Differential Induction of the Toll-Like Receptor 4–MyD88-Dependent and -Independent Signaling Pathways by Endotoxins

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The biological response to endotoxin mediated through the Toll-like receptor 4 (TLR4)-MD-2 receptor complex is directly related to lipid A structure or configuration. Endotoxin structure may also influence activation of the MyD88-dependent and -independent signaling pathways of TLR4. To address this possibility, human macrophage-like cell lines (THP-1, U937, and MM6) or murine macrophage RAW 264.7 cells were stimulated with picomolar concentrations of purified endotoxins. Harvested supernatants from previously stimulated cells were used to stimulate RAW 264.7 or 23ScCr (TLR4-deficient) macrophages (i.e., indirect induction). Neisseria meningitidis lipooligosaccharide (LOS) was a potent direct inducer of the MyD88-dependent pathway molecules tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 3α (MIP-3α), and the MyD88-independent molecules beta interferon (IFN-β), nitric oxide, and IFN-γ-inducible protein 10 (IP-10). Escherichia coli 55:B5 and Vibrio cholerae lipopolysaccharides (LPSs) at the same pmole/ml lipid A concentrations induced comparable levels of TNF-α, IL-1β, and MIP-3α, but significantly less IFN-β, nitric oxide, and IP-10. In contrast, LPS from Salmonella enterica serovars Minnesota and Typhimurium induced amounts of IFN-β, nitric oxide, and IP-10 similar to meningococcal LOS but much less TNF-α and MIP-3α in time course and dose-response experiments. No MyD88-dependent or -independent response to endotoxin was seen in TLR4-deficient cell lines (C3H/HeJ and 23ScCr) and response was restored in TLR4-MD-2-transfected human embryonic kidney 293 cells. Blocking the MyD88-dependent pathway by DNMyD88 resulted in significant reduction of TNF-α release but did not influence nitric oxide release. IFN-β polyclonal antibody and IFN-α/β receptor 1 antibody significantly reduced nitric oxide release. N. meningitidis endotoxin was a potent agonist of both the MyD88-dependent and -independent signaling pathways of the TLR4 receptor complex of human macrophages. E. coli 55:B5 and V. cholerae LPS, at the same picomolar lipid A concentrations, selectively induced the MyD88-dependent pathway, while Salmonella LPS activated the MyD88-independent pathway.

Bacterial endotoxins of human pathogens interact with macrophages and other cells via the Toll-like receptor 4 (TLR4)-MD-2 receptor complex (TLR4 complex) (13), resulting in cellular activation, the release of cytokines, chemokines, reactive oxygen species, nitric oxide (4) and, in some individuals, the clinical picture of sepsis. However, other endotoxins often found in commensal species act as antagonists of the TLR4 complex (14, 44).

Two signaling pathways have been described following TLR4 activation, the MyD88-dependent (9, 26) and -independent pathways (15, 33). These signaling pathways depend on Toll/interleukin-1 receptor adapter proteins including MyD88, Mal/TIRAP (10, 17), TRIF/TICAM-1 (32, 47), and TRAM/TICAM-2 (11, 48). Endotoxin activation of the MyD88-dependent pathway results in rapid NF-κB activation and release of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL-1)β, IL-6, and chemokines like monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 3α (MIP-3α), and IL-8. Endotoxin activation of the MyD88-independent pathway results in rapid activation of interferon regulatory factor 3 (IRF3) (22, 33) leading to beta interferon (IFN-β) release but delayed NF-κB activation (15). IFN-β binds to the type 1 IFN-α/β receptor, which activates STAT1 (1, 23, 36) and leads to type 1 IFN-α/β, IFN-α-inducible protein 10 (IP-10), MCP-5, RANTES, and nitric oxide release (22, 35, 46). Further, TLR4 activation by endotoxin via the MyD88-independent (TRIF/TICAM-1) pathway is important for dendritic cell maturation and provides an important link between the innate and adaptive immune responses (16).

How endotoxin interacts with the TLR4 complex molecules and recruits specific adaptor proteins remains poorly understood. Lipid A structure and configuration of endotoxin are known to determine the agonist or antagonist activity (24, 27, 37). For example, the KDO2-lipid A structure of meningococcal lipooligosaccharide (LOS) is necessary for the maximal agonist activation of macrophages via the TLR4 complex (51). The aim of the study was to further determine the influence of endotoxin structure on activation of the TLR4-MyD88-dependent and -independent signaling pathways.

MATERIALS AND METHODS

Reagents. RPMI 1640 medium, Dulbecco’s modified Eagle’s medium, fetal bovine serum, penicillin/streptomycin, sodium pyruvate, and nonessential amino acids.
acids were obtained from Cellgro Mediatech (Herndon, VA). Phorbol myristate acetate was purchased from GibcoBRL (Grand Island, NY). Human and mouse TNF-α, IFN-γ enzyme-linked immunosorbent assay (ELISA) kits were from R&D systems (Minneapolis, MN). The RA52 (MD-2 deficient) cell lines were purchased from the American Type Culture Collection. The

<table>
<thead>
<tr>
<th>N. meningitidis</th>
<th>LPS Lipid A phosphorylation</th>
<th>Fatty acyl chain symmetry</th>
<th>Fatty acyl chain number</th>
<th>3-OHC10:0</th>
<th>C12:0</th>
<th>3-OHC12:0</th>
<th>C14:0</th>
<th>3-OHC14:0</th>
<th>C16:0</th>
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<tr>
<td>E. coli 55:35</td>
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<td>Hexa-acylated</td>
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<td>1</td>
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<tr>
<td>S. enterica serovar Minnesota</td>
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<td>Asymmetrical</td>
<td>Hepta-acylated</td>
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<td>1</td>
<td>1</td>
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<td>4</td>
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<tr>
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<td>Hepta-acylated</td>
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<tr>
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*N. meningitidis and V. cholerae* lipid A are symmetrical hexa-acylated (29, 30), E. coli lipid A is asymmetrical hexa-acylated (31, 32), *Klebsiella pneumoniae*, *Salmonella enterica* serovars Typhimurium and Minnesota lipid A’s are asymmetrical and hepta-acylated (33–35), *Pseudomonas aeruginosa* lipid A is an asymmetrical penta-acylated structure (36) and *Serratia marcescens* lipid A is an asymmetrical hexa-acylated structure (37).
IFN-α/β receptor blocking. Freshly grown THP-1 cells were washed and adjusted to 10^6 cell/ml, resuspended in 200 μl of PBS final volume. IFN-α/β interferon receptors were blocked with 1, 2, 5, or 10 μg/10^6 cells of the anti-human IFN-α/β receptor 1 polyclonal antibody [anti-human IFN-α/β receptor 1 antibody was produced in goats immunized with purified recombinant human IFN-α/β receptor 1 extracellular domain and the specific IgG was purified by human IFN-α/β receptor 1 affinity chromatography (Sigma)]. The cell mixture was incubated for 30 min at 37°C and then the volume was adjusted to 1 ml with RPMI 1640 supplemented as noted above. Blocked and unblocked cells were stimulated with 1 pmol lipid A/ml of LPS or 10 pmol/ml of lipid A (acid hydrolyzed from meningococcal LOS) and incubated overnight; 100 μl from the harvested supernatants was used to measure the amount of released IP-10 and nitric oxide released in supernatants (from previously stimulated cells containing released cytokines) in the presence or absence of IFN-α (solid bars) and MIP-3α (dashed bars) induction in 10^6 THP-1 cells stimulated with highly purified LOS or LPS (0.56 pmol lipid A/ml) for 18 h is shown. Error bars represent standard deviation from the average of four readings. The figure is representative of six independent experiments.

**Strategies of cellular and molecular signaling events** were first assessed in THP-1 cells (10^6 cells/ml) constitutively expressing TLR4/MD-2 (Fig. 1 and 2). THP-1 cells were stimulated with highly purified endotoxins from N. meningitidis, E. coli 55:B5, Serratia marcescens, Klebsiella pneumoniae, and Pseudomonas aeruginosa LPSs. *P < 0.001. TNF-α (solid bars) and MIP-3α (dashed bars) induction in 10^6 THP-1 cells stimulated with highly purified LOS or LPS (0.56 pmol lipid A/ml) for 18 h is shown. Error bars represent standard deviation from the average of four readings. The figure is representative of six independent experiments. 

**RESULTS**

**Differential agonist activity of endotoxins.** The agonist activity of endotoxins for the MyD88-dependent and -independent signaling pathways was first assessed in THP-1 cells (10^6 cells/ml) constitutively expressing TLR4/MD-2 (Fig. 1 and 2). THP-1 cells were stimulated with highly purified endotoxins from N. meningitidis, E. coli 55:B5, Serratia marcescens, Klebsiella pneumoniae, Salmonella enterica serovar Typhimurium, Salmonella enterica serovar Minnesota, Pseudomonas aeruginosa, and V. cholerae. Cytokine (TNF-α, IL-1β, IP-10, and IFN-β) chemokine (MCP-1 and MIP-3α), and nitric oxide induction or activity was determined. TNF-α, IL-1β, and MIP-3α release reflected MyD88-dependent pathway activation while IFN-β, IP-10, and nitric oxide reflected MyD88-independent pathway activation (42).

**N. meningitidis** endotoxin was consistently the most potent activator of the MyD88-dependent TLR4 pathway (Fig. 1). Equal molar amounts of *V. cholerae* and *E. coli* 55:B5 LPS (0.56 pmol lipid A/ml [=1 ng/ml] concentrations of lipid A) also induced high levels of TNF-α and MIP-3α. *S. marcescens, K. pneumoniae, S. enterica serovar Typhimurium, S. enterica serovar Minnesota,* and *P. aeruginosa* endotoxins induced TNF-α and MIP-3α above controls, but those levels were significantly less than with *N. meningitidis, E. coli* 55:B5, and *V. cholerae* endotoxins (*P < 0.001). IL-1β and MCP-1 profiles (data not shown) gave results similar to those obtained for TNF-α.
Compared to other endotoxins, *N. meningitidis* LOS was the most active inducer of nitric oxide via TLR4 (Fig. 2A). Nitric oxide induction was highest from RAW 264.7 macrophages stimulated directly with 0.56 pmol lipid A/ml of meningococcal endotoxin or indirectly with supernatants (containing released cytokines) harvested from macrophages stimulated with 0.56 pmol lipid A/ml *N. meningitidis* LOS (Fig. 2A). The *Salmonella* LPSs were also significant inducers of nitric oxide compared to the other LPSs (*P* < 0.001) except *N. meningitidis*. In addition, *N. meningitidis* LOS and *Salmonella* LPSs induced significantly more IP-10 compared to other endotoxins (Fig. 2B). Similar results were seen in 23ScCr macrophages stimulated with 0.56 pmol lipid A/ml of highly purified endotoxins and incubated for 18 h. IP-10 release was quantified in harvested supernatants using an ELISA. Error bars represent standard deviation from the average of four readings. Data are representative of four independent experiments.

**The role of TLR4 and MyD88 in the selective induction of signaling pathways.** Significant direct cytokine, chemokine or nitric oxide responses were not seen with TLR4-deficient cell lines (C3H/HeJ, 23ScCr, or HEK293) even at very high doses of endotoxin, but responses were restored in TLR4-MD-2 transfected HEK293 cells (data not shown), indicating that the endotoxins utilized TLR4 for MyD88-dependent and independent activation. When the TLR4 and CD14 receptors on THP-1 cells were blocked with monoclonal antibodies prior to stimulation with *N. meningitidis* LOS or *Salmonella enterica* serovar Typhimurium, *E. coli* 55:B5, and *V. cholerae* LPSs, significant decreases in TNF-α induction (*P* < 0.001) were seen as expected (data not shown). To confirm the role of MyD88 in the proinflammatory cytokine and chemokine induction, the DNMyD88 construct was transfected into THP-1 cells and used to block the MyD88-dependent pathway. The presence of DNMyD88 resulted in significant reduction (*P* < 0.0001) of TNF-α release (Fig. 3A) but did not significantly influence indirect nitric oxide release (Fig. 3B). These data indicate that TNF-α production was largely MyD88-dependent and nitric oxide production (induced selectively by *N. meningitidis* and *S. enterica* serovar Typhimurium endotoxins) was the result of MyD88-independent signaling.

**Dose- and time course-dependent differential induction of the MyD88-dependent and -independent signaling pathways.** The differential induction of the MyD88-dependent and independent pathways by endotoxin was further investigated in dose and time course assays. Human macrophage-like cell lines (THP-1, U937, and MM6) or murine macrophage RAW 264.7 cells were stimulated with a range of concentrations of the endotoxins for 1, 2, 3, 4, 5, 8, and 24 h. Supernatants from
previously stimulated cells were used to indirectly stimulate RAW 264.7 or 23ScCr (TLR4-deficient) macrophages. N. meningitidis LOS (inducer of high levels of both TNF-α, nitric oxide and IP-10), S. enterica serovar Typhimurium LPS (inducer of high levels of nitric oxide and IP-10 but modest TNF-α and other proinflammatory cytokines), and E. coli 055:B5 and V. cholerae LPS (inducers of TNF-α and other proinflammatory cytokines but low levels of nitric oxide and IP-10) were studied in detail in these experiments.

N. meningitidis LOS, E. coli 55:B5, and V. cholerae LPS at 10 to 0.0046 pmol lipid A/ml concentrations induced TNF-α release in a dose-dependent manner (Fig. 4A). In contrast, S. enterica serovar Typhimurium LPS was a weak inducer of TNF-α even at high picomolar concentrations (significant differences, P < 0.006, from 0.156 pmol to 10 pmol lipid A/ml). N. meningitidis LOS and S. enterica serovar Typhimurium LPS induced high levels of nitric oxide release directly from RAW 264.7 cells (Fig. 4B) or indirectly with low concentrations of supernatants from previously stimulated THP-1 cells (Fig. 4C).

In comparison to N. meningitidis LOS and S. enterica serovar Typhimurium LPS, E. coli 55:B5 and V. cholerae LPS were poor inducers of direct nitric oxide release at concentrations below 5 pmol (V. cholerae) and 1.25 pmol (E. coli 55:B5). The amount of E. coli 55:B5 LPS required to directly induce equivalent nitrite (50 μM) was 34 to 74-fold more (range seen in nine different experiments) and the amount of lipid A of V. cholerae LPS required was 41 to 136-fold more.

High concentrations of supernatants from E. coli 55:B5 and V. cholerae LPS stimulated cells were required to indirectly induce nitric oxide (Fig. 4C).

In time course experiments, N. meningitidis LOS induced TNF-α release over 3 h (Figs. 5A). E. coli 55:B5 LPS and V. cholerae LPS also rapidly induced TNF-α within 3 h (Fig. 5A) but the supernatants of these activated cells induced less (P < 0.004) nitric oxide at all time points compared to supernatants from cells stimulated with N. meningitidis or S. enterica serovar Typhimurium LPS (Fig. 5B). S. enterica serovar Typhimurium LPS generated minimal TNF-α at all time points (Fig. 5A), but the supernatants of these cells induced maximal concentrations of nitric oxide (Fig. 5B).

For both dose-dependent and time course assays, similar results were obtained in indirect assays when TLR4-deficient macrophages 23ScCr cells were induced with supernatants from TLR4−/− cells previously stimulated with LPS to eliminate the contribution of residual LPS. Similar results were also obtained when U937 or MM6 macrophages were used (data not shown). These data indicate that at identical picomolar concentrations of lipid A, N. meningitidis LOS was a potent agonist of both the MyD88-dependent and -independent signaling pathways. Salmonella LPS selectively induced the MyD88-independent pathway, while E. coli 55:B5 and V. cholerae LPS induced the MyD88-dependent pathway. Further, a
FIG. 4. Dose-dependent induction of TNF-α and nitric oxide release by endotoxin. A: TNF-α induction from THP-1 cells stimulated with serial fold dilutions of N. meningitidis LOS and S. enterica serovar Typhimurium, E. coli 55:B5 and V. cholerae LPSs (10–0.0046 pmol/ml) and incubated for 18 h. B: Direct nitric oxide induction from RAW 264.7 cells stimulated with serial fold dilutions of endotoxins (10–0.0046 pmol lipid A/ml) and incubated for 18 h. C: Indirect nitric oxide induction in RAW 264.7 cells stimulated with serial fold dilutions of supernatants (50 μl to 0.046 μl/well) harvested from THP-1 cells previously stimulated with 0.56 pmol lipid A/ml LPS for 2 h. Error bars represent the ± standard deviation from the average of 4 readings. *, P values indicate significant differences compared to N. meningitidis LOS. This figure is representative of six independent experiments. N. meningitidis LOS, diamonds; S. enterica serovar Typhimurium LPS, squares; E. coli 55:B5 LPS, triangles; V. cholerae LPS, crossed squares.
soluble mediator other than endotoxin was present in supernatants from endotoxin-exposed cells and this mediator induced indirect nitric oxide release.

The role of IFN-β in the differential induction of the MyD88-independent (TRIF) pathway. Induction of the MyD88-independent pathway is postulated to act through IFN-β (11). Exogenous IFN-β when added to RAW 264.7 or 23ScCr macrophages induced nitric oxide in a dose-dependent manner (Fig. 6A). When the IFN-α/β receptor was blocked with IFN-α/β receptor 1 antibody, a significant reduction (P < 0.0001) in nitric oxide release was observed (Fig. 6B). In addition, anti-IFN-β polyclonal antibody when added to RAW 264.7 cells directly stimulated with N. meningitidis LOS at concentrations of 1 to 0.031 pmol lipid A/ml significantly reduced (P < 0.0005) nitric oxide induction (Fig. 6C). No decrease in nitric oxide release was seen when isotype control antibodies for human anti-IFN-α/β receptor 1 or mouse anti-IFN-β polyclonal antibodies were used (data not shown). When RAW 264.7 macrophages induced with 1 pmol LOS nitric oxide release was 66.2 ± 1.94 µM nitrite, but in the presence of 2, 4, or 6 µg/ml of anti-IFN-β polyclonal antibody, a significant decrease in nitric oxide release (47.8 ± 1.38, 39.8 ± 1.91, and 30.1 ± 2.4 µM nitrite, respectively) was observed. Similar results were seen when anti-IFN-β polyclonal antibody was added to 23ScCr cells and stimulated with supernatants from previously induced TLR4+/− cells (data not shown). Further, anti-IFN-β and anti-IFN-α polyclonal antibodies neutralized the effects of induced IFN-β and significantly reduced nitric oxide release (Fig. 6D). These data support a role for IFN-β release in the differential induction of the MyD88-independent pathway and nitric oxide release. The differential activation of the MyD88-independent pathway by N. meningitidis LOS and Salmonella LPS was likely due to enhancement of IFN-α/β induction downstream of TLR4 signaling and likely reflects an autocrine/paracrine amplification via the IFN-α/β receptor and STAT1 pathway activation (31).

**DISCUSSION**

Ligand (e.g., endotoxin)-triggered activation of TLRs involves cytoplasmic TIR domain-containing adaptor proteins that are essential for signal transduction. These TIR domain-containing adaptors (MyD88, Mal/TIRAP, TRIF, TRAM, and...
possibly others) provide specificity for the individual TLR-mediated signaling pathways (2, 41). For example, TLR2, -5, and -9 ligands utilize the MyD88-dependent pathway, while TLR3 activation is MyD88-independent. TLR2 and TLR4 MyD88-dependent activation requires Mal/TIRAP (33), TLR4-mediated MyD88-independent signaling requires TRIF and TRAM (11, 48), and TLR3 does not utilize MyD88 or TRAM but requires TRIF (3, 8).

Specifically, TLR4 activation of the MyD88-dependent pathway recruits MyD88 and Mal/TIRAP to the TLR4-TIR domain resulting in the activation of TRAF6, early phase activation of NF-κB and mitogen-activated protein kinase and the subsequent induction of proinflammatory cytokines such as TNF-α (18). TLR4-mediated activation of the MyD88-independent signaling recruits TRIF and TRAM that activate IRF3 and IRF7. This results in the rapid production of IFN-β and consequently IFN-α and other interferon-inducible genes such as IP-10 and nitric oxide and the late phase activation of NF-κB and mitogen-activated protein kinases (11).

In this study, differential induction of the TLR4-MD-2 MyD88-dependent and -independent signaling pathways with pathophysiologically important concentrations of endotoxin was demonstrated. TLR4 was required and N. meningitidis LOS was the most potent agonist of both the TLR4-MyD88-dependent and -independent signaling pathways. At equal molar lipid A concentrations, E. coli 55:B5 and V. cholerae LPS induced the MyD88-dependent pathway and released proinflammatory cytokines and chemokines such as TNF-α and MIP3α but induced significantly less IFN-β, IP-10, or nitric oxide compared to equal concentrations of meningococcal LOS. In addition, cytokines released in supernatants via the MyD88-dependent pathway by E. coli 55:B5 and V. cholerae LPSs induced significantly less nitric oxide as shown by the indirect induction experiments. In contrast, Salmonella LPS induced the MyD88-independent signaling pathway mediators IFN-β, IP-10, and nitric oxide but low amounts of the proinflammatory cytokines such as TNF-α. The differential activation of the MyD88-independent pathway was driven by IFN-α/β rather than the major proinflammatory cytokine TNF-α.

The differences in induction of MyD88-dependent and -independent signaling by endotoxin are suggested that the initial interaction of endotoxin with the extracellular domain of the TLR4 complex results in changes that lead to the recruitment of different adaptor proteins. Differences in the affinity of endotoxin binding to TLR4 and or MD-2 (40, 43) and resulting different conformational changes may create more or less binding sites for specific adaptor proteins on the TIR domain of TLR4, modulating the signaling pathway. Alternatively, the number of TLR4-MD-2 molecules aggregated by endotoxins may be different and lead to differences in adaptor protein binding to the cytoplasmic domain of TLR4.

The differential induction of MyD88-dependent and -independent signaling pathways is illustrated in FIG. 6. Induction of nitric oxide by endotoxin was IFN-β mediated. A: Nitric oxide induction in RAW 264.7 cells stimulated with two fold dilutions (3–0.19 ng/ml) of exogenous IFN-β. B: Nitric oxide induction in RAW 264.7 cells stimulated with 50 μl supernatants harvested from THP-1 cells (10⁶) blocked with 1.5, or 10 μg of anti-IFN-α/β receptor 1 polyclonal antibody and stimulated with 10 pmol/ml of N. meningitidis lipid A. Controls were supernatants (50 μl) from unstimulated THP-1 cells, unstimulated RAW 264.7 cells, and lipid A alone. C: Direct nitric oxide induction in RAW 264.7 cells stimulated with decreasing doses of wild-type N. meningitidis LOS in the presence or absence of murine anti-IFN-β polyclonal antibody (2 μg/ml) and THP-1 cell supernatants stimulated with 10 pmol/ml of N. meningitidis lipid A. Anti-IFN-α (2 μg/ml) or anti-IFN-β (2 μg/ml) or both antibodies were added directly into RAW 264.7 cells. Error bars represent the standard deviation from the average of four readings. This experiment figure is representative of three independent experiments.
pendent signaling by endotoxins are likely due to differences in lipid A fatty acyl structure or conformation and structure of the lipid A head groups. The number and length of the fatty acyl chains, the phosphorylation of the lipid A, and the linkage to ketodeoxyoctulosonic acid (KDO) are known to influence biological activity by changing the supramolecular conformation of lipid A (38–40, 43). A bisphosphorylated, C12 and C14, hexa-acylated lipid A structure linked to KDO is the most bioactive when the MyD88-dependent cytokine TNF-α is the measure of bioactivity (5, 6, 43).

In agreement with these data, *N. meningitidis* LOS, *E. coli* 55:B5, and *V. cholerae* LPS which are hexa-acylated with C12 and C14 chain length induced significantly more TNF-α than hepta-acylated *Salmonella* and *Klebsiella* LPS or the penta-acylated *P. aeruginosa* LPS. Differences in lipid A structure also influence the binding affinity to the TLR4-MD-2 receptor complex (40, 43) and, as shown here, modulate the MyD88-independent (TRIF) signaling pathway. Interestingly, the hexa-acylated lipid A structure of *N. meningitidis* LOS and the hepta-acylated lipid A structure of *Salmonella* LPS both dramatically activate the MyD88-independent (TRIF) pathway. Preliminary fluorescence resonance energy transfer measurements data indicate that *N. meningitidis* LOS and *Salmonella* LPS clustered or recruited significantly more TRIF adaptor proteins in lipid rafts of THP-1 cell membrane compared to *E. coli* 55:B5 and *V. cholerae* (personal communication, Kathy Triantafilou, Sussex University, United Kingdom).

The differential induction by the endotoxins was not due to impurities of the endotoxin preparations, as the effects were only seen when TLR4 was present or functional. These highly purified preparations were free of protein, contaminating phospholipids, DNA or RNA and were carefully standardized based on the same number of lipid A molecules. The different effects of the same concentration of the *Salmonella* endotoxins on the MyD88-dependent and -independent signaling pathways in the assays further support these conclusions. Lipid A extracted by acid hydrolysis is a weaker agonist (28) compared to unhydrolyzed LPS in activating the TLR4-MyD88-dependent and -independent signaling pathways, and thus LPS was used in this study to reflect the differential induction of TLR4. Moreover, meningococcal KDO-lipid A was the minimal structure required for optimal TLR4 complex activation (49). In support, a deep rough *Salmonella enterica* serovar Minnesota Re595 LPS (KDO₂-lipid A) used in this study was compared to a different *S. enterica* serovar Minnesota Re595 LPS preparation (gift from Frank Neumann, Bioaxxus, United Kingdom) and to *Salmonella enterica* serovar Typhimurium Ra LPS, which consists of complete core sugars. All *Salmonella* LPSs induced similar large amounts of nitric oxide but little TNF-α. Thus, the length and structure of oligosaccharide chains beyond KDO₂ had no significant effect on activating macrophages.

Differential induction of the TLR4-MyD88-dependent and -independent signaling pathways is supported by earlier in vivo and in vitro studies (29, 30). Netea et al. found that TNF knockout mice were as susceptible as wild type controls to *S. enterica* serovar Typhimurium LPS, but they were significantly more resistant to lethal endotoxemia induced by *E. coli* 055:B5 or *K. pneumoniae* LPS (29, 30). The studies also demonstrated a delayed cytokine response in TNF knockout mice challenged with *S. enterica* serovar Typhimurium LPS but not in mice challenged with *E. coli* LPS (29). In their mouse model, the lethal effects of *S. enterica* serovar Typhimurium LPS were mediated through cytokines such as IFN-γ and IL-18. Mathiak et al. (25) found lipopolysaccharides from different bacterial sources elicit disparate effects on cytokine responses in whole blood assays. Further, *S. enterica* serovar Typhimurium expressing a mutant lipid A (lacking a secondary fatty acyl chain) has been shown to induce less TNF-α, IL-1β, inducible nitric oxygen species than wild-type *S. enterica* serovar Typhimurium (45). However, both the wild-type and the mutant *Salmonella* strains were able to induce murine dendritic cell maturation in a multiplicity of infection-independent manner (21).

Hoebe et al. demonstrated that LPS-TLR4 dendritic cell maturation and upregulation of costimulatory molecules is TRIF-IFN-β axis dependent (16). Studies in TRIF-deficient mice indicate that TRIF is not directly involved in the LPS-induced activation of the MyD88-dependent signaling, however, TRIF-deficient mice are impaired in LPS-induced inflammatory cytokine production (47). MyD88-deficient mice demonstrated late activation of NF-κB and mitogen-activated protein kinases but no impairment in dendritic cell activation or maturation (9, 20). Interaction of the MyD88-dependent and -independent signaling is required for the overall TLR4-mediated inflammatory cytokine response induced by endotoxin (41).

IFN-α/β release appears to be a key molecule in the differential induction of the MyD88-independent (TRIF) signaling pathway by *N. meningitidis* LOS and *Salmonella* LPS. Polyclonal antibodies to IFN-α and IFN-β and the blocking of the IFN-α/β receptor with anti IFN-α/β receptor 1 antibody markedly decreased direct and indirect nitric oxide induction. Further, when MyD88-dependent signaling was blocked using DNMd88, significant reduction in TNF-α release was seen (7), but no effect on nitric oxide release was observed. These data suggest that IFN-α/β was responsible for the differential induction of the MyD88-independent (TRIF) signaling pathway by *N. meningitidis* and *Salmonella* endotoxins, and is in agreement with previous reports showing TLR4 mediated STAT1 phosphorylation was abolished in IFN-α/β receptor 1-deficient mice (8), and nitric oxide response was impaired in STAT1-deficient mice (8, 31).

In conclusion, *Salmonella* LPSs were found to enhance the activation of the TLR4-MyD88-independent pathway, while with *E. coli* 55:B5 and *V. cholerae* LPSs, the TLR4-MyD88-dependent pathway was dominant. *N. meningitidis* LOS in equal molar lipid A concentrations was a potent inducer of both pathways. The differential induction of the TLR4-MyD88-independent pathway was IFN-β-mediated. The different activities of these endotoxins may be influenced by affinity of binding to LBP, CD14, or MD-2 (upstream TLR4 signaling), differences in recruitment of TIR adaptor proteins and activation of the IFN-α/β transcription factors like IRF-3 and IRF-7 or other downstream TLR4 signaling molecules such as TNF-receptor-associated factor 6 (TRAF6), IL-1 receptor-associated kinases, transforming growth factor beta-activated kinase (TAK1), and TAK1-binding protein (TAB1), possibilities that are under current investigation. The contribution of the recruitment of other receptors or unknown adaptor proteins in the differential induction of the
TLR4 signaling pathways is also an explanation for the results. Triantafillou and Triantafillou (45) have identified a receptor signaling complex for LPS including TLR4, the integrins CD11c and/or CD18, as well as CD55, HSP70 and -90, and CXCR4, and growth differentiation factor 5. However, the contribution of coreceptors is dependent upon TLR4, since no responses to endotoxin are seen in the absence of TLR4. Differences in MyD88-dependent and –independent signaling by endotoxins may help explain different clinical features of bacteremia caused by gram-negative organisms and differences in immune responses, and may prove useful in the design of adjuvants for vaccines.

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