

Bacterial Lipopolysaccharide Activates Nuclear Factor- κ B through Interleukin-1 Signaling Mediators in Cultured Human Dermal Endothelial Cells and Mononuclear Phagocytes*

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Bacterial lipopolysaccharide (LPS)-mediated immune responses, including activation of monocytes, macrophages, and endothelial cells, play an important role in the pathogenesis of Gram-negative bacteria-induced sepsis syndrome. Activation of NF- κ B is thought to be required for cytokine release from LPS-responsive cells, a critical step for endotoxic effects. Here we investigated the role and involvement of interleukin-1 (IL-1) and tumor necrosis factor (TNF- α) signal transducer molecules in LPS signaling in human dermal microvessel endothelial cells (HDMEC) and THP-1 monocytic cells. LPS stimulation of HDMEC and THP-1 cells initiated an IL-1 receptor-like NF- κ B signaling cascade. In transient cotransfection experiments, dominant negative mutants of the IL-1 signaling pathway, including MyD88, IRAK, IRAK2, and TRAF6 inhibited both IL-1- and LPS-induced NF- κ B-luciferase activity. LPS-induced NF- κ B activation was not inhibited by a dominant negative mutant of TRAF2 that is involved in TNF signaling. LPS-induced activation of NF- κ B-responsive reporter gene was not inhibited by IL-1 receptor antagonist. TLR2 and TLR4 were expressed on the cell surface of HDMEC and THP-1 cells. These findings suggest that a signal transduction molecule in the LPS receptor complex may belong to the IL-1 receptor/toll-like receptor (TLR) super family, and the LPS signaling cascade uses an analogous molecular framework for signaling as IL-1 in mononuclear phagocytes and endothelial cells.

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Lipopolysaccharide (LPS),¹ or endotoxin, is the major component of the outer surface of Gram-negative bacteria. LPS is a potent activator of cells of the immune and inflammatory systems, including macrophages, monocytes, and endothelial cells, and contributes to systemic changes known as septic shock (1, 2). LPS-induced activation of monocytes/macrophages is mediated through a cell surface receptor glycoprotein, known as membrane CD14 (mCD14). The binding of LPS to mCD14 is enhanced by LPS-binding protein, a plasma protein (3). On the other hand, vascular endothelial cells do not express mCD14 and respond to LPS only in the presence of soluble CD14 (4–6).

We (7) and others (8–12) have shown that protein tyrosine phosphorylation and activation of ERK1, ERK2, p38 mitogen-activated protein kinase, and c-Jun N-terminal kinase appear to be important for LPS-induced cellular activation. LPS rapidly induces nuclear factor- κ B (NF- κ B) in both monocytic (13, 14) and endothelial cells (15). Activation of NF- κ B is required for release of proinflammatory cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF) (16, 17). However, the molecular mechanisms of the signaling cascade induced by LPS to activate NF- κ B are unknown. Furthermore, the signaling LPS receptor is still unidentified.

The toll gene controls dorsoventral pattern formation during the early embryonic development of *Drosophila melanogaster* (18). Toll initiates a signaling pathway homologous to the mammalian NF- κ B activation cascade (18). The toll family of receptors is defined by homology to the *Drosophila* toll protein (19, 20). The mammalian IL-1 receptor is a member of the toll family (18). Five other mammalian family members (toll-like receptors 1 through 5, TLR1–5) have been identified, but their function is uncertain. Several TLRs, similar to IL-1R, have been observed to signal through the NF- κ B pathway (19–22). LPS signaling also leads to activation of NF- κ B, and recent studies suggested that a toll-like receptor (TLR) might be a signaling receptor that is activated by LPS (22, 23). In these reports, expression of TLR2 in LPS-unresponsive human embryonic kidney cells (293 cells) enabled these cells to respond to LPS stimulation (22, 23). These investigators observed that LPS binds to a TLR2 extracellular domain and suggested that TLR2 is a candidate for a long sought LPS receptor, although the data were generated from a transfected and normally LPS-unresponsive cell line (22, 23). A recent study in the LPS-resistant C3H-HeJ mice has implicated another toll homologue (TLR4) as a signal-transducing component in the LPS receptor complex (24).

It is known that the IL-1 signaling pathway in mammals is strikingly similar to the toll signaling pathway in *Drosophila* (19–22). The molecular events linking the IL-1 receptor (IL-1R) signaling complex to the induction of NF- κ B have been recently characterized. Upon binding of IL-1 to its receptors (IL-1R), IL-1R associates with the IL-1 receptor accessory protein (IL-1RAcP) (26, 27). The complex then recruits and activates an

¹ The abbreviations used are: LPS, lipopolysaccharide; HDMEC, human dermal microvessel endothelial cells; IL-1, interleukin-1; IL-1R, IL-1 receptor; IL-1RAcP, IL-1R accessory protein; IL-6, interleukin-6; IRAK, IL-1 receptor-associated kinase; MyD88, myeloid differentiation protein; NF- κ B, nuclear factor- κ B; NIK, NF- κ B-inducing kinase; RT-PCR, reverse transcription-polymerase chain reaction; TLR, toll-like receptor; TNF, tumor necrosis factor; TRAF, tumor necrosis factor receptor-associated factor; PAGE, polyacrylamide gel electrophoresis.

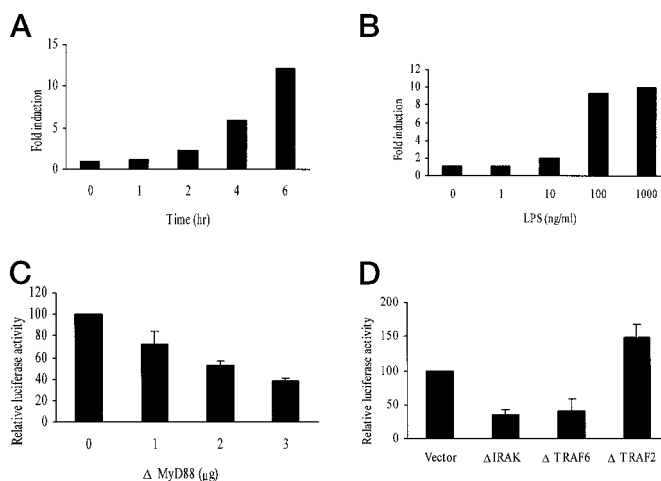


FIG. 1. Dominant negative versions of MyD88 and TRAF6 but not TRAF2 inhibited LPS-induced NF- κ B activity in human THP-1 cells. Human THP-1 cells were transiently transfected with NF- κ B-luciferase and β -galactosidase reporter vectors and dominant negative mutants of MyD88, TRAF6, and TRAF2 for 24 h. The total amount of DNA was kept constant with pcDNA3 vector. The cells were then stimulated with LPS (100 ng/ml) for different periods of time (A) or increasing amounts of LPS for 6 h (B). Some cells were transfected with different amounts of MyD88 mutants and stimulated with 100 ng/ml LPS for 6 h (C). Cells were transfected with TRAF2 and TRAF6 mutants and then stimulated with 100 ng/ml LPS for 6 h (D). Luciferase and β -galactosidase assays were performed as described under "Experimental Procedures." The time and dose response experiments (A and B) are one representative of two independent experiments with similar qualitative results. C and D were graphed with means and standard deviations of three or more experiments.

adapter protein myeloid differentiation protein (MyD88) (28, 29). MyD88 in turn recruits two distinct putative serine-threonine kinases, namely IL-1 receptor-activated kinase (IRAK) and IRAK2, to the receptor complex (30). IRAK and IRAK2 interact subsequently with the adapter molecule, TNF receptor-associated factor 6 (TRAF6) (31, 32), which links them to the protein kinase NF- κ B-inducing kinase (NIK) (33). NIK activates the I κ B kinase complex (IKK α and IKK β) that directly phosphorylates I κ B (34–36). The phosphorylation of I κ B initiates ubiquitin-proteasome-mediated degradation and liberates and activates NF- κ B (16, 17). On the other hand, TNF and its receptor complex activate TRAF2, but not TRAF6 (37, 38). Downstream signaling pathways of IL-1 and TNF distal to TRAF6 and TRAF2 converge (16, 17).

In this study, we tested the hypothesis that LPS activates NF- κ B through IL-1 signaling molecules, namely MyD88, IRAK, IRAK2, and TRAF6 in two LPS-responsive cell types. Dominant negative mutants of these signaling elements were transfected into human monocytes (THP-1 cells) and human dermal endothelial cells together with a NF- κ B-responsive reporter gene, and LPS-induced NF- κ B luciferase activity was measured. Our results indicate that LPS transduces signals of NF- κ B activation utilizing the IL-1 signaling molecules in both monocytic and endothelial cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Human THP-1 cells (from ATCC) were cultured in RPMI medium with 10% fetal calf serum. The immortalized human dermal endothelial cells (generous gift of Dr. Candal of the Center for Disease Control, Atlanta) were cultured in MCDB-131 medium with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 100 μ g/ml penicillin and streptomycin in 6-well plates.

Expression Vectors and Transfection of THP-1 Cells—Dominant negative expression vectors of MyD88, IRAK, IRAK2, TRAF2, TRAF6, and NIK have been characterized and described before (30, 32, 33). Cells were used for transfection with FuGene 6 Transfection Reagent (Boehringer Mannheim) following the manufacturer's instructions in RPMI

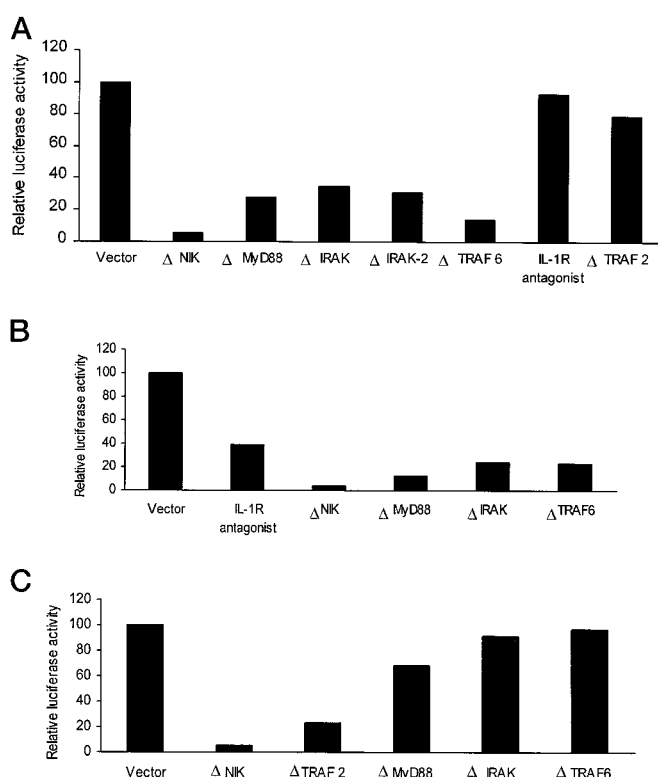


FIG. 2. Dominant negative versions of MyD88, IRAK, IRAK2, TRAF6, and NIK but not TRAF2 inhibited LPS-induced NF- κ B activity in HDMEC. Human dermal endothelial cells grown on 6-well plates were transfected with dominant negative mutants of MyD88, IRAK, IRAK2, TRAF6, TRAF2, and NIK as well as reporter genes for 24 h. The amount of total DNA was kept constant with pcDNA3 vector. The cells were treated with 100 ng/ml LPS (A), 400 units/ml human IL-1 β (B), 200 units/ml human TNF- α (C) for 6 h. Some cells were also treated with 100 ng/ml IL-1 receptor antagonist for 6 h (A and B). NF- κ B luciferase activity was obtained with luciferase assay and normalized with β -galactosidase activity. Data shown are one representative experiment of two experiments with similar qualitative results.

with 10% serum. Reporter genes pCMV- β -galactosidase (0.5 μ g) and ELAM-NF- κ B-luciferase (2 μ g) and pcDNA3 empty vector or dominant negative mutants of MyD88, IRAK, TRAF6, TRAF2, and NIK (3 μ g each) were used. After a 24-h transfection, cells were stimulated for 6 h with 100 ng/ml LPS. Cells were then lysed, and luciferase activity was measured with a Promega kit (Promega, Madison, WI) and a luminometer. β -Galactosidase activity was determined by the calorimetric method to normalize transfection efficiency as described earlier (30). Data shown are means of two independent experiments.

Transfection of Human Dermal Endothelial Cells—Transfection was carried out using the same method described above. The amount of NF- κ B luciferase construct DNA was 1.5 μ g, and empty vector and various dominant negative constructs were 2 μ g each. Cells were transfected for 24 h and stimulated for 6 h with 100 ng/ml LPS, human TNF- α (200 units/ml, Genzyme, Cambridge, MA), recombinant human IL-1 β (400 units/ml, Genzyme), and recombinant IL-1 receptor antagonist (100 ng/ml, R&D Systems, Minneapolis, MN) in 2 ml of serum-containing MCDB-131 medium.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis—Total RNA was isolated from HDMEC and THP-1 cells using a Qiagen kit (Valencia, CA) and treated with RNase-free DNase I. For RT reaction, the SuperScriptTM Preamplification system (Life Technologies, Inc.) was applied. PCR amplification was performed with Taq polymerase (Qiagen, Valencia, CA) for 28 cycles at 95 $^{\circ}$ C for 40 s, 54 $^{\circ}$ C for 40 s, and 72 $^{\circ}$ C for 1 min. PCR primers for TLR2 were 5'-GC-CAAAGTCTTGATTGATTGG and 5'-TTGAAGTTCTCCAGTCCTG. PCR primers for TLR4 were 5'-TGGATACGTTTCCTTATAAG and 5'-GAAATGGAGGCACCCCTTC. GAPDH primers were obtained from CLONTECH.

Immunostaining and Immunoblotting—Smeared THP-1 cell and cultured HDMEC on slides were fixed with acetone for 5 min and then stained with rabbit anti-TLR2 and TLR-4 antibody (1:100) and rabbit

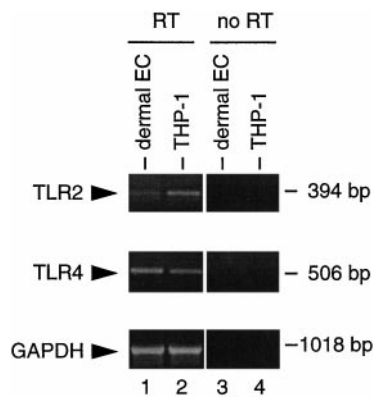


FIG. 3. Expression of TLR2 and TLR4 in HDMEC and THP-1 cells. Expression of TLR2 mRNA (upper panel, 347 base pairs) and TLR4 mRNA (middle panel, 548 base pairs) in HDMEC (lanes 1 and 3) and THP-1 cells (lanes 2 and 4) was analyzed by PCR following reverse transcription (RT; lanes 1 and 2) or without RT (lanes 3 and 4). RT-PCR analysis of GAPDH expression was used as control (lower panel, 983 base pairs). Labels of base pairs at the right are DNA standard markers.

IgG following the instructions on a DACO immunostaining kit. The anti-TLR2 and anti-TLR4 antisera were raised against synthetic peptides (extracellular domains of TLR2 and TLR4) by BABCO (Richmond, CA). The sequence of the synthetic peptide for TLR2 was a 27-amino acid peptide, starting at amino acid residue 277 of the mature hTLR2 (FRASDNDVRVIDPGKVETLTIRRLHIPR), whereas the peptide for TLR4 was a 23-amino acid peptide, starting at amino acid 201 of mature hTLR4 (FKEIRHKLTLRNNFDLSLNVMTK). Following immunoperoxidase staining, the representative fields were photographed.

THP-1 and HDMEC cells were lysed in Laemmli buffer and separated with a 10% SDS-PAGE gel. The protein was then transferred onto a polyvinylidene difluoride membrane, and then the membrane was probed with anti-TLR2, anti-TLR4 antibodies, and prebleeds corresponding to each antibody (1:2,000). After incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody, the membrane was developed with an enhanced chemiluminescence ECL detection kit.

RESULTS AND DISCUSSION

A series of defense mechanisms are triggered in vertebrates and invertebrates in response to Gram-negative bacterial infections by sensing the presence of LPS (1–3). LPS-induced signal relay is thought to be initiated following its binding to specific cellular receptors which then triggers intracellular signaling pathways leading to the activation of NF- κ B (4–12) in various LPS-responsive cell types. To date, the identification of a functional, signal-transducing component of the putative LPS receptor complex and the signaling pathways involved in LPS-induced activation of NF- κ B have remained elusive. Recent findings suggested that LPS might use signaling molecules of the TLR and IL-1R superfamilies to transduce signals (23, 24).

To investigate the potential involvement of IL-1 and TNF signal transducers in LPS signaling in two LPS-responsive cell types, HDMEC and THP-1 cells, we cotransfected dominant negative constructs of various components of the NF- κ B signaling cascades for IL-1 and TNF together with NF- κ B-luciferase reporter gene. LPS induced the activation of NF- κ B in a time- (Fig. 1A) and dose-dependent (Fig. 1B) manner in THP-1 cells. Activation of NF- κ B reached a maximum at a LPS concentration of 100 ng/ml and when cells were stimulated with LPS for 6 h (Fig. 1, A and B). Similar results were also obtained from endothelial cells.

A mutant version of MyD88 (Δ MyD88), encoding only for the COOH-terminal toll-IL-1R-like domain, which abrogates IL-1R-induced NF- κ B activation (30), inhibited both LPS- and IL-1-mediated NF- κ B activation (Figs. 1C and 2, A and B) but not TNF-induced NF- κ B activation (Fig. 2C). IRAK and IRAK-2 are two additional proximal mediators of the IL-1R

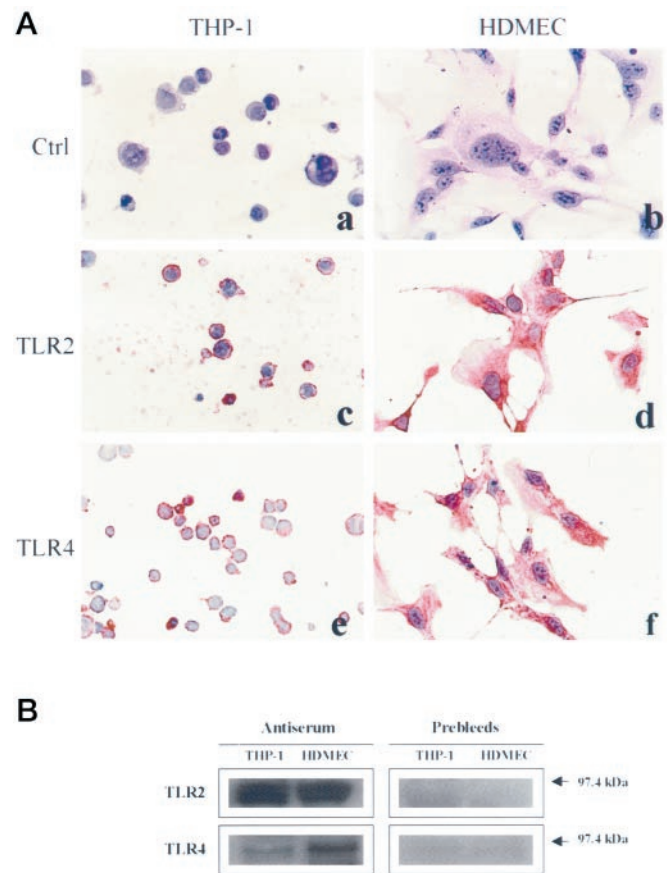


FIG. 4. Immunostaining and immunoblotting. In panel A, THP-1 cells (a, c, and e) and HDMEC cells (b, d, and f) were immunostained with rabbit IgG (a and b), anti-TLR2 (c and d), and anti-TLR4 (e and f) (1:100). In panel B, THP-1 and HDMEC cell lysates were resolved with SDS-PAGE and probed with anti-TLR2, anti-TLR4 antibodies, and corresponding prebleeds. Molecular mass markers are indicated at the right. The apparent molecular masses for TLR2 and TLR4 were 85 kDa and 92 kDa, respectively.

signaling complex (30). Dominant negative constructs of IRAK (Δ IRAK) and IRAK2 (Δ IRAK2) inhibited both LPS- (Figs. 1D and 2A) and IL-1R-mediated NF- κ B activation (Fig. 2B), but not TNF-induced NF- κ B activation (Fig. 2C).

NF- κ B activation induced by various cytokine receptors is mediated by members of the TRAF adapter family. While TRAF2 plays a crucial role in NF- κ B activation by TNFR-1 and TNFR-2 (37, 38), TRAF6 has been implicated in IL-1 signaling (31, 32). Therefore, we next determined whether dominant negative versions of TRAF6 (Δ TRAF6) or TRAF2 (Δ TRAF2) could act to inhibit LPS-induced NF- κ B activity. Δ TRAF-6 but not Δ TRAF2 significantly impaired LPS-induced NF- κ B activation, suggesting that TRAF6 may act as an additional downstream mediator of LPS-induced NF- κ B activation cascade (Figs. 1D and 2A). Δ TRAF2 blocked TNF-induced NF- κ B activation in endothelial cells (Fig. 2C), but not LPS-induced NF- κ B activation (Figs. 1D and 2A). Because the pathways for IL-1 and TNF- α signaling converge at the level of NIK for NF- κ B activation, we next investigated whether dominant negative NIK mutant (Δ NIK) would block LPS-induced, as well as IL-1- and TNF-induced, NF- κ B activation. As expected, Δ NIK blocked NF- κ B activation induced by LPS, IL-1, and TNF- α (Fig. 2).

IL-1 receptor antagonist had no effect on LPS-induced NF- κ B activation (Fig. 2A) but inhibited IL-1-induced NF- κ B activation in endothelial cells (Fig. 2B). This observation suggests that NF- κ B activation that we measured following 6 h of

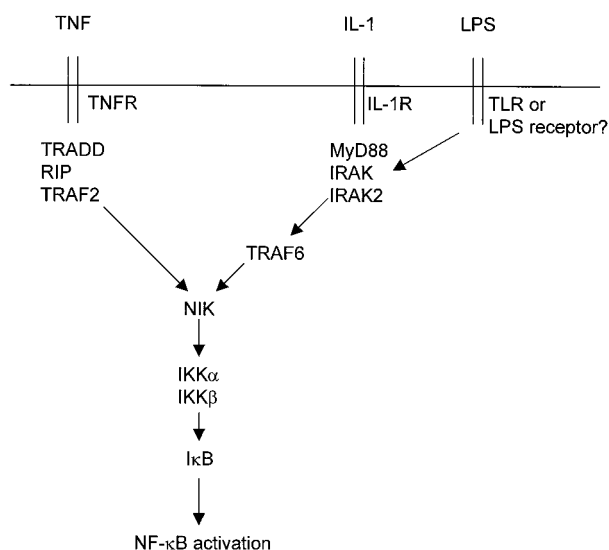


FIG. 5. **Schematic diagram of signaling cascades for LPS, IL-1, and TNF.** The diagram illustrates that LPS utilizes IL-1 signaling molecules (MyD88, IRAK, IRAK2, and TRAF6) but not TNF signaling molecules (TRAF2).

LPS stimulation of cells is not due to an autocrine effect such as LPS-induced IL-1 release from endothelial cells.

These data suggest that LPS stimulation of endothelial cells and THP-1 cells triggers an IL-1R-like signal relay leading to activation of NF- κ B (Fig. 5). Further support for this concept is provided by the observation of a 15-year-old girl with recurrent infections who was found to be resistant to both LPS and IL-1 stimulation *in vivo* and *in vitro* (39). The authors suggested that resistance to LPS and IL-1 was due to a defect very early in the common signaling pathway for LPS and IL-1 (39).

The experiments with IL-1R antagonist also suggest that LPS does not use IL-1 receptor to transduce signals in endothelial cells. Although recent findings imply that TLR2 or other members of the TLR family, which use the IL-1R signaling pathway, might be an important mediator for LPS signaling (23, 24), further studies are needed to identify naturally existing LPS receptors in LPS-responsive cells. Beutler and coworkers (25) recently reported that TLR4 is the protein encoded by the LPS gene, which is mutated in the LPS-non-responsive C3H-HeJ mice. To investigate the expression of the TLR2 and TLR4 message in HDMEC and THP-1 cells, we used RT-PCR. Human THP-1 and HDMEC were found to express significant levels of both TLR2 and TLR4 mRNA (Fig. 3). Expression levels of TLR2 appeared to be stronger in THP-1 whereas the expression level of TLR4 appeared to be stronger in HDMEC. Expression of TLR2 and TLR4 was confirmed with Northern analysis in THP-1 cells. Immunohistochemistry and immunoblotting data demonstrate that TLR2 and TLR4 proteins are expressed on THP-1 cells and HDMEC (Fig. 4). Staining was absent in THP-1 and HDMEC incubated without the first antibody or incubated with rabbit IgG. These results suggest that TLR2 and TLR4 are expressed in endothelial and monocytic cells and may represent a signaling component of a cellular receptor for LPS which signals through an IL-1-like pathway (Fig. 5).

In summary, we have demonstrated in endothelial and THP-1 cells that LPS-induced NF- κ B activation is mediated by IL-1R signaling molecules, namely MyD88, IRAK, IRAK2, and TRAF6, but not the TNF signaling molecule, TRAF2. We have also shown that TLR2 and TLR4 are expressed on the cell surface of two LPS-responsive cell types, endothelial cells and THP-1 cells. These data strongly suggest that a crucial signaling component in the LPS receptor complex may belong to the

IL-1 receptor/TLR superfamily, and the LPS signaling cascade uses an analogous molecular framework for signaling as IL-1. MyD88 appears to represent the most upstream mediator of the LPS-mediated signaling cascade, which ultimately activates NF- κ B, thus driving transcriptional activation of several cytokines. Thus, MyD88 may represent a potentially useful therapeutic target to control the molecular switch from innate to the adaptive immune response.

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