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Hexa-Acylated Lipid A Is Required for Host Inflammatory Response to *Neisseria gonorrhoeae* in Experimental Gonorrhea

Xiyou Zhou,[a] Xi Gao,[a] Peter M. Broglie,[a] Chahnaz Kebaier,[a]† James E. Anderson,[a] Natalie Thom,[a] Michael A. Apicella,[b] Gregory D. Sempowski,[c] Joseph A. Duncan[a,d,e]

Department of Medicine, Division of Infectious Diseases, University of North Carolina, Chapel Hill, North Carolina, USA[a]; Departments of Microbiology and Internal Medicine, The Carver College of Medicine, The University of Iowa, Iowa City, Iowa, USA[b]; Departments of Medicine and Pathology and the Duke Human Vaccine Institute, Duke University Medical Center, Durham, North Carolina, USA[c]; Department of Pharmacology, University of North Carolina, Chapel Hill, North Carolina, USA[d]; Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA[e]

***Neisseria gonorrhoeae* causes gonorrhea, a sexually transmitted infection characterized by inflammation of the cervix or urethra. However, a significant subset of patients with *N. gonorrhoeae* remain asymptomatic, without evidence of localized inflammation. Inflammatory responses to *N. gonorrhoeae* are generated by host innate immune recognition of *N. gonorrhoeae* by several innate immune signaling pathways, including lipooligosaccharide (LOS) and other pathogen-derived molecules through activation of innate immune signaling systems, including toll-like receptor 4 (TLR4) and the interleukin-1β (IL-1β) processing complex known as the inflammasome. The lipooligosaccharide of *N. gonorrhoeae* has a hexa-acylated lipid A. *N. gonorrhoeae* strains that carry an inactivated *msbB* (also known as *lpxL1*) gene produce a penta-acylated lipid A and exhibit reduced biofilm formation, survival in epithelial cells, and induction of epithelial cell inflammatory signaling. We now show that *msbB*-deficient *N. gonorrhoeae* induces less inflammatory signaling in human monocytic cell lines and murine macrophages than the parent organism. The penta-acylated LOS exhibits reduced toll-like receptor 4 signaling but does not affect *N. gonorrhoeae*-mediated activation of the inflammasome. We demonstrate that *N. gonorrhoeae msbB* is dispensable for initiating and maintaining infection in a murine model of gonorrhea. Interestingly, infection with *msbB*-deficient *N. gonorrhoeae* is associated with less localized inflammation. Combined, these data suggest that TLR4-mediated recognition of *N. gonorrhoeae* LOS plays an important role in the pathogenesis of symptomatic gonorrhea infection and that alterations in lipid A biosynthesis may play a role in determining symptomatic and asymptomatic infections.**

Lipopolysaccharide (LPS) is an importance virulence factor in Gram-negative bacteria. LPS consists of a complex polysaccharide attached to a core saccharide consisting of 3-deoxy-D-mannooctulosonic acid (keto-deoxyoctulosonate [KDO]) and an acylated diglucosamine moiety known as lipid A. *Neisseria* species (as well as some other commensal and pathogenic bacteria) make LPS with a truncated polysaccharide structure, which is termed lipooligosaccharide (LOS). LOS is a potent mediator of inflammatory signaling and is known to activate several host innate immune signaling systems, including toll-like receptor 4 (TLR4) through the lipid A molecule, C-lectin receptors (particularly MGL and DC-SIGN) through the oligosaccharide structure, and the caspase-1 activating complex known as the NLRP3-inflammasome through mechanisms yet to be determined (1–4).

Lipid A synthesis is largely carried out by a highly conserved set of enzymatic steps that include the acylation of glucosamine-based precursors followed by condensation of these acylated glucosamine structures into the tetra-acylated disaccharide backbone that is the core structure of lipid A. This core is further decorated by the addition of two KDOs (2) and a variety of other additional modifications of the lipidated disaccharide (5). Lipid A is known to influence virulence of numerous bacterial pathogens and has been studied well in *Neisseria meningitidis*. Deletion of *lpxA*, the acyltransferase that catalyzes the initial lipidation of UDP-glucosamine, in *N. meningitidis* leads to a bacterium that does not generate LOS (6). *N. meningitidis* without LOS induces less inflammatory signaling through TLR4 than the wild-type *N. meningitidis*, but the two strains have been found to be roughly equal in virulence in a murine sepsis model (7). *N. meningitidis* also has two lipid A acyltransferases (*lpxL1* and *lpxL2*), which are responsible for adding secondary acyl chains to lipid A. Deletion of *lpxL1* (*msbB*) and *lpxL2* leads to *N. meningitidis* with penta- or tetra-acylated lipid A, respectively. Both penta- and tetra-acylated LOS demonstrate reduced potency in the activation of human TLR4 (8, 9). However, an *lpxL2* mutant demonstrates increased virulence, while an *lpxL1* mutant has dramatically reduced virulence in a mouse model of meningococcal sepsis (10). Interestingly, naturally occurring mutations in *lpxL1* have been discovered in ~13% of all strains of *N. meningitidis* recovered from patients with invasive *N. meningitidis* infection (11, 12). Patients with these strains had a significantly reduced severity of thrombocytopenia and petechial rash and a nonsignificant reduction in hypotension and septic shock, suggestive of reduced virulence in human infection as well (12). Further, *N. meningitidis* strains carrying *lpxL1* mutations make up the majority of strains isolated from patients with chronic meningococcemia, which presents with a prolonged dis-

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Editor: A. J. Bäumler
Address correspondence to Joseph A. Duncan, jaduncan@med.unc.edu.
† Deceased 25 May 2012.

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ease course that lacks the symptoms of severe septic shock-associated acute meningococcal infections (13).

The phenotype of a *Neisseria gonorrhoeae* strain with disrupted *msbB* has also been examined (14–16). Lipid A from the LOS of these mutant bacteria is penta-acylated (14). The bacteria show reduced capacity to produce biofilms and are more susceptible to intracellular killing by epithelial cells (14–16). They also induce less production of the cytokine interleukin-8 (IL-8) from epithelial cells (15). While epithelial cells are the initial targets of *N. gonorrhoeae* in early infection, infected sites are later infiltrated by host phagocytic cells, which largely make up the inflammatory exudates characteristic of infection with *N. gonorrhoeae*. We sought to further investigate the role of lipid A acylation in inflammatory signaling during gonococcal infection by studying the inflammatory signaling in monocyte-derived cells and the pathogenesis of infection using a well-established murine model of gonococcal infection (17).

MATERIALS AND METHODS

Ethics statement. Experiments describing the generation of bone marrow-derived immune cells and the use of the murine model of gonorrhea were conducted in accordance with National Institutes of Health guidelines for the care and use of laboratory animals under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina at Chapel Hill (UNC IACUC protocols 09-229.0 and 12-207.0).

***N. gonorrhoeae* strains and culture.** *N. gonorrhoeae* strain 1291, isogenic *msbB*-deficient strain (designated 1291-Δ*msbB* in this article; relevant genotype, *msbB*::*kan*^{r}), and the 1291-Δ*msbB* strain complemented in *trans* by insertion of the spectinomycin resistance marker and *msbB* gene into the *proB* gene (designated 1291-Δ*msbB*;*msbB*$^+$ in this article; relevant genotype, *msbB*::*kan*^{r} *proB*::*spc*^{r}+*msbB*) have all been described previously (14, 16). Piliated (pil+), opaque (opa+) populations of each strain were cultured and prepared to inoculate immune cell cultures or mice as previously described (4). Briefly, the strains were grown on Difco GC medium base (GCB; Becton Dickinson, Sparks, MD) agar plates overnight (16 h to 18 h) at 37°C in 5% CO_2, and the bacteria were harvested using sterile cotton swabs and transferred into complete cell culture media (for cell culture experiments) or phosphate-buffered saline (PBS) (for murine inoculation). The quantity of CFU in the suspensions was estimated by measuring the optical density at 600 nm (OD_{600}), and the actual administered doses were determined by colony counting after plating serial dilutions of the suspended *N. gonorrhoeae* cells.

Culture of THP1 monocytic cells and BMDM. The human monocytic cell line THP1 was propagated in RPMI 1640 plus 10% fetal bovine serum (FBS) as previously described (4). Mouse bone marrow-derived macrophage (BMDM) were prepared from 8- to 12-week-old C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) (18). Femurs and tibiae were harvested from euthanized mice, and the marrow was flushed from each bone using RPMI 1640 (Invitrogen). The marrow cells were dispersed, washed, and cultured in cytokine-supplemented medium. To generate BMDM, the bone marrow cells were cultured in medium containing 30% L929 conditioned medium as a source of granulocyte colony-stimulating factor (G-CSF). At day 7, the BMDM were collected and adjusted to a concentration of 1×10^6 cells/ml and plated for further treatment.

Murine *N. gonorrhoeae* infection. The protocol for murine vaginal infection with *N. gonorrhoeae* was utilized as described by Jerse et al. (17). Basically, 4- to 6-week-old female BALB/c (Jackson laboratory) mice in proestrus stage were treated with water-soluble β-estradiol (Sigma) and streptomycin, vancomycin, and trimethoprim (Sigma) on day −2, as described. On day zero, the mouse vagina was inoculated with an estimated 10^6 CFU of the indicated strain of *N. gonorrhoeae* suspended in PBS. For the subsequent 10 days, vaginal swabs were collected from each mouse in the morning. The population of *N. gonorrhoeae* was determined by plating of serial dilutions of the suspended material obtained from the vaginal swab on GCB agar supplemented with vancomycin, colistin, nystatin, trimethoprim, and streptomycin (VCNTS-GCB). The quantity of vaginal neutrophils as a fraction of recovered nucleated cells was determined each day by differential counting of the Giemsa-stained smears from the vaginal swab. Inflammatory gene expression was monitored as described by Packiam et al. using the mouse antibacterial response RT^2 Profiler PCR array (SA Bioscience, Frederick, MD) (19, 20). Vaginal lavage was performed after the vaginal swab collection was carried out on the fourth day of infection by repeated flushing (20 times) of the vaginal cavity with 50 μl of PBS. Cells and debris from the lavage were removed by centrifugation at $700 \times g$ for 10 min and saved for RNA extraction, and the supernatant was processed for cytokine measurement. Competitive infection experiments were carried out as an adaption of that published by Warner et al. (21). An inoculum was prepared containing roughly a 1:1 ratio of two *N. gonorrhoeae* strains (strains 1291 and 1291-Δ*msbB* or 1291-Δ*msbB*;*msbB*$^+$). Vaginal swabs were obtained every other day, and the quantity of viable *N. gonorrhoeae* organisms was determined by plating serial dilutions from resuspended swabs on VCNTS-GCB agar. The ratio of each strain was then determined by picking 48 individual colonies from the VCNTS-GCB plates and replica plating these under appropriate antibiotic selection.

Measurement of cytokines, chemokines, and Caspase-1 activation. IL-1β, tumor necrosis factor alpha (TNF-α), and macrophage inflammatory protein 1 alpha (MIP-1α) secreted from THP1 or BMDM were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' protocols: the MIP-1α ELISA kit was from R&D system (Minneapolis, MN), and the IL-1β and TNF-α kits were from BD biosciences (San Diego, CA). IL-1β and TNF-α from BMDM were measured using AlphaLisa cytokine detection kits from PerkinElmer (Waltham, MA). The cytokines and chemokines from vaginal lavage supernatants were measured with the Bio-plex system (Bio-Rad, Hercules, CA) in the Duke Human Vaccine Institute Immune Reconstitute and Biomarker Facility. Caspase-1 activation was assessed by immunoblot analysis using antibodies directed to the p10 subunit of Caspase-1 as described by Craven et al. (22).

Treatment of HEK-Blue-hTLR4 cells, THP-1 cells, and BMDM with LOS or *N. gonorrhoeae*. Purified LOS from *N. gonorrhoeae* strains 1291 and 1291-Δ*msbB* were prepared as previously described (15). HEK-Blue-hTLR4 cells (Invivogen) were treated with the indicated concentration of purified LOS for 16 h, and secreted alkaline phosphatase (SEAP) was assayed using a colorimetric assay (quanti-Blue; Invivogen) according to the manufacturer's protocol. To determine the level of LOS-induced SEAP activity, the level of SEAP activity was measured in cells that were not treated with LOS, and this basal level of SEAP activity was subtracted from the relative SEAP activity measured in each treated sample. THP1 cells (and BMDM) were treated with LOS from *N. gonorrhoeae*, and cell culture supernatants were harvested for cytokine measurements as previously described (4).

RNA isolation and qRT-PCR from murine vaginal washes. Cell-containing pellets from vaginal lavage samples obtained from infected mice were stored at −80°C, and RNA was isolated using the RNeasy isolation kit (Qiagen) according to the manufacturer's protocol. The isolated RNA was DNase treated to remove residual DNA contamination, and total RNA was then used in a quantitative reverse transcription-PCR (qRT-PCR) assay to measure inflammation-related RNA expression on a mouse antibacterial response RT^2 Profiler PCR array (SA Bioscience, Frederick, MD) according to the manufacturer's protocol (20).

Statistical analysis. Prism Graphpad was used for statistical analysis. Student's *t* test was used to compare groups when experimental design dictated two groups. Analysis of variance (ANOVA) was used in conjunction with a *post hoc* Bonferroni test for multiple comparisons to analyze experiments with more than two test groups. *P* values of <0.05 were considered significant.

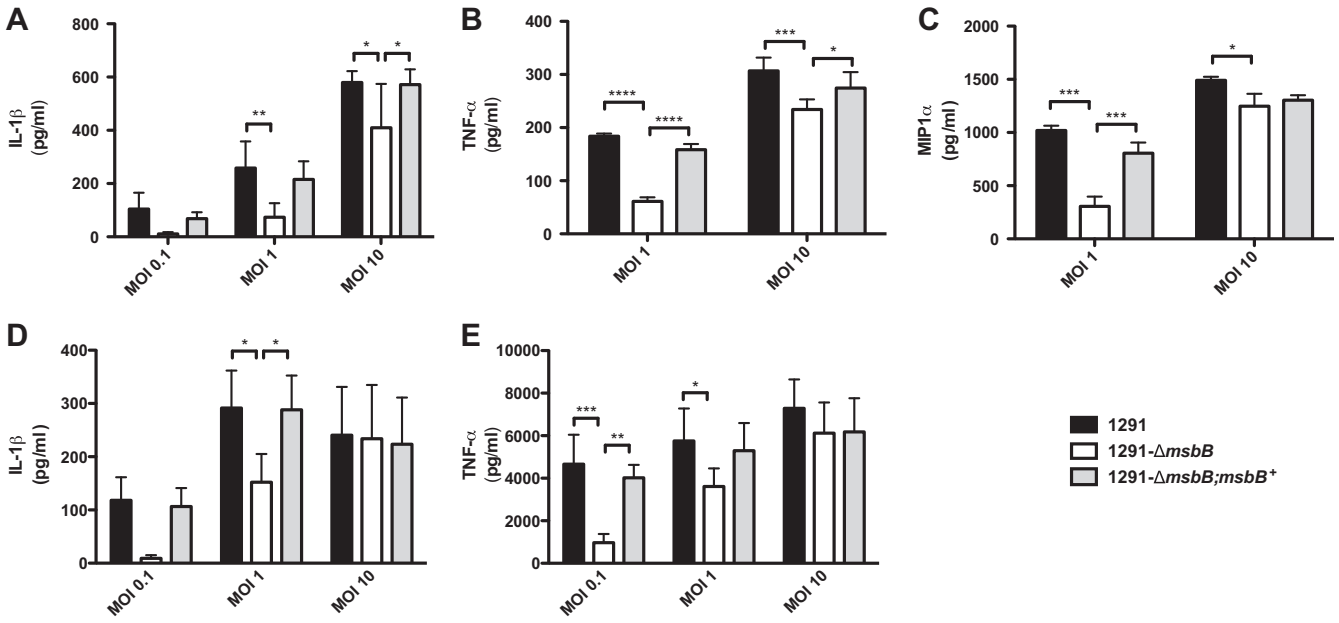

FIG 1 *N. gonorrhoeae msbB* controls induction of host inflammatory cytokines in human-derived cells and mouse primary macrophages. (A to C) THP1 cells were treated with the indicated doses of *N. gonorrhoeae* (MOI, 0.1,1, or 10) for 4 h, and culture supernatants were collected. Secreted IL-1β (A), TNF-α (B), and MIP-1α (C) were measured with ELISA. (D and E) Mouse bone marrow-derived macrophages were generated and exposed to the indicated doses (MOI) of the indicated *N. gonorrhoeae* strains as described in Materials and Methods. Secreted IL-1β (D) and TNF-α (E) were measured in cell culture supernatants as described in Materials and Methods. The mean values ± standard deviations from five (A, B, C) or three (D, E) independent experiments are plotted. Statistical analysis with two-way ANOVA and Bonferroni's *post hoc* test for multiple comparisons was carried out; *, $P < 0.05$; **, $P < 0.05$ and $P > 0.01$; ***, $P < 0.01$ and $P > 0.005$.

RESULTS

In order to determine whether *N. gonorrhoeae msbB* influenced the level of inflammatory cytokine response in phagocytic cells, THP1 cells were treated with increasing doses of *N. gonorrhoeae* strain 1291, strain 1291-Δ*msbB*, or the complemented mutant, 1291-Δ*msbB*;*msbB*$^+$. The secretion levels of IL-1β, TNF-α, and MIP-1α were examined in the culture supernatants of these bacterium-exposed THP1 cells (Fig. 1A to C). The 1291-Δ*msbB* strain caused less secretion of each of these cytokines than the parental 1291 strain when a low multiplicity of infection (MOI; the ratio of bacteria to cells) was used. At higher MOI, the difference between the parental strain and the *msbB*-deficient strain was lost, suggesting that mutant bacteria had reduced potency in cytokine induction that could be overcome by sufficiently high inoculation. Alternatively, and not exclusive of a simple reduction of signaling potency in a single innate immune signaling pathway, higher doses of *N. gonorrhoeae* might lead to the activation of additional inflammatory signaling pathways that could compensate for the pathway or pathways affected by loss of the *msbB* gene. The reduced potency in induction of host cytokine production was reversed in the complemented *msbB* mutant strain. Similar effects on IL-1β secretion and TNF-α secretion were observed when primary bone marrow-derived macrophages from mice were treated with these *N. gonorrhoeae* strains (Fig. 1D and E). This indicated that the effects on host inflammatory signaling by disruption of *msbB* were similar between species. When isolated lipooligosaccharide from the parental and 1291-Δ*msbB* strains was used to stimulate THP1 cells, this penta-acylated LOS showed a >3-log decrease in its potency in inducing TNF-α (Fig. 2). These data demonstrate that lipid A hexa-acylation plays an important role in induction of host inflammatory cytokine production by *N. gonorrhoeae*.

Two signaling pathways are known to contribute to host production of IL-1β. First, activation of TLR4 leads to production of pro-IL-1β as well as the inflammasome component NLRP3 (23). NLRP3 is also nontranscriptionally primed for activation in response to TLR4 stimulation through regulated deubiquitination (24). Second, NLRP3 inflammasome activation and subsequent Caspase-1 activation are triggered by a variety of second signals

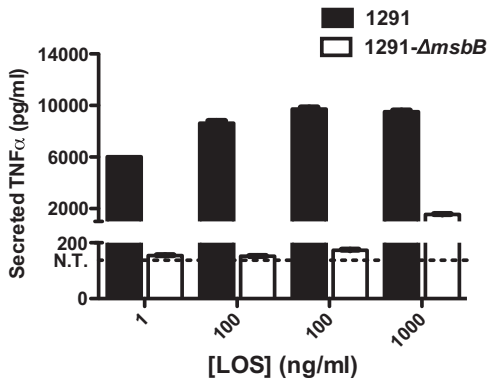

FIG 2 *N. gonorrhoeae msbB* alters the host cytokine response through effects on purified LOS. LOS was isolated from *N. gonorrhoeae* strains 1291 and 1291-Δ*msbB*. THP1 cells were treated with the indicated doses of LOS (1 ng/ml to 1,000 ng/ml) for 4 h, and secreted TNF-α was measured as described in Materials and Methods. Results depicted are means of triplicate measurements in a single experiment and are representative of at least three independent experiments.

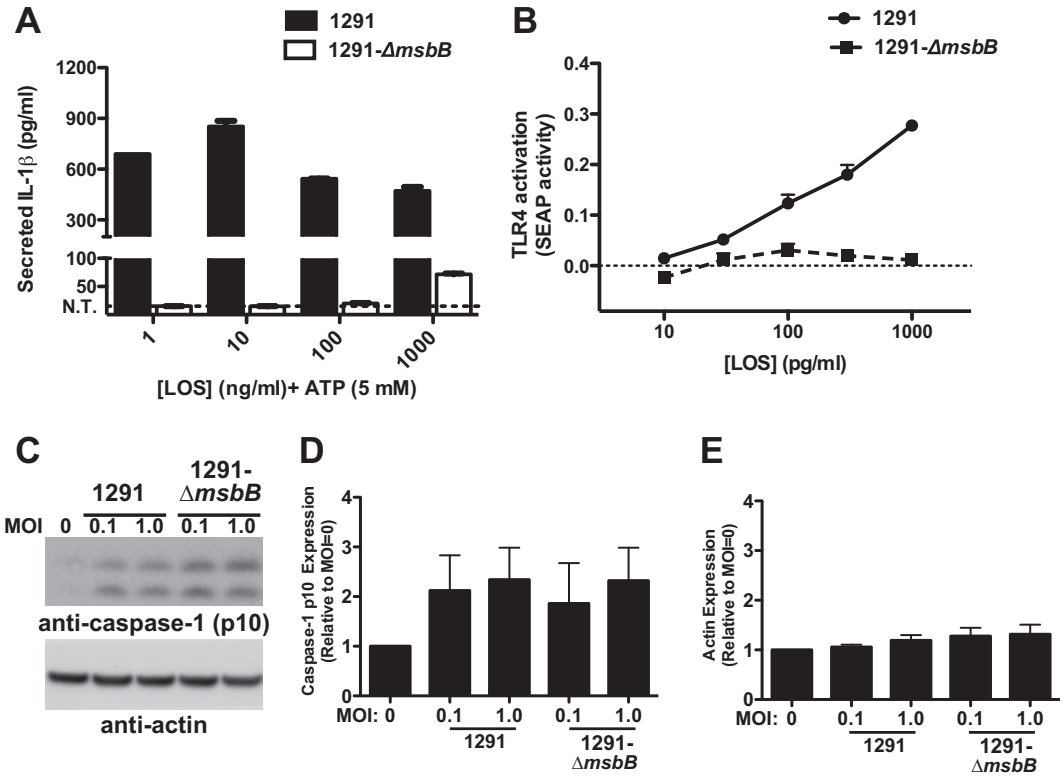

FIG 3 *N. gonorrhoeae msbB* influences TLR4 activation but not NLRP3 inflammasome activation in host cells. (A) THP1 cells were treated with the indicated doses of LOS (1 ng/ml to 1,000 ng/ml) for 3 h, 5 mM ATP was added for an additional hour to induce inflammasome activation, and secreted IL-1β was measured as described in Materials and Methods. (B) The indicated concentrations of purified LOS from *N. gonorrhoeae* strains 1291 and 1291-Δ*msbB* were incubated with HEK-blue TLR4 cells for 24 h, and secreted alkaline phosphatase activity was measured as described in Materials and Methods. (C to E) THP-1 cells were treated with the indicated doses of *N. gonorrhoeae* strains 1291 and 1291-Δ*msbB* as described for Fig. 1. Cell pellets were harvested, and cell lysates were generated and analyzed by immunoblot analysis with antibodies directed to Caspase-1 p10 subunit and actin. Representative results are shown in panel C. Densitometry was performed on three independent experiments, and the mean band density normalized to the untreated sample is reported for activated Caspase-1 (C) and actin (D).

that can include activation of the purogenic receptor P2X7 by extracellular ATP, disruption of phagocytic vacuoles, or insertion of pore-forming toxins into the cell membrane. The "second signals" that are provided by *N. gonorrhoeae* to induce NLRP3 activation have yet to be determined. The ability of *N. gonorrhoeae* 1291-Δ*msbB* to activate these signaling pathways was assessed. First, bone marrow macrophages were primed with LOS derived from either 1291 or 1291-Δ*msbB* and subsequently treated with ATP to induce inflammasome activation. As previously noted for TNF-α secretion, the 1291-Δ*msbB* LOS demonstrated at least a 3-log decrease in its potency in contributing to IL-1β secretion (Fig. 3A). Isolated LOS from *N. gonorrhoeae* 1291 and 1291-Δ*msbB* were tested for the ability to stimulate TLR using a TLR4 reporter cell line that expresses human TLR4/MD-2 and an NF-κB-responsive secreted alkaline phosphatase. LOS from the parental *N. gonorrhoeae* strain caused the induction of alkaline phosphatase activity in the 10- to 100-pg/ml concentration range, while isolated penta-acylated LOS from the *msbB* mutant failed to activate TLR4 at even 1,000 pg/ml (Fig. 3B). In a second set of experiments, Caspase-1 activation was examined in THP1 cells treated with each *N. gonorrhoeae* strain using immunoblotting to detect the p10 subunit of activated Caspase-1. While TLR4 was differentially activated by 1291 and 1291-Δ*msbB*, Caspase-1 activation was equally activated by each strain (Fig. 3C to E). Consequently, the hexa-acyl lipid A appears to exert the majority of its effect on host inflammatory signaling, including IL-1β secretion, through effects on TLR4 activation.

To test the role of lipid A hexa-acylation in gonococcal infection, a murine model of vaginal *N. gonorrhoeae* infection was used. We first sought to determine whether *msbB* deficiency led to a relative fitness defect in *N. gonorrhoeae* by carrying out a competitive infection. The *N. gonorrhoeae* strain with a disrupted *msbB* gene had a profound fitness deficit compared to the parental strain or the isogenic strain with complemented *msbB* expression (Fig. 4A and B). Though 1291-Δ*msbB* has previously been reported to have no growth deficit in *in vitro* culture compared to its isogenic parental strain, 1291, we also confirmed that both strains maintained themselves in equal proportion when grown as mixed culture *in vitro* (data not shown) (14). We also sought to determine the effect of gonococcal *msbB* on the pathogenesis of infection by studying BALB/c mice inoculated with single *N. gonorrhoeae* strains, either 1291, 1291-Δ*msbB*, or 1291-Δ*msbB*;*msbB*$^+$. The number of *N. gonorrhoeae* colonies recovered from daily vaginal swabs of each infected mouse was assessed. Despite the notable fitness defect caused by deletion of *msbB* in competitive infections, no difference between persistence of infection was noted between any of the tested strains (Fig. 4C). In the mice with persistent infection, no significant difference in the quantity of *N. gonor-*

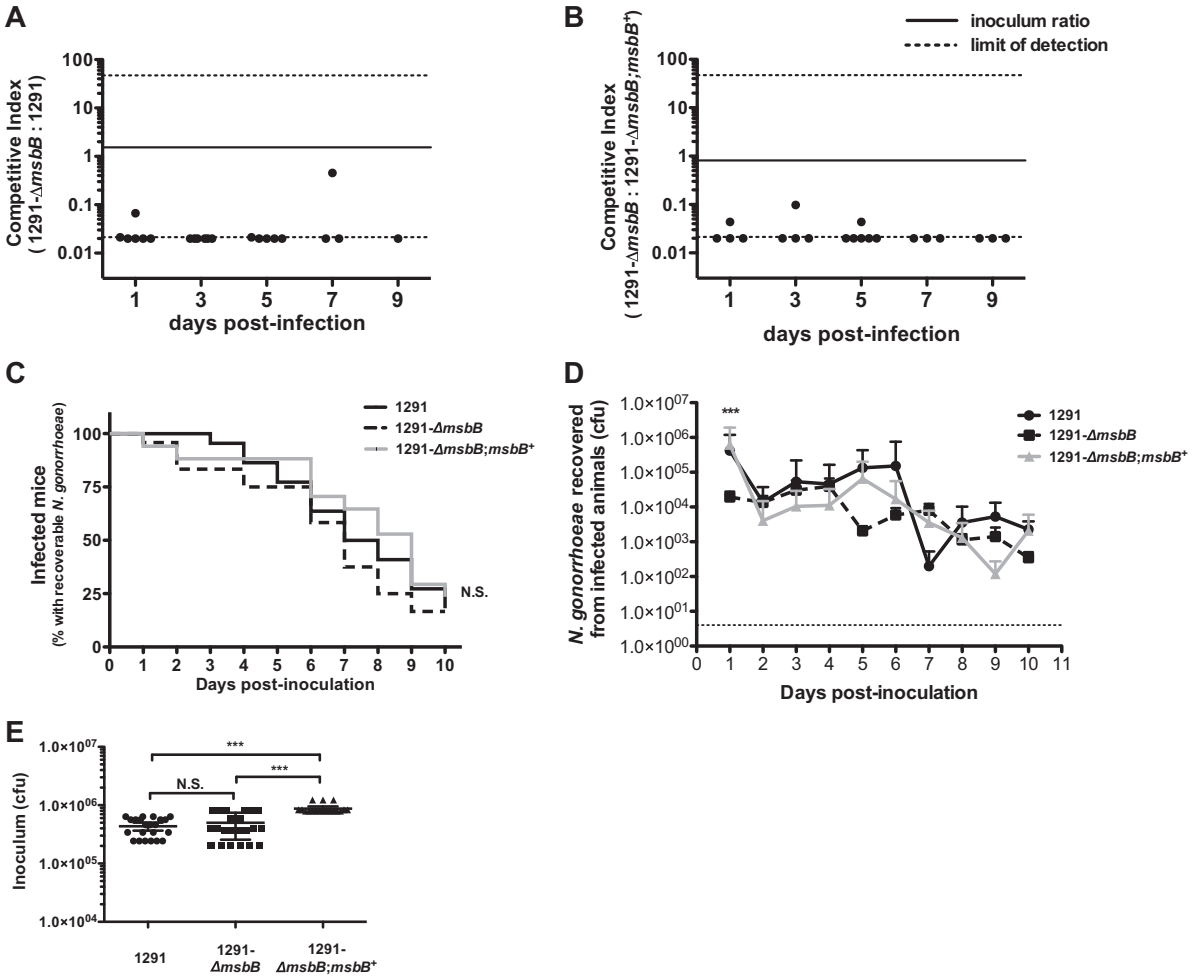

FIG 4 *N. gonorrhoeae msbB* is not required to establish or maintain vaginal infection in the murine model of *N. gonorrhoeae* but influences fitness in competitive infection. Four- to 7-week-old BALB/c mice were infected with *N. gonorrhoeae* strains 1291, 1291-Δ*msbB*, and 1291-Δ*msbB*;*msbB*$^+$ as indicated. (A and B) Mice were inoculated with a combination of 1291 and 1291-Δ*msbB* (A) or 1291-Δ*msbB* and 1291-Δ*msbB*;*msbB*$^+$ (B), and the relative quantity of each strain is plotted for each mouse on the indicated day postinoculation. The hashed lines indicate the limit of detection for the ratio of bacteria (48 colonies from each mouse were replica plated to determine the strain of each colony). The solid black line indicates the ratio of the strains in the inoculum. The plot is representative of two independent experiments. (C and D) Mice were infected with single *N. gonorrhoeae* strains 1291 ($n = 22$), 1291-Δ*msbB* ($n = 24$), and 1291-Δ*msbB*;*msbB*$^+$ ($n = 17$) as indicated, and persistence of infection (C) and quantity (D) of *N. gonorrhoeae* present on vaginal swabs were determined as described in Materials and Methods. Plots show combined results from 4 independent experimental infections. Vaginal *N. gonorrhoeae* burdens were analyzed using two-way ANOVA, and Bonferroni's *post hoc* test for multiple comparisons was carried out; ***, $P < 0.01$ and $P > 0.005$; N.S., not significant. The inoculum dose for each mouse in all experiments is plotted in panel E.

rhoeae colonies recovered was noted between these strains except on the first day postinoculation (Fig. 4D). Despite having no significant difference in the quantity of live bacteria in the CFU cultured from the initial inoculum compared to the parental strain, 1291, the 1291-Δ*msbB* strain exhibited a significant 1.5-log reduction in recovered *N. gonorrhoeae* on day 1 (Fig. 4D and E). The 1291-Δ*msbB* strain also demonstrated a similar 1.5-fold reduction in the first-day *N. gonorrhoeae* recovery compared to *N. gonorrhoeae* in which *msbB* expression was complemented, though the average inoculum for the complemented strain was slightly higher than for the mutant strain (Fig. 4E). These data suggested that while *msbB* is not required for *N. gonorrhoeae* to initiate or persist in the genital tract, it does impart a competitive advantage in vaginal infection compared to *N. gonorrhoeae* isolates that lack an active *msbB* gene.

In the murine model of gonococcal infection, the mouse vagina develops a neutrophilic influx several days after inoculation (17). This is not unlike the onset of cervical or urethral inflammation that accompanies symptomatic gonococcal infection in humans. We found that on most days after inoculation the quantity of vaginal neutrophils was lower in mice infected with 1291-Δ*msbB*; however, this finding did not meet statistical significance (Fig. 5A). We also quantitated the total neutrophil influx over the course of the infection by calculating the area under the curve for the percentage of vaginal neutrophils plotted against time for each individual infected mouse and designated this area under the curve the neutrophil flux AUC. The mean neutrophil influx AUC for mice infected with the parental strain was significantly greater than the mean neutrophil influx AUC found in mice inoculated with PBS, while the mean neutrophil influx AUC of the mice infected with the 1291-Δ*msbB* strain was not significantly different from that of PBS-treated mice (Fig. 5B). To confirm that the lack

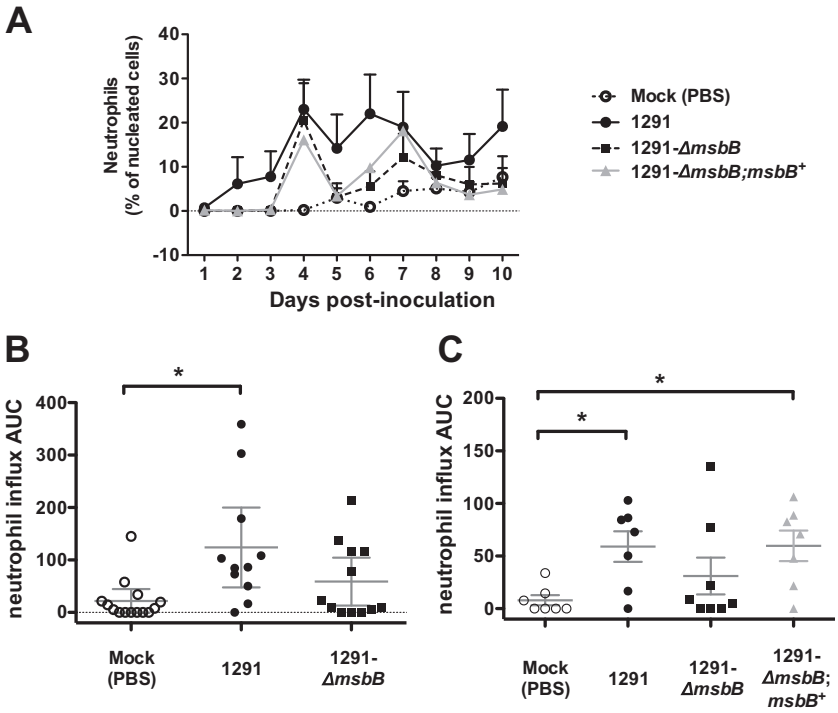

FIG 5 *N. gonorrhoeae msbB* is required to induce significant vaginal inflammation in the murine model of gonorrhea. Mice were infected with the indicated *N. gonorrhoeae* strain as described in the legend to Fig. 4. Neutrophils and total nucleated cells present on vaginal swabs were enumerated as described in Materials and Methods, and the relative frequency (mean ± standard deviation [SD]) of neutrophils was plotted for each day (A). A plot of relative vaginal neutrophils against day postinoculation was generated for each infected animal, and the area under the curve was calculated as the neutrophil influx AUC for that animal. Results from 3 experiments carried out comparing mock infection ($n = 15$) to infection with *N. gonorrhoeae* strains 1291 ($n = 11$) and 1291-Δ*msbB* ($n = 12$) (B) and from 3 experiments comparing mock infection ($n = 7$) and infection with 1291 ($n = 7$), 1291-Δ*msbB* ($n = 8$), and 1291-Δ*msbB*;*msbB*$^+$ ($n = 7$) (C) are shown. Statistical analysis comparing the mean values with two-way ANOVA and Bonferroni's *post hoc* test for multiple comparisons was carried out; *, statistically significant difference ($P < 0.05$).

of neutrophil influx during infection with 1291-Δ*msbB* was attributable to the loss of *msbB*, cohorts of mice were inoculated with PBS, 1291, 1291-Δ*msbB*, or 1291-Δ*msbB*;*msbB*$^+$. The induction of vaginal neutrophil influx was restored (Fig. 5C). Vaginal inflammation was also characterized by assessing mRNA expression of genes involved in host antimicrobial response and levels of inflammatory cytokines present in vaginal lavage fluid during infection. The inflammatory cytokines TNF-α, MIP-1α, and IL-1β were measured in vaginal lavage fluid from mice infected with each strain of *N. gonorrhoeae* on day 4 postinoculation. Vaginal TNF-α and MIP-1α were both induced by infection with the wild type and the complemented *msbB* mutant but not by infection with strain 1291-Δ*msbB* (Fig. 6A and B). A similar trend was noted in vaginal IL-1β, although the majority of mice did not have detectable quantities of IL-1β in the vaginal lavage at day 4 (Fig. 6C). Limitations in the quantity of cells, and consequently mRNA, recovered from the vaginal lavage fluid and a relatively low abundance of expression of many of the host antimicrobial response genes resulted in the inability to fully evaluate the expression of many of these genes during infection (data not shown). However, *Cxcl1* and *Naip1* were both found to be diminished in their expression in mice infected with the 1291-Δ*msbB* mutant strain compared to those infected with wild-type 1291, while those infected with 1291-Δ*msbB*;*msbB*$^+$ were not significantly different from those infected with 1291 (Fig. 6D).

DISCUSSION

Our findings suggest that the state of lipid A acylation status can play a significant role in the pathogenesis of *N. gonorrhoeae* infection. Deletion of the *msbB* gene resulted in three primary phenotypic changes related to pathogenesis, the reduction of TLR4-induced inflammatory signaling *in vitro*, reduced vaginal inflammation during infection, and a reduced fitness in competitive infections in the murine gonorrhea model.

Previous work studying this strain of *N. gonorrhoeae* with a disrupted *msbB* gene has demonstrated that both live bacteria with a disrupted *msbB* gene and LOS isolated from these bacteria have diminished capacity to induce IL-6 and IL-8 production by epithelial cells (15). Epithelial cells incubated with *N. meningitidis* carrying a deletion of *lpxL1* (also *msbB*) secreted less TNF-α than cells treated with the parental wild-type strain (8, 9). Further studies demonstrated that penta-acylated lipid A-containing *N. meningitidis* LOS (from the Δ*lpxL1* strain) has reduced potency in activation of murine and hamster TLR4 while actually acting as a TLR4 antagonist or partial agonist for human TLR4 (8, 9). In monocyte-derived cells, *Neisseria* LOS had been implicated in activation of two signaling pathways: TLR4 and the caspase-1 activating NLRP3 inflammasome pathway (4, 25). We now present data demonstrating that *N. gonorrhoeae msbB* is required for robust inflammatory signaling by *N. gonorrhoeae*. This effect is primarily dependent on the ability of the hexa-acylated LOS to stimulate

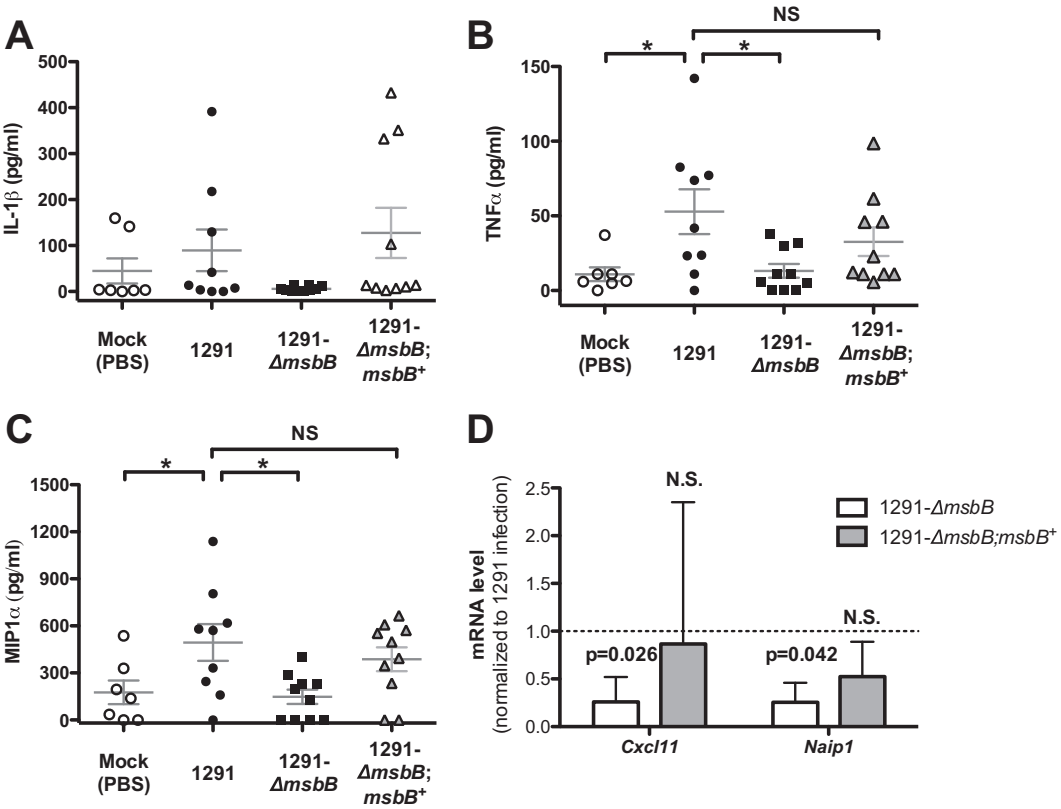

FIG 6 *N. gonorrhoeae msbB* controls vaginal cytokine accumulation in the murine model of vaginal infection. Mice were infected with the indicated *N. gonorrhoeae* strains as described for Fig. 4. The indicated cytokines, i.e., TNF-α (A), MIP-1α (B), and IL-1β (C), were measured in day 4 vaginal lavage fluid as described in Materials and Methods. (D) Expression levels of host infection response genes in cells from the vaginal swabs of mice infected with 1291, 1291-Δ*msbB*, and 1291-Δ*msbB*;*msbB*$^{+}$ were measured using an RT-PCR array as described in Materials and Methods. The relative expressions (normalized to strain 1291-infected mice) of *cxcl2* and *naip1* are reported. Statistical analysis with two-way ANOVA and Bonferroni's *post hoc* test for multiple comparisons was carried out; *, statistically significant difference ($P < 0.05$); N.S., not significant.

TLR4, with no detectable contribution to NLRP3 inflammasome activation.

A functional *Tlr4* allele in mice has been shown to be important in the control of the bacterial load of *N. gonorrhoeae* in the murine vaginal infection model (20). Surprisingly, the Δ*msbB* strain of *N. gonorrhoeae*, which has reduced TLR4 activation, does not exhibit extended persistence or higher loads in the murine model. In fact, the bacterial load after infection with 1291-Δ*msbB* was lower on the day immediately after inoculation (Fig. 4D). The early reduction in bacterial load seen in the Δ*msbB* strain appears to be a phenotype stemming from the penta-acylation of lipid A because the phenotype is restored in the complemented mutant strain in which hexa-acylated lipid A is restored. The mechanism underlying these phenotypes may reflect increased susceptibility to host antimicrobial defenses. The deletion of *msbB* has been shown to increase susceptibility to host cell killing, polymyxin, and other antimicrobial peptides in *N. meningitidis* as well as other pathogenic bacteria, including *Yersinia* and *Vibrio* species (26–28). However, if this susceptibility to host factors does play a role in the initial reduction in bacterial load, it is inadequate to allow clearance of the *N. gonorrhoeae* inoculum. Deletion of the *msbB* gene did not affect the ability of *N. gonorrhoeae* to persist in the mouse vaginal infection model or the quantity of bacteria present in the infected mice over the duration of the infection. It is possible that because deletion of *msbB* in *N. gonorrhoeae* results in reduced TLR4 stimulation, there are reduced levels of antimicrobial peptides present in mice infected only with 1291-Δ*msbB* compared to those infected with a combination of wild-type 1291 and 1291-Δ*msbB*. This combination of increased susceptibility to innate defenses and reduced stimulation of host innate immunity afforded by disruption of *msbB* could explain the observation that the *msbB* mutant *N. gonorrhoeae* demonstrated a profound fitness deficit in mixed infection and almost no deficit in single infection. Other possible explanations exist for the reduced recovery of the *msbB* mutant *N. gonorrhoeae* strain from the murine vaginal cavity 1 day after inoculation, such as diminished biofilm formation or diminished epithelial invasion, both of which are known phenotypes of 1291-Δ*msbB* in *ex vivo* culture experiments (15, 16). Further studies are required to better understand the mechanisms that contribute to the initial drop in *N. gonorrhoeae* bacteria after inoculation in this model.

Paralleling the finding that *N. gonorrhoeae* strains that lack a functional *msbB* induced limited inflammatory signaling in phagocytic cells, the *msbB* mutant of *N. gonorrhoeae* also induces less vaginal inflammation than its wild-type parental strain in a murine gonorrhea model. These findings are in line with observations for the role of *msbB* in other bacterial pathogens, including *Shigella flexneri* and *N. meningitidis*. In murine and rabbit models of shigella enteritis, deletion of the two *msbB* genes and the resulting lipid A hypoacylation are associated with reduced inflamma-

tion and increased host survival (29). Deletion of *lpxL1* (the *N. meningitidis* equivalent of *N. gonorrhoeae msbB*) in *N. meningitidis* results in an avirulent meningococcus in a murine bacteremia model, with rapid bacterial clearance compared to that seen in the parental *N. meningitidis* strain (10). The clearance of *N. meningitidis* Δ*lpxL1* is compared with enhanced host survival and a dramatic reduction in clinical severity of disease scores. The increased clearance in this bacteremia model suggests that the protection against host antimicrobial peptides that is afforded by a hexa-acylated LOS may be more important for survival in blood than at the mucosal surface of the vagina, where *N. gonorrhoeae* Δ*msbB* appears able to maintain colonization. Because penta-acylated LOS is a TLR4 antagonist in human cells and a low-potency agonist in murine cells, the reduced inflammatory response seen in the murine gonorrhea model in response to *msbB*-deficient *N. gonorrhoeae* may actually be further accentuated in a human infection.

N. gonorrhoeae induces localized inflammation that is responsible for symptoms associated with infection by this bacterium. However, in one-half of the cases of gonococcal infection of the female genitourinary tract, patients experience no noticeable symptoms. The factors that dictate symptomatic infection rather than asymptomatic infection have yet to be determined. However, the data we have presented suggest that variations in lipooligosaccharide structure may play an important role in this phenomenon. The role of lipid A hexa-acylation directed by *msbB* in human disease is of particular interest given the identification of mutations in this gene in another pathogenic *Neisseria* species, which are associated with milder disease presentation (12, 13). Though mutations that eliminate a functional *lpxL1* gene (the homolog of *N. gonorrhoeae msbB*) in *N. meningitidis* have been identified in clinical isolates, these have yet to be identified in *N. gonorrhoeae* isolates. No evidence of phase-variable expression has been observed in *N. gonorrhoeae msbB* in *in vitro* culture. However, the gene does contain a homopolymeric tract of six thymidine residues nine bases downstream of the initiating methionine codon that could potentially be a site for slipped strand mispairing. Such mispairing would lead to phase-variable expression of *msbB* by placing the gene out of frame. Phase-variable expression in *Neisseria* species is more often associated with longer homopolymeric runs of guanine bases, but there is one report of phase variation resulting from a polyadenine tract in the promoter of the *porA* gene in *N. meningitidis* (30). Recent studies have also found associations between lipid A phosphorylation, diphosphorylation, and phosphotidyl ethanolamine (PEA) decoration and the potency of TLR4 activation and host inflammatory cytokine production (25, 31–33). Interestingly, PEA decoration is carried out by an enzyme (LptA) that is encoded in the genomes of both pathogenic *Neisseriaceae* but not the commensal *Neisseriaceae*. As reported by John et al., for *N. meningitidis*, we have found that LOS from *N. gonorrhoeae* that lacks *lptA* also has reduced potency in TLR4 activation and host inflammatory cytokine induction (32; X. Zhou and J. A. Duncan, data not shown). Like hexa-acylation of lipid A, PEA decoration has also been shown to afford resistance to host antimicrobial peptides (34, 35). Combined with the data we have presented on the activity of penta-acylated LOS from *N. gonorrhoeae* with disrupted *msbB*, these findings all suggest that the host may create a selective pressure for the maintenance of a proinflammatory lipid A; however, this hypothesis requires further evaluation. Additionally, a detailed examination of the lipid A structure from gonococcal clinical isolates may demonstrate that alterations of multiple pathways in lipid A biosynthesis may contribute to the development of symptomatic and asymptomatic *N. gonorrhoeae* infections.

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REFERENCES

1. **Pridmore AC, Jarvis GA, John CM, Jack DL, Dower SK, Read RC.** 2003. Activation of toll-like receptor 2 (TLR2) and TLR4/MD2 by Neisseria is independent of capsule and lipooligosaccharide (LOS) sialylation but varies widely among LOS from different strains. Infect. Immun. **71:**3901–3908. http://dx.doi.org/10.1128/IAI.71.7.3901-3908.2003.
2. **van Vliet SJ, Steeghs L, Bruijns SC, Vaezirad MM, Snijders Blok C, Arenas Busto JA, Deken M, van Putten JP, van Kooyk Y.** 2009. Variation of Neisseria gonorrhoeae lipooligosaccharide directs dendritic cell-induced T helper responses. PLoS Pathog. **5:**e1000625. http://dx.doi.org/10.1371/journal.ppat.1000625.
3. **Zhang P, Schwartz O, Pantelic M, Li G, Knazze Q, Nobile C, Radovich M, He J, Hong SC, Klena J, Chen T.** 2006. DC-SIGN (CD209) recognition of Neisseria gonorrhoeae is circumvented by lipooligosaccharide variation. J. Leukoc. Biol. **79:**731–738. http://dx.doi.org/10.1189/jlb.0405184.
4. **Duncan JA, Gao X, Huang MT, O'Connor BP, Thomas CE, Willingham SB, Bergstralh DT, Jarvis GA, Sparling PF, Ting JP.** 2009. Neisseria gonorrhoeae activates the proteinase cathepsin B to mediate the signaling activities of the NLRP3 and ASC-containing inflammasome. J. Immunol. **182:**6460–6469. http://dx.doi.org/10.4049/jimmunol.0802696.
5. **Raetz CR, Reynolds CM, Trent MS, Bishop RE.** 2007. Lipid A modification systems in gram-negative bacteria. Annu. Rev. Biochem. **76:**295–329. http://dx.doi.org/10.1146/annurev.biochem.76.010307.145803.
6. **Steeghs L, Jennings MP, Poolman JT, van der Ley P.** 1997. Isolation and characterization of the Neisseria meningitidis lpxD-fabZ-lpxA gene cluster involved in lipid A biosynthesis. Gene **190:**263–270. http://dx.doi.org/10.1016/S0378-1119(97)00005-X.
7. **Pridmore AC, Wyllie DH, Abdillahi F, Steeghs L, van der Ley P, Dower SK, Read RC.** 2001. A lipopolysaccharide-deficient mutant of Neisseria meningitidis elicits attenuated cytokine release by human macrophages and signals via toll-like receptor (TLR) 2 but not via TLR4/MD2. J. Infect. Dis. **183:**89–96. http://dx.doi.org/10.1086/317647.
8. **Steeghs L, Keestra AM, van Mourik A, Uronen-Hansson H, van der Ley P, Callard R, Klein N, van Putten JP.** 2008. Differential activation of human and mouse Toll-like receptor 4 by the adjuvant candidate LpxL1 of Neisseria meningitidis. Infect. Immun. **76:**3801–3807. http://dx.doi.org/10.1128/IAI.00005-08.
9. **Sprong T, Ley P, Abdollahi-Roodsaz S, Joosten L, Meer J, Netea M, Deuren M.** 2011. Neisseria meningitidis lipid A mutant LPSs function as LPS antagonists in humans by inhibiting TLR 4-dependent cytokine production. Innate Immun. **17:**517–525. http://dx.doi.org/10.1177/1753425910383999.
10. **Fransen F, Hamstra HJ, Boog CJ, van Putten JP, van den Dobbelsteen GP, van der Ley P.** 2010. The structure of Neisseria meningitidis lipid A determines outcome in experimental meningococcal disease. Infect. Immun. **78:**3177–3186. http://dx.doi.org/10.1128/IAI.01311-09.

11. **Ladhani SN, Lucidarme J, Newbold LS, Gray SJ, Carr AD, Findlow J, Ramsay ME, Kaczmarski EB, Borrow R.** 2012. Invasive meningococcal capsular group Y disease, England and Wales, 2007–2009. Emerg. Infect. Dis. **18:**63–70. http://dx.doi.org/10.3201/eid1801.110901.
12. **Fransen F, Heckenberg SG, Hamstra HJ, Feller M, Boog CJ, van Putten JP, van de Beek D, van der Ende A, van der Ley P.** 2009. Naturally occurring lipid A mutants in Neisseria meningitidis from patients with invasive meningococcal disease are associated with reduced coagulopathy. PLoS Pathog. **5:**e1000396. http://dx.doi.org/10.1371/journal.ppat.1000396.
13. **Brouwer MC, Spanjaard L, Prins JM, van der Ley P, van de Beek D, van der Ende A.** 2011. Association of chronic meningococcemia with infection by meningococci with underacylated lipopolysaccharide. J. Infect. **62:**479–483. http://dx.doi.org/10.1016/j.jinf.2011.03.010.
14. **Harvey HA, Post DM, Apicella MA.** 2002. Immortalization of human urethral epithelial cells: a model for the study of the pathogenesis of and the inflammatory cytokine response to Neisseria gonorrhoeae infection. Infect. Immun. **70:**5808–5815. http://dx.doi.org/10.1128/IAI.70.10.5808-5815.2002.
15. **Post DM, Phillips NJ, Shao JQ, Entz DD, Gibson BW, Apicella MA.** 2002. Intracellular survival of Neisseria gonorrhoeae in male urethral epithelial cells: importance of a hexaacyl lipid A. Infect. Immun. **70:**909–920. http://dx.doi.org/10.1128/IAI.70.2.909-920.2002.
16. **Steichen CT, Shao JQ, Ketterer MR, Apicella MA.** 2008. Gonococcal cervicitis: a role for biofilm in pathogenesis. J. Infect. Dis. **198:**1856–1861. http://dx.doi.org/10.1086/593336.
17. **Jerse AE, Wu H, Packiam M, Vonck RA, Begum AA, Garvin LE.** 2011. Estradiol-Treated female mice as surrogate hosts for Neisseria gonorrhoeae genital tract infections. Front. Microbiol. **2:**107. http://dx.doi.org/10.3389/fmicb.2011.00107.
18. **Fields RC, Osterholzer JJ, Fuller JA, Thomas EK, Geraghty PJ, Mule JJ.** 1998. Comparative analysis of murine dendritic cells derived from spleen and bone marrow. J. Immunother. **21:**323–339.
19. **Packiam M, Veit SJ, Anderson DJ, Ingalls RR, Jerse AE.** 2010. Mouse strain-dependent differences in susceptibility to Neisseria gonorrhoeae infection and induction of innate immune responses. Infect. Immun. **78:**433–440. http://dx.doi.org/10.1128/IAI.00711-09.
20. **Packiam M, Wu H, Veit SJ, Mavrogiorgos N, Jerse AE, Ingalls RR.** 2012. Protective role of Toll-like receptor 4 in experimental gonococcal infection of female mice. Mucosal Immunol. **5:**19–29. http://dx.doi.org/10.1038/mi.2011.38.
21. **Warner DM, Folster JP, Shafer WM, Jerse AE.** 2007. Regulation of the MtrC-MtrD-MtrE efflux-pump system modulates the in vivo fitness of Neisseria gonorrhoeae. J. Infect. Dis. **196:**1804–1812. http://dx.doi.org/10.1086/522964.
22. **Craven RR, Gao X, Allen IC, Gris D, Bubeck Wardenburg J, McElvania-Tekippe E, Ting JP, Duncan JA.** 2009. Staphylococcus aureus alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells. PLoS One **4:**e7446. http://dx.doi.org/10.1371/journal.pone.0007446.
23. **Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, Fernandes-Alnemri T, Wu J, Monks BG, Fitzgerald KA, Hornung V, Latz E.** 2009. Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. J. Immunol. **183:**787–791. http://dx.doi.org/10.4049/jimmunol.0901363.
24. **Lopez-Castejon G, Luheshi NM, Compan V, High S, Whitehead RC, Flitsch S, Kirov A, Prudovsky I, Swanton E, Brough D.** 2013. Deubiquitinases regulate the activity of caspase-1 and interleukin-1beta secretion via assembly of the inflammasome. J. Biol. Chem. **288:**2721–2733. http://dx.doi.org/10.1074/jbc.M112.422238.
25. **John CM, Liu M, Jarvis GA.** 2009. Profiles of structural heterogeneity in native lipooligosaccharides of Neisseria and cytokine induction. J. Lipid Res. **50:**424–438. http://dx.doi.org/10.1194/jlr.M800184-JLR200.
26. **van der Ley P, Steeghs L, Hamstra HJ, ten Hove J, Zomer B, van Alphen L.** 2001. Modification of lipid A biosynthesis in Neisseria meningitidis lpxL mutants: influence on lipopolysaccharide structure, toxicity, and adjuvant activity. Infect. Immun. **69:**5981–5990. http://dx.doi.org/10.1128/IAI.69.10.5981-5990.2001.
27. **Matson JS, Yoo HJ, Hakansson K, Dirita VJ.** 2010. Polymyxin B resistance in El Tor Vibrio cholerae requires lipid acylation catalyzed by MsbB. J. Bacteriol. **192:**2044–2052. http://dx.doi.org/10.1128/JB.00023-10.
28. **Reines M, Llobet E, Llompart CM, Moranta D, Perez-Gutierrez C, Bengoechea JA.** 2012. Molecular basis of Yersinia enterocolitica temperature-dependent resistance to antimicrobial peptides. J. Bacteriol. **194:**3173–3188. http://dx.doi.org/10.1128/JB.00308-12.
29. **D'Hauteville H, Khan S, Maskell DJ, Kussak A, Weintraub A, Mathison J, Ulevitch RJ, Wuscher N, Parsot C, Sansonetti PJ.** 2002. Two msbB genes encoding maximal acylation of lipid A are required for invasive Shigella flexneri to mediate inflammatory rupture and destruction of the intestinal epithelium. J. Immunol. **168:**5240–5251. http://www.jimmunol.org/content/168/10/5240.
30. **Alcala B, Salcedo C, Arreaza L, Abad R, Enriquez R, De La Fuente L, Uria MJ, Vazquez JA.** 2004. Antigenic and/or phase variation of PorA protein in non-subtypable Neisseria meningitidis strains isolated in Spain. J. Med. Microbiol. **53:**515–518. http://dx.doi.org/10.1099/jmm.0.05517-0.
31. **John CM, Liu M, Jarvis GA.** 2009. Natural phosphoryl and acyl variants of lipid A from Neisseria meningitidis strain 89I differentially induce tumor necrosis factor-alpha in human monocytes. J. Biol. Chem. **284:**21515–21525. http://dx.doi.org/10.1074/jbc.M109.004887.
32. **John CM, Liu M, Phillips NJ, Yang Z, Funk CR, Zimmerman LI, Griffiss JM, Stein DC, Jarvis GA.** 2012. Lack of lipid A pyrophosphorylation and functional lptA reduces inflammation by Neisseria commensals. Infect. Immun. **80:**4014–4026. http://dx.doi.org/10.1128/IAI.00506-12.
33. **Liu M, John CM, Jarvis GA.** 2010. Phosphoryl moieties of lipid A from Neisseria meningitidis and N. gonorrhoeae lipooligosaccharides play an important role in activation of both MyD88- and TRIF-dependent TLR4-MD-2 signaling pathways. J. Immunol. **185:**6974–6984. http://dx.doi.org/10.4049/jimmunol.1000953.
34. **Lewis LA, Choudhury B, Balthazar JT, Martin LE, Ram S, Rice PA, Stephens DS, Carlson R, Shafer WM.** 2009. Phosphoethanolamine substitution of lipid A and resistance of Neisseria gonorrhoeae to cationic antimicrobial peptides and complement-mediated killing by normal human serum. Infect. Immun. **77:**1112–1120. http://dx.doi.org/10.1128/IAI.01280-08.
35. **Tzeng YL, Ambrose KD, Zughaier S, Zhou X, Miller YK, Shafer WM, Stephens DS.** 2005. Cationic antimicrobial peptide resistance in Neisseria meningitidis. J. Bacteriol. **187:**5387–5396. http://dx.doi.org/10.1128/JB.187.15.5387-5396.2005.