Induction of Endotoxin Tolerance by Pathogenic Neisseria Is Correlated with the Inflammatory Potential of Lipooligosaccharides and Regulated by MicroRNA-146a

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Induction of Endotoxin Tolerance by Pathogenic Neisseria Is Correlated with the Inflammatory Potential of Lipooligosaccharides and Regulated by MicroRNA-146a

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In this article, we report that retreatment of human monocytic THP-1 cells and primary monocytes with pathogenic Neisseria or with purified lipooligosaccharides (LOS) after previous exposure to LOS induced immune tolerance, as evidenced by reduced TNF-α and IL-1β cytokine expression. LOS that we have previously shown to vary in their potential to activate TLR4 signaling, which was correlated with differences in levels of lipid A phosphorylation, had similarly variable ability to induce tolerance. Efficacy for induction of tolerance was proportional to the level of lipid A phosphorylation, as LOS from meningococcal strain 891 with the highest degree of phosphorylation was the most tolerogenic following retreatment with LOS or whole bacteria, compared with LOS from gonococcal strains 1291 and GC56 with reduced levels of phosphorylation. Hydrogen fluoride treatment of 891 LOS to remove phosphates rendered the LOS nontolerogenic. Tolerance induced by the more highly inflammatory meningococcal LOS was correlated with significantly greater downregulation of p38 activation, greater induction of the expression of A20 and of microRNA-146a, and greater reductions in IL-1R-associated kinase 1 and TRAF6 levels following LOS retreatment of cells. The role of miR-146a in regulation of induction of TNF-α was confirmed by transfecting cells with an inhibitor and a mimic of miR-146a. Our results provide a mechanistic framework for understanding the variable pathophysiology of meningococcal and gonococcal infections given that after an initial exposure, greater upregulation of microRNA-146a by more highly inflammatory LOS conversely leads to the suppression of immune responses, which would be expected to facilitate bacterial survival and dissemination. The Journal of Immunology, 2014, 192: 1768–1777.

Neisseria meningitidis is the leading cause of epidemic meningitis and fatal sepsis in otherwise healthy individuals (1). More than 500,000 cases of meningococcal infection occur annually worldwide, leading to ~50,000 deaths, and large-scale epidemic outbreaks occur that can cause periodic sharp increases in the disease incidence. N. meningitidis is a frequent colonizer of the human upper respiratory tract; however, in some individuals the bacterium spreads to the bloodstream, causing sepsis, which is a serious condition with high morbidity and mortality (2). Meningococcal sepsis represents the prototypical human Gram-negative septic syndrome, characterized by extremely high concentrations of lipooligosaccharide (LOS) and cytokines (1, 3). It is well known that the induction of cytokines and the subsequent pathophysiological events that occur during meningococcal sepsis are mainly induced by the endotoxic LOS component of the bacterial outer membrane (3–5).

In general, bacterial sepsis involves an extremely complex pathological state resulting from a dysregulated inflammatory response by the innate immune system with an underlying disease pathogenesis that remains poorly understood. It was only recently that sepsis was characterized by an early phase of systemic hyperinflammatory cytokine responses, followed by a later hyporesponsive phase during which patients with sepsis commonly develop anti-inflammatory cytokines and become refractory to further endotoxin challenge by a phenomenon widely referred to as endotoxin tolerance (6, 7). The monocytes isolated from patients during the refractory phase demonstrate a dramatic decrease in the production of proinflammatory cytokines like TNF-α, IL-6, IL-1α, and IL-1β when they were challenged with lipopolysaccharide (LPS) ex vivo, whereas anti-inflammatory cytokines like IL-10, TGF-β, and IL-1RA showed enhanced expression (7). Recently, endotoxin tolerance in sepsis has gained significant interest because most clinical trials aiming to control the acute inflammatory response at the onset of infection have failed (6, 8).

Our understanding of the mechanism of endotoxin tolerance primarily involves studies of tolerance induced by LPS. LPS-induced tolerance involves many factors, which are mainly related to the inhibition of IL-1R–associated kinase 1 (IRAK1) and TNFR-associated factor 6 (TRAF6) signaling components of the TLR4 pathway (9). One of the mechanisms known to regulate endotoxin tolerance to LPS is mediated through the induction of microRNA, a small non-coding RNA that posttranscriptionally regulates gene expression (9). Mature microRNAs can specifically bind to 3′-UTRs of target mRNA and trigger mRNA degradation or inhibition of translation (10). The impact of microRNA on innate immune signaling was first observed when proinflammatory stimuli such as LPS, TNF-α, and IL-1β induced the expression of specific microRNAs, miR-146a and miR-155, and downregulated TLR4 and IL-1R signaling pathways in monocytes and macrophages (11, 12). In particular, significant downregulation of IRAK1 and TRAF6 protein during endotoxin-induced tolerance has been correlated to the induction
of miR-146a, which is expressed in response to a variety of inflammatory stimuli, including IL-1β and TNF-α, ligands for TLR2 and TLR5, and Gram-positive and Gram-negative bacteria (9).

Endotoxin tolerance induced by the LOS of \textit{N. meningitidis} has not been well studied. The initial evidence for endotoxin tolerance in meningococcal infection is derived from studies by Westendorp et al. (13, 14) of TNF-α and IL-10 levels in first-degree relatives of patients with meningococcal disease. Using a whole-blood ex vivo endotoxin stimulation assay, they observed that families with low TNF-α production and high IL-10 production had the greatest risk of a fatal outcome. More recent studies of severe septic shock caused by \textit{N. meningitidis} demonstrated that despite high proinflammatory cytokine levels, anti-inflammatory cytokine responses such as IL-10 that are regulatory in nature are significantly upregulated (15–17). However, immunomodulatory strategies have not been used in patients with meningococcal disease because, as for patients with sepsis resulting from other Gram-negative bacteria, these strategies have been predicted to offer limited protection or possibly induce harmful effects (18, 19).

In contrast to disease caused by \textit{N. meningitidis}, that produced by \textit{Neisseria gonorrhoeae} is predominantly a mucosal infection that disseminates only on rare occasions. In general, gonococcal infection is characterized as a localized and mild disease, and gonococcal infection rarely induces protective immunity against future infection, as evidenced by repetitive infections of individuals with the same strain (20, 21). We recently demonstrated that immunosuppression, or immune tolerance, elicited by gonococci was associated with the selective inhibition of Th1 and Th2 cells through the induction of TGF-β along with the enhancement of Th17 cell development in response to TLR4 signaling by LOS (22).

As the main virulence factor in the pathogenesis of neisserial infections, LOS is one of the primary components of the organism that contribute to host proinflammatory cytokine responses (3). We have previously shown that differing levels of phosphoryl substitutions on the lipid A (LA) component of neisserial LOS, which lack the repeating O-polysaccharide component of LPS from \textit{Escherichia coli}, underlie different potencies for the activation of NF-κB by LOS. We found that in monocytes, LOS with the most phosphoryl substitutions on the LA induced the highest levels of cytokine expression by signaling through TLR4, resulting in activation of both the MyD88-dependent and TRIF-dependent pathways (23).

The main goals of the current study were to explore whether neisserial LOS induces tolerance in human monocytes and to determine whether variability in phosphorylation of the LA component affects the potency of LOS for inducing tolerance. These studies provide new insight into our understanding of the pathogenesis of disease caused by \textit{N. meningitidis} and \textit{N. gonorrhoeae}.

Materials and Methods

\textbf{Neisserial strains and LOS}

\textit{N. meningitidis} serogroup C strain 891 and \textit{N. gonorrhoeae} strains 1291 and GC56 are clinical isolates that have been described previously (23–25). LOS was extracted and purified by a modification of the hot phenol-water method (26), and LOS LA structures for the strains have been reported previously (23, 25). Hydrogen fluoride (HF) treatment of 891 LOS to remove phosphoethanolamine (PEA) and eliminate phosphate from pyrophosphate (PP) was performed as described previously (24).

\textbf{Cytokine expression and LOS-induced tolerance in human monocytes}

The cell model for LOS-induced tolerance was adapted using the human monocytic cell line THP-1 and primary human monocytes, as described by Nahid et al. (27). THP-1 cells were propagated in RPMI 1640 supplemented with 10% FBS at 37°C in a 5% CO₂ atmosphere, and differentiated with 10 ng/ml PMA (Sigma-Aldrich, St. Louis, MO) for 12–18 h. Stimulation of cells was performed in growth medium supplemented with 10% FBS as a source for LPS-binding protein. Cells were seeded into 12-well plates at 1 × 10⁶ cells per well, and either medium only or medium containing LOS at a concentration of 100 ng/ml was added and the cells were incubated for 18 h. Cells were then washed twice with PBS, and either 100 ng/ml of the same or different LOS or whole viable bacteria (multiplicity of infection of 1) in RPMI 1640 with 10% FBS was added to the cells, followed by incubation for 4 h or for the indicated period. The amount of LOS and bacteria was based on our previous studies of their relative bioactivities (23). The supernatants were collected and stored at −80°C for subsequent cytokine assays. For primary human monocytes, freshuffy blood (Blood Centers of the Pacific, San Francisco, CA) was diluted 1:1 with PBS, overlaid on Ficoll-Paque (GE Healthcare Biobiosciences, Piscataway, NJ), and centrifuged at 1000 rpm for 30 min. The interphase containing the monocytes was collected and washed twice with PBS, and CD14+ monocytes were purified using MACS beads according to the instructions of the manufacturer (Miltenyi Biotec, Auburn, CA). Purified primary monocytes were treated with LOS in similar fashion to THP-1 cells, and supernatants were collected for cytokine assays. For both THP-1 and primary monocytes, cell viability following LOS treatment was > 95%, as determined by trypan blue dye (Life Technologies, Carlsbad, CA) exclusion cell counts. For quantification of TNF-α in cell culture supernatants, an ELISA kit (eBioscience, San Diego, CA) was used according to the instructions of the manufacturer. IL-1β levels were determined using a bead-based assay (Life Technologies). Samples of supernatants (50 μl each) were processed as recommended by the manufacturer and analyzed using a Bio-Plex 200 system (Bio-Rad, Hercules, CA).

\textbf{Western blot analyses}

For p38 and A20 analyses, PMA-differentiated THP-1 cells were distributed into 12-well plates (1 × 10⁶ cells per well) and treated with 100 ng/ml LOS for 18 h and then retreated with 100 ng/ml LOS for increasing periods as indicated. For TRAP6 and IRAK1 analyses, cells were distributed into 12-well plates (1 × 10⁶ cells per well) and treated with increasing concentrations of LOS for 18 h. After washing with PBS, cells were lysed on ice for 10 min in 100 μl lysis buffer (50 mM HEPES, pH 7.6, containing 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 20 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 mM NaF, 1 mM benzamidine, 5 mM p-nitrophenyl phosphate, 1 mM DTT, 1 mM PMSF, and complete protease inhibitor mixture from Roche Diagnostics [Indianapolis, IN]). Aliquots of 20 μl of each sample were heated at 100°C for 5 min, and samples were resolved in a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane for immunoblotting. The membranes were blocked for 1 h at room temperature with 5% nonfat milk in PBS/0.05% Tween 20. Phosphorylated and nonphosphorylated proteins were detected by incubation at 4°C overnight with A20 phospho-A20 or β-actin Ab, followed by horseradish peroxidase-conjugated anti-mouse IgG secondary Ab (Cell Signaling, Danvers, MA), and proteins were visualized using ECL Plus (GE Healthcare Biobiosciences). The relative intensity of each band was determined by densitometry using the Image J software available from the National Institutes of Health, after which the intensity values were normalized by calculating the ratio of the phosphorylated band intensity to that of the nonphosphorylated band (for phosphorylation of p38) or β-actin control band (for A20, IRAK1, and TRAF6). The data were presented as fold changes compared with controls.

\textbf{Analyses of LOS-induced microRNA expression by quantitative real-time PCR}

PMA-differentiated THP-1 cells were distributed into 12-well plates at 1 × 10⁶ cells per well in RPMI 1640 with 10% FBS. LOS was added to the cells at 100 ng/ml, followed by an incubation for 18 h. Cells were harvested, and the total RNA from treated and untreated cells was prepared using the mirVana miRNA Isolation Kit (Life Technologies) following the protocol of the manufacturer. RNA yield and purity were determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technology, Wilmington, DE), and equal amounts of each RNA were used for reverse transcription using TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantification of mature miRNA expression was performed using the TaqMan Universal PCR Master Mix and TaqMan microRNA assay primers for human miRNAs (Applied Biosystems). The cycle threshold values corresponding to the PCR cycle number at which fluorescence emission reaches a threshold above baseline emission were determined, and miRNA expression values were calculated after normalization to mammalian abundant RNU48 as an endogenous control (Applied Biosystems) following the 2^ΔΔCt method (28).
**Results**

LOS induces endotoxin tolerance in relation to the degree of LA phosphorylation

LOS has been shown to induce a diverse array of inflammatory mediators, including TNF-α, both in vitro and in vivo (25, 29–32). We have previously shown that the LA phosphoryl moieties of LOS play an important role in determining the potency of LOS for cytokine induction through activation of both the MyD88- and TRIF-dependent pathways (23). To investigate whether LOS induces endotoxin tolerance, as has been demonstrated with LPS, and whether phosphorylation differences affect the immune modulation, we treated THP-1 cells with the LOS from meningococcal strain 89I and gonococcal strains 1291 and GC56 for 18 h. Cells were then retreated with the same LOS for increasing periods up to 4 h and supernatants were analyzed for TNF-α expression. As shown in Fig. 1, cells treated initially with medium only for 18 h were induced to produce significant amounts of TNF-α following the subsequent LOS treatment. Consistent with our previous report (23), 89I LOS with the most phosphoryl moieties induced the highest level of TNF-α followed by the LOS from 1291 and GC56, which displayed medium to low degrees of phosphorylation, respectively. In contrast, the cells expressed significantly lower levels of TNF-α when treated with LOS for 18 h and then retreated with the homologous LOS, suggesting that LOS was able to induce immune tolerance to itself.

To determine the effect of differences in phosphoryl substitution on the otherwise structurally identical LA, we compared the ability of heterologous LOS to influence endotoxin tolerance. THP-1 cells were treated with 89I, 1291, and GC56 LOS for 18 h; retreated with heterologous LOS for 4 h; and assayed for the induction of both TNF-α and IL-1β. We found that 89I, which has the highest degree of phosphorylation and induced the most TNF-α, also induced the highest level of tolerance. As shown in Fig. 2A, although treatment with 89I, 1291, and GC56 LOS was able to significantly reduce TNF-α induction following retreatment with heterologous LOS, the degree of reduction was highest by 89I LOS followed by 1291 and GC56. Similar results were observed when the induction of IL-1β was tested (Fig. 2B). Treatment with 89I LOS significantly reduced the expression of IL-1β following retreatment with 1291 and GC56 LOS, whereas IL-1β expression was reduced to a lesser degree when cells were treated with 1291 and GC56 LOS and subsequently retreated with a heterologous LOS.

Next we tested whether LOS induced tolerance when whole bacteria were used to retreat the cells. As shown in Fig. 2C, treatment of cells with 89I LOS significantly reduced the induction of TNF-α by whole bacteria for both the homologous 89I bacteria and the heterologous 1291 and GC56 bacteria. By comparison, treatment of cells with 1291 and GC56 LOS had a less significant effect on the induction of TNF-α by homologous or heterologous bacteria, with 1291 LOS only reducing TNF-α induction by the GC56 bacteria, and GC56 LOS not affecting the level of TNF-α induction by any of the three bacterial strains. These results demonstrated that LOS with the highest degree of LA phosphorylation also had the highest potential for inducing tolerance to further challenge by both LOS and bacteria, suggesting a positive correlation between the degree of LA phosphorylation and the strength of tolerance induction by LOS.

To further confirm that LA phosphorylation plays an important role in tolerance induced by LOS, we tested the potential of HF-treated 89I LOS to induce tolerance, as we have previously reported that HF treatment removes PEA and eliminates phosphate from PP groups from LOS and significantly reduces TNF-α induction in THP-1 cells (24). As shown in Fig. 2D, Although 89I LOS induced strong TNF-α expression by itself and tolerance against retreatment by LOS, HF-treated LOS induced a significantly lower
level of TNF-α and generated no tolerance against further LOS treatment, thereby providing further evidence of the importance of LA phosphorylation in the induction of tolerance by LOS.

Results similar to those observed for THP-1 cells were found using primary human monocytes. As seen in Fig. 2E, monocytes treated with 89I LOS were significantly tolerized against TNF-α induction following retreatment with homologous and heterogeneous LOS. However, as with THP-1 cells, 89I LOS treated with HF and lacking PEA and PP moieties was not able to induce tolerance in monocytes.

LOS treatment blocks p38 activation in THP-1 cells

NF-κB and MAPKs are critical for the expression of cytokines and chemokines (33). We have previously shown that LOS treatment differentially induced p65 phosphorylation in THP-1 cells, and that 89I LOS with the highest degree of phosphorylation induced the greatest degree of p65 activation (23). It is known that p38 MAPK regulates TNF-α expression through modulation of NF-κB and that the p38 pathway regulates TNF-α and IL-1β expression by posttranscriptionally stabilizing their mRNA (34, 35). Therefore, we next explored whether phosphorylation of LA influenced p38 activation during the induction of tolerance. THP-1 cells were treated with LOS for 18 h and then retreated with LOS for increasing periods, and cells were harvested for p38 phosphorylation analyses. Consistent with the concept that LA with greater degrees of phosphorylation induce greater quantities of cytokines and chemokines, we observed that treatment with 89I LOS induced significant p38...
phosphorylation at 15 min (80-fold), which peaked at 30 min (131-fold) (Fig. 3). Conversely, the activation of p38 was relatively modest in response to 1291 LOS (9-fold and 9.4-fold at 30 and 60 min, respectively) and minimal in response to GC56 LOS (5.3-fold and 2-fold at 30 and 60 min, respectively). Overall, treatment with 89I LOS significantly inhibited p38 phosphorylation after cells were retreated with the same LOS, whereas retreatment of cells with 1291 and GC56 LOS showed similar patterns of negligible p38 phosphorylation, as the two gonococcal LOS were much weaker agonists for p38 activation, consistent with their lower ability to induce pro-inflammatory cytokines. These results suggest that treatment with LOS inhibited p38 activation and that the level of inhibition was correlated with the potency of LOS to induce a proinflammatory response.

**LOS treatment induces A20 expression in THP-1 cells**

A20 is a zinc finger protein with ubiquitin-editing activity that removes K63-linked polyubiquitin chains and has C-terminal ubiquitin ligase activity that facilitates target protein degradation (36). It has been shown that A20 can target TRAF6 and receptor-interacting protein kinase 1 and downregulate NF-κB activity. In addition, A20−/− mice develop severe intestinal inflammation early in life, suggesting that A20 is important for the inhibition of innate immune responses (37). To explore whether LOS affects the expression of A20, which may subsequently contribute to LOS-induced tolerance, we treated THP-1 cells with different LOS and analyzed A20 expression following retreatment with LOS for increasing periods. As shown in Fig. 4, A20 expression was barely detectable at 4 h of LOS treatment without prior LOS treatment. However, after 18 h of treatment with LOS, A20 expression was significantly enhanced following retreatment with 89I LOS (21-fold increase at 4 h), which contains the highest degree of phosphorylation of its LA. In contrast, a similar retreatment with 1291 LOS induced significantly less A20 expression (10-fold increase at 4 h), and GC56 LOS, which has the lowest degree of phosphorylation, had a minimal effect on A20 expression (5-fold increase at 4 h). These results suggest that the degree of LA phosphorylation is positively correlated with the expression of A20, which likely contributes to LOS-induced tolerance through downregulation of NF-κB activity.

**LOS treatment induces TLR4 inhibitor miR-146a**

To understand the mechanism of how phosphorylation of the LA of LOS influenced tolerance induction, we explored the effect of 89I, 1291, and GC56 LOS on the expression of miR-146a. Initially, miR-146a was identified as an LPS-inducible gene in response to the TLR4 signaling pathway through microarray analysis of THP-1 monocytes (11) and was later reported to play a critical role in LPS-induced cross-tolerance through a negative feedback pathway (9). To this end, we tested whether neisserial LOS induced miR-146a in THP-1 cells and whether expression correlated with the degree of phosphorylation of LA. Cells were treated with LOS and subsequently retreated with the same LOS, and qRT-PCR was performed to determine the expression of miR-146a. As shown in Fig. 5A, although the expression of miR-146a was increased by 12-, 10-, and 8-fold in response to a 4-h treatment with 89I, 1291, and GC56 LOS, respectively, in the absence of an 18-h initial treatment, the level of expression was significantly higher after the 18-h treatment followed by the 4-h retreatment with LOS. Consistent with its level of LA phosphorylation, 89I induced the highest level of expression of miR-146a, followed by 1291 and GC56 LOS (40-, 27-, and 25-fold, respectively). To confirm the contribution of LA phosphorylation to the induction of miR-146a, 89I LOS was treated with HF to remove PEA and PP groups and was used to treat THP-1 cells for 18 h, after which miR-146a expression was analyzed by qRT-PCR. As shown in Fig. 5B, treatment of cells with 89I LOS resulted in a significantly greater increase in the level of miR-146a compared with that seen with HF-treated LOS. Taken together,

**FIGURE 3.** Induction of tolerance downregulates p38 activation. THP-1 cells were treated for 18 h either with medium only or with medium containing 89I, 1291, or GC56 LOS. Cells were washed and then retreated with the same LOS for 0, 5, 15, 30, or 60 min. Cell lysates were analyzed for the phosphorylation of p38 and total p38 by Western blot (top panel). The bottom panel displays the densitometry analyses of the corresponding treatments showing the fold increase in phosphorylation of p38.
these results indicate that the ability of LOS to induce miR-146a expression is correlated with the degree of LA phosphorylation. Studies of LPS have shown that miR-146a targets the expression of MyD88 downstream signal proteins IRAK1 and TRAF6, which are required for subsequent cytokine induction by LPS (9, 11). Thus, we next tested whether the expression of IRAK1 and TRAF6 was affected by the treatment of cells with LOS. THP-1 cells were treated with LOS for 18 h and then retreated for 4 h with increasing concentrations of LOS, and the levels of IRAK1 and TRAF6 were determined by Western blot. As shown in Fig. 6, the protein level of TRAF6 was reduced significantly by 89I LOS at a concentration of 100 ng/ml and was barely detected at the highest dose of 1000 ng/ml (inhibition levels of 70 and 93%, respectively). 1291 LOS also reduced the expression of TRAF6 at the two highest concentrations; however, expression remained moderate at both levels. In contrast, GC56 LOS had little impact on TRAF6 expression, with an inhibition of TRAF6 expression of only 4% at the highest concentration. For IRAK1, although treatment with 10 ng/ml of 89I LOS had no effect on expression, concentrations of 100 and 1000 ng/ml significantly reduced IRAK1 expression (inhibitions of 43 and 73%, respectively), whereas both 1291 and GC56 LOS inhibited expression only ~10–20% at the two highest concentrations.

To confirm that miR-146a plays an important role in the induction of tolerance by neisserial LOS, we transfected THP-1 cells with an miR-146a inhibitor and treated the cells with 89I LOS and assessed the expression of TNF-α. As shown in Fig. 7, transfection with the miR-146a inhibitor significantly increased the expression of TNF-α in transfected cells compared with untransfected cells. We next transfected THP-1 cells with an miR-146a mimic and then treated the cells with either LOS or whole bacteria for 4 h and analyzed the supernatants for TNF-α expression. As seen in Fig. 8, although LOS and whole bacteria induced significant amounts of TNF-α expression in untransfected cells, the induction of TNF-α was significantly reduced in cells that were transfected with the miR-146a mimic prior to treatment with LOS or bacteria. These results provide further evidence that miR-146a plays a critical role in the induction of tolerance by neisserial LOS and whole bacteria.

**Discussion**

We have previously shown that the potency of neisserial LOS to induce cytokine expression through both the MyD88- and TRIF-dependent pathways is correlated with the degree of phosphorylation of the LA (23, 24). In this study, we found that meningococcal 89I LOS, which is the most efficacious in induction of inflammatory responses and has the highest degree of phosphorylation of its LA, also is the most efficacious in the induction of tolerance to further exposure to LOS in human THP-1 monocytic cells and primary monocytes. Monocytes pretreated with 89I LOS not only were effectively prevented from responding to further challenge by the homologous LOS but were also strongly inhibited from responding to heterologous LOS or to whole bacteria. By comparison, treatment of monocytes with LOS from two gonococcal strains, 1291 or GC56, which are mild TLR4 agonists with a lower level of phosphor content on the LA, prevented induction of inflammatory cytokines by monocytes retreated with LOS from the same strain but was significantly less capable of reducing cytokine induction by 89I LOS or by whole bacteria. HF-treated 89I LOS lacking PEA and PP groups exhibited no tolerance-inducing potential, thus confirming the importance of phosphorylation in tolerance induction. These results using neisserial LOS with well-characterized structures clearly demonstrate that differences in the phosphor substitution on the LA that we previously have correlated with their relative efficacy for cytokine induction are also a determinant of the relative capability of LOS to induce tolerance in human monocytes (24, 25).

Although the phenomenon of endotoxin tolerance in which the response to LPS challenge is reduced following an initial exposure to LPS was first described more than 65 y ago (38), the mechanism...
neithertheless remains incompletely understood. Our findings provide evidence that several signaling pathways are differentially induced by neisserial LOS to tolerize human monocytes. One of the pathways, the p38 MAPK pathway, mediates many of the proinflammatory events that are induced by LPS stimulation (39–41). In particular, TNF-α and IL-1β expression is partly regulated by p38 MAPK, which posttranscriptionally stabilizes their mRNA, and a reduction in their expression is characteristic of endotoxin tolerance induced by LPS rechallenge (34, 35). Two postulates have been put forth regarding the involvement of p38 in LPS-induced tolerance. One is that LPS activation of p38 enhances the expression of macrophage scavenger receptor-A that can bind to and reduce the circulating levels of LPS by facilitating cellular internalization and thereby reducing inflammation (42). The other is that LPS-mediated induction of the dual-specific phosphatase, MKP-1, inhibits p38 activation and leads to the reduction in TNF-α expression and anti-inflammatory signaling (43). We observed that in the absence of LOS pretreatment for 18 h, p38 was significantly activated by 89I LOS within 15 min of LOS pretreatment, whereas the activation level of p38 was much lower with 1291 LOS and was minimal with GC56 LOS, both of which have lower levels of LA phosphorylation. In contrast, pretreatment of THP-1 cells with either the 89I or the 1291 LOS inhibited p38 activation upon retreatment of the cells, supporting the postulate that the p38 pathway is involved in LOS-mediated tolerance.

A negative regulator of TLR4, A20, is an ubiquitin-editing enzyme thought to play a key role in the development of LPS-induced tolerance through downregulation of p38, c-Jun, and NF-kB (44). When induced by LPS and enteric bacteria, A20 targets various signaling molecules for degradation, and induction of A20 coincides with the establishment of hyporesponsiveness to repeated stimulation with LPS. Consistent with these studies, we demonstrated a significant induction of A20 in cells retreated with 89I LOS, but only a minimal induction in cells retreated with 1291 and GC56 LOS.

Recently, expression of miR-146a was found to be critical for LPS-induced tolerance and to act by a negative feedback mechanism that targets IRAK1 and TRAF6 downstream of the TLR4 signaling pathway (9, 11, 27). It has been suggested that upregulation of miR-146a by adapter kinases prevents the destructive consequences of an uncontrolled inflammatory reaction caused by overstimulation of TLR4 signaling. We hypothesized that neisserial LOS, as a TLR4 ligand, induces miR-146a expression, which then negatively regulates the TLR4 pathway. Indeed, we observed that neisserial LOS induced miR-146a in THP-1 cells after treatment and that the miR-146a expression level was closely correlated to the degree of tolerance of the cells. We further demonstrated that LOS treatment downregulated the expression of IRAK1 (89I LOS) and TRAF6 (89I and 1291 LOS), which are the downstream signaling adapters for NF-kB, resulting in the downregulation of both TNF-α and IL-1β expression. The effect of miR-146a on the LOS induction of cytokines was confirmed by the up- or downregulation of TNF-α expression in THP-1 cells transfected with either a miR-146a inhibitor or a miR-146a mimic, respectively. Importantly, the ability of LOS to induce miR-146a expression was correlated with its ability to induce an inflammatory response and to the degree of LA phosphorylation. Our results demonstrate that meningococcal 89I LOS, which has the highest degree of LA phosphorylation, induced the highest level of miR-146a. Conversely, gonococcal 1291 and GC56 LOS, which have lower level phosphorylation of LA, induced lower levels of miR-146a.

Fulminant meningococcal sepsis is an extremely complex pathological state resulting from a dysregulated inflammatory response by the innate immune system to systemic meningococcal infection (1, 45). Septic shock and multiple organ failure occur in ≤30% of patients with infections caused by meningococcal serogroups B and C and are the direct cause of death in 90% of patients who succumb to meningococcal infections in Western countries (15). In clear contrast, disseminated gonococcal infection (DGI) begins with a localized mucosal infection that progresses to bacteremia in only an estimated 0.5–3% of infected persons. DGI is characterized by dermatitis, tenosynovitis, and septic arthritis, with symptomatic manifestations of arthritis occurring most frequently in ~42–85% of DGI patients (46, 47). Unlike the prognosis for meningococcal sepsis, that for patients with DGI and its associated sequelae is very favorable; only rarely do serious complications such as endocarditis or meningitis occur, and mortality is exceedingly uncommon. The dramatic differences in the prevalence of and outcome from bacteremia and sepsis from N. meningitidis and N. gonorrhoeae infections appear to mirror the respective ability of these two pathogenic species to induce immune tolerance—which we have shown to be correlated with the level of LA phosphorylation.

The severity of meningococcal disease is related to bacterial endotoxin levels in the plasma as well as to the degree of induction of host cytokines, especially TNF-α, by LOS and other bacterial cell wall components and inflammatory mediators (5, 48). Our
data are consistent with the view that LOS from \textit{N. meningitidis} induces a more potent TLR4 response than that induced by LOS from \textit{N. gonorrhoeae} (23). The greater response in turn induces a higher level of immune tolerance by homeostatic regulation in the later phase of infection, which could enable a virtually unchecked expansion of the meningococcal population and eventually a potentially overwhelming infection and inflammatory response (49), features characteristic of the progression of this often deadly infection. As such, the data presented in this study indicate that a rational strategy for managing meningococcal disease could be to modulate, rather than to inhibit, the production of proinflammatory mediators to provide beneficial effects by preventing the induction of endotoxin tolerance. Thus, determining the impact of structural variation of the neisserial LOS on the induction of meningitis and sepsis may be critical for understanding the molecular mechanisms of the disease and for establishing new targets for treatment. This concept is supported by a recent report of naturally occurring meningococcal \textit{LpxL1} LA mutants with reduced TLR4 activation isolated from patients who had infections with more mild symptoms and less systemic inflammation (45). In contrast, \textit{N. gonorrhoeae}, which causes much more mild symptoms or can be asymptomatic, expresses LOS that induces a weaker inflammatory response, as characterized by less TNF-\textalpha, NF-\kappaB, and p38 activation, and leads to less immune tolerance, thereby creating less opportunity for gonococci to evade the immune system (50, 51). Using structurally well-defined LOS from \textit{N. meningitidis} and \textit{N. gonorrhoeae}, we were able to demonstrate molecular features that are correlated with the variable ability of the organisms to induce immune tolerance and with the differences in the natural courses of infection by these two pathogens.

In summary, this study demonstrated that the degree of phosphorylation on the LA moiety of neisserial LOS is correlated with...
its efficacy in inducing endotoxin tolerance. LOS induced endotoxin tolerance via mediators of several pathways that regulate TLR4 signaling. In particular, LOS downregulated p38 activation, which directly affects inflammatory cytokine expression, as well as upregulated the expression of A20 and miR-146a, both of which act as negative regulators of TLR4 that target p38, IRAK1, TRAF6, and eventually downregulate NF-κB, resulting in reduced expression of TNF-α and induction of endotoxin tolerance. Our results for the first time, to our knowledge, link structural differences in the degree of phosphorylation of LA with the capacity of neisserial LOS to induce immune tolerance. These findings have significance for understanding differences in the natural course of infections due to N. meningitidis and N. gonorrhoeae, as well as implications for developing new treatments for these infections.

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