34–36). Other innate defenses include complement, phagocytes, and hydrophobic antimicrobial substances, such as bile salts and fatty acids, that bathe mucosal surfaces. Here we sought in vivo evidence that PEA decoration of lipid A, which we previously showed increases resistance to the bacterially derived CAMP PMB, benefits N. gonorrhoeae during experimental genital tract infection of female mice. In support of our hypothesis that PEA-decorated lipid A is important for gonococcal survival during infection, we showed that a mutant devoid of the 4’ lipid A PEA moiety had a survival disadvantage during murine infection that was not observed when cocultured with the wild-type strain in broth. CRAMP-38, SLPI, and BPI are expressed in genital tract tissues in N. gonorrhoeae-infected BALB/c mice (H. Wu, A. Marinelli, and A. E. Jerse, unpublished observation), and from this observation, we propose that this modification protects N. gonorrhoeae from one or more of these CAMPS. In both the human urethral-challenge model (17) and experimental murine infection, we have consistently observed attenuation of lptA mutant gonococci during competitive infections with the wild-type strain, since the mutant is not significantly attenuated during non-competitive infection of mice. The host factors that challenge the lptA mutant during competitive infection experiments may therefore be a component(s) of the inflammatory response induced by the wild-type strain. Alternatively, protection mediated by PEA-decorated lipid A against innate effectors present in the absence of inflammation may be too subtle to be detected by noncompetitive infection, which is less sensitive than competitive infections.

The presence of other lipid A isoforms must also be considered for these results. We previously reported four different lipid A isoforms in strain FA19 that differ in PEA modifications at the 4’ position and in hydroxylation or phosphorylation at the 1 and 4’ positions; a minor species being decorated with PEA at both positions is also produced by strain FA19 (14). We previously found that the lptA null mutant used here lacks the PEA-decorated species at the 4’ position but maintains the phosphate or hydroxyl groups at position 1. Thus, while complementation with the wild-type lptA gene reversed the influence on fitness and PMB susceptibility imparted by the null mutation in lptA that results in loss of PEA decoration at the 4’ position, we cannot formally rule out the possibility that the 1-phosphate group contributes somewhat to binding CAMPS or determining fitness.

During human infections, PEA lipid A decoration of gonococcal LOS may have an even more profound effect on gonococcal survival by also protecting against complement-mediated bacterial activity (14). This mechanism of complement resistance was recently described and is due to increased binding of human C4b-binding protein (C4BP), a negative regulator of the classical pathway of complement activation, to wild-type gonococci compared to its binding to gonococcal devoid of this modification (37). Human and murine C4BP are not conserved, and a host restriction in the binding of human C4BP to gonococcal porin has been documented (38). Therefore, it is unlikely that increased resistance of wild-type gonococci to complement-mediated bacteriolysis is responsible for the fitness advantage that we observed during competitive murine infections with the lptA mutant. Experimental infection of BALB/c mice therefore allows us to focus on antimicrobial factors other than complement that challenge N. gonorrhoeae in vivo. Interestingly, PEA decoration of lipid A also protects Salmonella enterica serovar Typhimurium (39), Acinetobacter baumannii (40), and N. meningitidis from CAMPS (16). In N. gonorrhoeae, CAMPS are also expelled through the gonococcal MtrC-MtrD-MtrE active efflux pump (26, 41), which is a critical virulence factor in the mouse infection model (23).

The second hypothesis that we wanted to test was that PEA-decorated lipid A is differentially recognized by the innate receptors within the host to induce an inflammatory response. To test this hypothesis, we examined vaginal concentrations of proinflammatory cytokines and chemokines in mice infected with wild-type, lptA mutant, or C’lptA mutant bacteria. Despite similar colonization loads, only the lptA mutant failed to induce proinflammatory responses. This result suggests that this modification is critical for the induction of inflammation in N. gonorrhoeae. Our findings are in agreement with recent work that demonstrated structural heterogeneity in the lipid A of LOS within a set of N. gonorrhoeae and N. meningitidis clinical isolates and showed that both phosphoryl and PEA lipid A modifications positively correlate with greater activation of the NF-κB pathway and enhanced induction of proinflammatory cytokines in human cells (20).

The mechanistic basis for differences in signal transduction and induction of inflammation due to lipid A PEA modifications remains unknown but is likely due to differential recognition by the TLR4 receptor complex. Our demonstration that wild-type LOS, but not LOS from the lptA mutant, induced high NF-κB expression in reporter cell lines expressing only the human TLR4 receptor complex is consistent with this hypothesis. The implications of these findings are particularly important for understand-
ing the pathophysiology of gonorrhea. In this respect, N. gonorrhoae signals host cells through TLR4 and TLR2 to induce proinflammatory cytokines in vivo (20, 42). While TLR2, but not TLR4, was detected on endocervical cells (43), TLR4-expressing immune cells are present in a whole model system, and signaling through TLR4 is likely to play a role in gonococcal genital tract infection based on evidence that TLR4 controls the colonization of experimentally infected mice (11) and is required for inducing Th17 responses (8, 11). Additionally, reproductive hormones and commensal flora can influence TLR4 expression on genital tract epithelial cells (44, 45). Interestingly, heat-inactivated serum from TLR4-defective mice was less bactericidal for N. gonorrhoeae than heat-inactivated serum from TLR4-sufficient mice, suggesting soluble effectors from TLR4, and Th17 pathways may challenge the induction of inflammation by soluble effectors from TLR4, and Th17 pathways may challenge the induction of inflammation by TLR4-defective mice was less bactericidal for N. gonorrhoeae (11). These factors may include CAMPs and may be responsible for the reduced viability of the lptA mutant when it is incubated with MEFs. We also found that preincubation of the LOS species with host-derived CAMPs results in differential neutralization of the LOS due to increased binding of the CAMPs to unmodified lipid A. This finding suggests that CAMPs further immunomodulated the reduced capacity of unmodified lipid A in inducing inflammation during infection by preferentially binding to the lptA mutant.

Finally, an intriguing question raised by this work is the possibility that the induction of inflammation by N. gonorrhoeae must confer an advantage to the pathogen; otherwise, this lipid A modification would not have been evolutionarily maintained. While independent gonococcal infections are common, particularly in the female genital tract, pharynx, and rectum, many infections are symptomatic, especially in males. It is possible that inflammation confers a transmission advantage; the presence of viable extracellular gonococci within exudates and PMNs (46) further supports this hypothesis. The intense inflammatory response seen in gonorrhea may also clear inhibitory commensal flora and thus open new colonization niches for the gonococcus. Continued research in these areas is needed to further elucidate the complex interactions between this highly successful pathogen and the innate immune system.

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We declare that we have no conflict of interest with this work.

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