Toll-like Receptor-4 Mediates Lipopolysaccharide-induced Signal Transduction*

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TLR4 is a member of the recently identified Toll-like receptor family of proteins and has been putatively identified as Lps, the gene necessary for potent responses to lipopolysaccharide in mammals. In order to determine whether TLR4 is involved in lipopolysaccharide-induced activation of the nucleotide factor-κB (NF-κB) pathway, HEK 293 cells were transiently transfected with human TLR4 cDNA and an NF-κB-dependent luciferase reporter plasmid followed by stimulation with lipopolysaccharide/CD14 complexes. The results demonstrate that lipopolysaccharide stimulates NF-κB-mediated gene expression in cells transfected with the TLR4 gene in a dose- and time-dependent fashion. Furthermore, E5531, a lipopolysaccharide antagonist, blocked TLR4-mediated transgene activation in a dose-dependent manner (IC₅₀ ~30 nM). These data demonstrate that TLR4 is involved in lipopolysaccharide signaling and serves as a cell-surface co-receptor for CD14, leading to lipopolysaccharide-mediated NF-κB activation and subsequent cellular events.

Lipopolysaccharide (LPS),¹ a component of the outer membrane of Gram-negative bacteria, is a potent activator of a variety of mammalian cell types (1, 2). Activation by LPS constitutes the first step in a cascade of events believed to lead to the manifestation of Gram-negative sepsis, a condition that results in approximately 20,000 annual deaths in the United States (3). Activation of LPS-responsive cells, such as monocytes and macrophages, occurs rapidly after LPS interacts with circulating LPS-binding protein and CD14, a glycosylphosphatidylinositol-linked cell surface glycoprotein necessary for sensitive responses to LPS (1, 2). LPS has been shown to initiate multiple intracellular signaling events (4), including the activation of NF-κB, which ultimately leads to the synthesis and release of a number of proinflammatory mediators, including interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-α (1). However, since CD14 is not a transmembrane protein, it lacks the ability to transduce cytoplasmic signals (2), and before the recent discovery of Toll-like receptors (TLRs), the identity of a transmembrane protein that could relay LPS-induced signals across the cell-surface membrane remained elusive.

Toll is a transmembrane receptor in Drosophila that is involved in dorsal-ventral patterning in embryos and in the induction of an anti-fungal response (5, 6). Activation of the Toll receptor by its ligand Spätzle results in the interaction and stimulation of several signaling molecules that are homologous to proteins involved in NF-κB activation by the IL-1 receptor in mammalian cells (7, 8). The cloning of a family of human receptors structurally related to Drosophila Toll revealed five proteins that have extracellular domains that contain multiple leucine-rich repeats and cytoplasmic domains with sequence homology to the intracellular portion of the IL-1 receptor (9). Furthermore, constitutively active mutants of TLR2, TLR4, and TLR5 can induce the activation of NF-κB (10, 11), and the active form of TLR4 increases the expression of NF-κB-regulated genes for the inflammatory cytokines IL-1, IL-6, and IL-8 (11).

Several lines of evidence suggest that one or more members of the TLR family is the cell-surface receptor for LPS, the prototypical activator of NF-κB and other proinflammatory responses. TLR2 and TLR4 are highly expressed in cells that respond to LPS, such as peripheral blood leukocytes, macrophages, and monocytes (11, 12). Also, heterologously expressed TLR2 mediates LPS-induced NF-κB activation and IL-8 mRNA expression in HEK 293 cells (12, 13). However, TLR2 is not the only potential LPS signal transducer. The C3H/HeJ mouse is a spontaneous LPS resistant mutant. Poltorak et al. (14) mapped the Lps gene, which has been shown previously to be necessary for LPS responses in LPS nonresponder C3H/HeJ mice, to TLR4. TLR4 from the C3H/HeJ mouse has a single point mutation at amino acid 712 (Pro to His) that changes the function of the receptor dramatically (14); furthermore, the LPS-resistant C57/10ScCr mouse appears to be null for the TLR4 locus. These observations strongly support the concept that TLR4, and not TLR2, is the dominant LPS receptor in mammals and the hypothesis that TLR4 is a cell-surface component of the LPS signaling pathway. Thus, the present study was conducted to investigate whether TLR4 is involved in mediating the actions of LPS.

**Experimental Procedures**

**Materials**—Human TLR4 cDNA was provided by Dr. Charles A. Janeway, Jr. (Yale University). The ELAM-1-luciferase reporter plasmid, pELAM-luc, was generated by cloning a fragment (~241 to ~54 base pairs) of the human E-selectin promoter into the pG3 reporter plasmid (Promega, Inc.). All plasmid constructs were confirmed by automated sequencing analysis. Lipopolysaccharide was purchased from Sigma. The human embryonic kidney cell line HEK 293 (CRL-1573) was from American Type Culture Collection (Rockville, MD), and the Chinese hamster ovary (CHO) cell line expressing CD14 was engineered and maintained as described (15). The LPS antagonist (E5531) was synthesized as described previously (16). Plasmid DNA was isolated with Qiagen Endo-free™ Maxi-prep columns (Chatsworth, CA). MEM-18 anti-CD14 antibody was purchased from Accurate Chemical & Scientific Corp. (Westbury, NY). Phorbol 12-myristate 13-acetate

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TLR4 Mediates LPS-induced Signaling

To determine whether TLR4 mediates LPS-induced activation of NF-κB, HEK 293 cells were transiently transfected with TLR4 cDNA or vector DNA and an NF-κB-dependent ELAM-1-luciferase reporter plasmid (pELAM-luc). Quantitative data are presented as mean ± S.E. from three independent experiments. Means with different superscripts are significantly different from one another, p < 0.05.

**Results**

FIG. 1. LPS increases TLR4-mediated NF-κB activation via a CD-14 dependent mechanism. HEK 293 cells were plated at a density of 3 × 10⁵ cells/well in 12-well plates and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum for 24 h. Cells were transiently transfected with TLR4 cDNA or vector DNA and the ELAM-1-luciferase reporter gene described above. *Experimental Procedures.* Cells were either left untreated or stimulated with LPS (1 μg/ml), or the combination of CD14 plus LPS for 6 h. After cell lysis, luciferase activity was assayed. These data represent the mean ± S.E. from seven independent experiments. Means with different superscripts are significantly different from one another, p < 0.05.

FIG. 2. LPS increases TLR4-mediated NF-κB activation in a dose- and time-dependent manner. HEK 293 cells were transiently transfected with TLR4 cDNA or vector DNA and pELAM-luc. Cells were either left untreated or exposed to sCD14 (10 nM) and the indicated concentration of LPS for 6 h (A) or 1 μg of LPS/ml for the indicated amount of time (B) as described in the legend to Fig. 1. After cell lysis, the amount of luciferase activity in each sample was quantified. These data represent the mean ± S.E. from three independent experiments. Means with different superscripts are significantly different from one another, p < 0.05.

Purification of Soluble CD14 (sCD14) from Transfected CHO Cells—CHO cells expressing CD14 were transiently cultured in suspension under serum-free conditions (EX-CELL 301 medium supplemented with I-glutamine). The culture supernatant was collected, filtered through a 0.22-μm nitrocellulose filter, and concentrated 5-fold in a protein concentrator (Amicon Diaflo, PM30) with a 30-kDa cut-off filter under pressure at 4 °C. This concentrate was then loaded onto an anti-CD14 affinity column. The column was washed twice with wash buffer before eluting with 0.1 M glycine, pH 2.8. The fractions were immediately neutralized with 1 M Tris-HCl, pH 9.5, and an aliquot of each fraction was kept and the remainder was eluted with 0.1 M glycine, pH 2.8. The fractions were immediately neutralized with 1 M Tris-HCl, pH 9.5, and an aliquot of each fraction was kept and the remainder was eluted with 0.1 M glycine, pH 2.8. This concentrate was then loaded onto an anti-CD14 affinity column. The column was washed twice with wash buffer before eluting with 0.1 M glycine, pH 2.8. The fractions were immediately neutralized with 1 M Tris-HCl, pH 9.5, and an aliquot of each fraction was kept and the remainder was eluted with 0.1 M glycine, pH 2.8. The fractions were immediately neutralized with 1 M Tris-HCl, pH 9.5, and an aliquot of each fraction was kept and the remainder was eluted with 0.1 M glycine, pH 2.8. These data represent the mean ± S.E. from three independent experiments. Means with different superscripts are significantly different from one another, p < 0.05.

**Cell Culture and Transfections—**HEK 293 cells (ATCC, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium (ATCC, Rockville, MD) supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Cells were plated in 12-well tissue culture plates (3 × 10⁵ cells/well) and maintained in the above medium for 24 h. Cells were transfected using the CalPhos Maximizer protocol (CLONTECH) with 250 ng of TLR4 cDNA or vector DNA (pcDNA3, Invitrogen, Inc.) and 100 ng of pELAM-luc. All cells were also transfected with a β-galactosidase control plasmid for normalizing transfection efficiencies. After transfection, cells were maintained in Dulbecco's modified Eagle's medium supplemented with 1% fetal bovine serum overnight (18 h). The following day, cells were either left untreated or incubated with the indicated amount of ligand and/or compound E5531. After the indicated treatment period, cells were harvested in lysis buffer and assayed for luciferase activity per the manufacturer's protocol. The amount of luciferase activity in each sample was quantified by a Wallac 1450 MicroBetaTrilux counter.

**Purification of Soluble CD14 (sCD14) from Transfected CHO Cells—**CHO cells expressing CD14 were transiently cultured in suspension under serum-free conditions (EX-CELL 301 medium supplemented with I-glutamine). The culture supernatant was collected, filtered through a 0.22-μm nitrocellulose filter, and concentrated 5-fold in a protein concentrator (Amicon Diaflo, PM30) with a 30-kDa cut-off filter under pressure at 4 °C. This concentrate was then loaded onto an anti-CD14 affinity column. The column was washed twice with wash buffer before eluting with 0.1 M glycine, pH 2.8. The fractions were immediately neutralized with 1 M Tris-HCl, pH 9.5, and an aliquot of each fraction was mixed with 2 × SDS loading buffer (Novex, San Diego, CA) and heated for 5 min at 95 °C. Expression and purification of sCD14 was verified by SDS-polyacrylamide gel electrophoresis followed by silver staining and immunoblotting with MEM-18 anti-CD14 antibody. Immunoreactive proteins were visualized using enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech).

**Statistical Analysis—**Quantitative data are presented as mean ± S.E. and analyzed using a statistical model based on a one-way classification analysis of variance. Tests of significance for all possible comparisons were determined by Student Newman-Keuls test or unpaired t test (GraphPad Prism, version 2.0a).

**Results**

To determine whether TLR4 mediates LPS-induced activation of NF-κB, HEK 293 cells were transiently transfected with TLR4 cDNA or empty vector control (pcDNA3) and an NF-κB-dependent ELAM-1-luciferase reporter plasmid (pELAM-luc). Twenty-four hours post-transfection, cells were left untreated or incubated with either LPS (1 μg/ml or indicated concentrations), sCD14 (10 nM) or both for an additional 6 h. Cells were then lysed and assayed for luciferase activity. Expression of TLR4 in HEK 293 cells induced activation of the NF-κB reporter gene 2.5-fold above controls (cells transfected with empty vector and the pELAM-luc reporter gene) in the absence of stimuli (Fig. 1). Soluble CD14 alone did not have a significant effect on NF-κB activity in the presence or absence of TLR4. LPS treatment alone (1 μg/ml) was sufficient to elicit an increase (1.6-fold) in luciferase activity after incubation with TLR4 transfected cells, and this increase was not observed in vector controls (Fig. 1). However, when cells were stimulated with LPS in the presence of sCD14, there was a marked increase in TLR4-mediated activation of NF-κB that was not observed in cells treated with LPS or CD14 alone (Fig. 1). Stimulation of TLR4-mediated NF-κB activation by LPS plus CD14 was 5-fold higher than levels produced by TLR4 expression in unstimulated controls or with CD14 alone. LPS-induced reporter activity occurred in a dose-dependent fashion, increasing 2-fold above controls at 10 ng/ml LPS and reaching maximal levels at 1 μg/ml LPS (Fig. 2A). Furthermore, stimulation of NF-κB activity by LPS was time-dependent and reached maximal levels at 18–24 h after LPS addition (Fig. 2B).

In order to further examine whether TLR4 is involved in LPS signaling at the cell surface, HEK 293 cells were transfected with TLR4 or empty vector and the NF-κB reporter plasmid. Subsequently, cells were co-incubated with LPS...
IL-1 receptor. These properties include the activation of the transcription factor NF-κB and induction of mRNA for several proinflammatory cytokines (11), both of which were also observed when responsive cells are activated with LPS (1). Second, reports by Yang et al. (12) and Kirschning et al. (13) demonstrate the ability of TLR2 to signal in the presence of LPS and CD14, strongly suggesting a role for this protein in LPS action. Kirschning et al. (13) also reported that TLR1 and TLR4 failed to increase NF-κB reporter activity in the presence of LPS, further supporting the notion that TLR2 is a specific component of the cellular receptor for LPS. However, the recent report by Poltorak et al. (14), which identifies the genetic lesion in the LPS-resistant C3H/HeJ mice as a mutation in the tlr4 gene (14), prompted us to examine the role of TLR4 in LPS signaling.

In the experiments presented here, transfection of HEK 293 cells using a construct containing the full-length cDNA for TLR4 was sufficient to elicit a significant response with LPS in the presence of soluble CD14. The reporter construct utilized in these experiments contained a region of the promoter for the E-selectin gene, which is absolutely dependent upon NF-κB activation for activity (17). Thus, LPS-induced stimulation of our ELAM-1 reporter gene in TLR4 expressing cells is predominantly mediated via the NF-κB signaling pathway. Previous studies have shown that constitutively active TLR4 constructs can activate proximal components (MyD88, IL-1 receptor-associated kinase, tumor necrosis factor receptor-associated factor-6, and NF-κB inducing kinase) of the IL-1 signaling pathway that lead to NF-κB activation (18, 19). Although we demonstrate a link between LPS signaling and TLR4 expression in this study, it remains to be determined whether the effects of LPS utilize the same subsequent signaling proteins as the IL-1 receptor (4).

Kirschning et al. (13) failed to observe LPS-inducible NF-κB activation in HEK 293 cells transfected with human TLR4. The reasons for this are unclear, but might reflect the differences inherent in HEK 293 cell lines. For example, we have found that one stock of HEK 293 cells responded to LPS, as evidenced by inducible NF-κB translocation, in the absence of transfection. In order to perform these studies, we were forced to locate an alternative stock of HEK 293 cells, as this particular lot, acquired directly from the distributor (ATCC, Rockville, MD), expressed high levels of TLR2 mRNA. Like the IL-1 receptor, TLRs might require the formation of heterodimeric signaling complexes with highly homologous proteins (such as another TLR) upon ligand binding (20). A testable hypothesis that might explain the differences in outcome between seemingly identical experiments is that the background expression of TLRs determines which heterodimers can be formed after gene transfer and that all strains of HEK 293 cells are not equivalent in this respect. Other experimental differences might also affect results obtained in these types of experiments, such as the nature of the TLR4 construct, the amount of DNA utilized in transfections, or other conditions that may influence TLR4 expression. For example, we have shown that the amount of TLR4 expression vector transfected into cells influences the regulation of pELAM-luc activity by LPS. This was presumably due to elevated basal-specific activity of pELAM-luc that was increased when higher amounts of TLR4 were expressed. A similar effect has been reported in HEK 293 cells expressing IκB kinase-β (21). In support of Yang et al. (12) and Kirschning et al. (13), we have shown that our HEK 293 cells transfected

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**Fig. 3.** E5531, an LPS antagonist, inhibits TLR4-mediated NF-κB activation by LPS. HEK 293 cells were transiently transfected as described in the legend to Fig. 1. The next day, cells were stimulated with 10 ng/ml CD14 plus 1 μg/ml LPS (A and B) or 400 ng/ml PMA or 20 ng/ml IL-1β (B) in the absence or presence of the indicated concentration of E5531 for 6 h. Cells were lysed, and the amount of luciferase activity in each sample was quantified. These data represent the mean ± S.E. from three independent experiments. Means with different superscripts are significantly different from one another, p < 0.05.

**DISCUSSION**

Although it has been proposed previously that LPS interacts with a transmembrane receptor or recognition molecules on the surface of plasma membranes of responsive cell types (2), strong evidence for this hypothesis has only recently become available. First, Medzhitov et al. (11) identified a human homolog of Drosophila Toll, later designated TLR4 (9), with signaling properties similar to those observed for the

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**Footnotes:**

1. E. Lien and D. T. Golenbock, unpublished observations.
2. J. C. Chow and F. Gusovsky, unpublished observations.
with a construct containing TLR2 are also responsive to LPS plus CD14.5

E5531 is a potent synthetic lipid A analog that acts as an antagonist of LPS-induced activation (16). The compound inhibits the effects of LPS in monocytes, macrophages, animal models of sepsis and infection (16), as well as the effects of low amounts of LPS administered to humans (22). Based on LPS binding studies, it is believed that E5531 antagonizes LPS activity at its cell-surface receptor, leading to inhibition of transmembrane signal transduction (16). Consistent with this hypothesis, E5531 inhibits the stimulation of TLR4 transfected cells by LPS plus CD14 in a dose-dependent manner with an IC50 of ~30 nM, comparable with its effects in other cell based assays. Since E5531 did not affect activation of the NF-κB reporter gene induced by IL-1β or the actions of interferon-γ in murine macrophages (16), this indicates that the inhibitory activity of E5531 is closely linked to and specific to cell-surface components utilized by LPS. In these experiments, TLR4 is the only protein whose expression can account for the LPS responsiveness observed in transfected cells. Therefore, based on these results, it is very likely that TLR4 is a receptor for LPS and that E5531 acts as an antagonist of this interaction.

In light of the recent identification of the lesion in C3H/HeJ and C57Bl/10ScCr LPS-resistant mice as a mutation in the tlr4 gene (14), the results presented here are particularly significant. These data demonstrate that TLR4 behaves as a functional LPS receptor when transfected into cells that are otherwise LPS-insensitive. This cell-based result is consistent with the observation made by Poltorak et al. (14) in C3H/HeJ mice. These data do not exclude a role for TLR2 in LPS signal transduction under certain conditions or in specific cell types, but support the hypothesis that TLR4 is essential in LPS signaling in vitro and in vivo. Finally, the characterization of TLR4 as a receptor for the LPS antagonist is an important development for understanding the mechanistic steps and will enable further improvements in the discovery of anti-endotoxin agents.

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