Cutting Edge: Repurification of Lipopolysaccharide Eliminates Signaling Through Both Human and Murine Toll-Like Receptor 2

Matthew Hirschfeld, Ying Ma, John H. Weis, Stefanie N. Vogel and Janis J. Weis

*J Immunol* 2000; 165:618-622; doi: 10.4049/jimmunol.165.2.618

http://www.jimmunol.org/content/165/2/618
Cutting Edge: Repurification of Lipopolysaccharide Eliminates Signaling Through Both Human and Murine Toll-Like Receptor 2

Matthew Hirschfeld,* Ying Ma,* John H. Weis,* Stefanie N. Vogel,† and Janis J. Weis2*

Toll-like receptor (TLR) 2 has recently been associated with cellular responses to numerous microbial products, including LPS and bacterial lipoproteins. However, many preparations of LPS contain low concentrations of highly bioactive contaminants described previously as “endotoxin protein,” suggesting that these contaminants could be responsible for the TLR2-mediated signaling observed upon LPS stimulation. To test this hypothesis, commercial preparations of LPS were subjected to a modified phenol re-extraction protocol to eliminate endotoxin protein. While it did not influence the ability to stimulate cells from wild-type mice, repurification eliminated the ability of LPS to activate cells from C3H/HeJ (Lps2) mice. Additionally, only cell lines transfected with human TLR4, but not human or murine TLR2, acquired responsiveness to both re-extracted LPS and to a protein-free, synthetic preparation of lipid A. These results suggest that neither human nor murine TLR2 plays a role in LPS signaling in the absence of contaminating endotoxin protein. The Journal of Immunology, 2000, 165: 618–622.

A common and serious consequence of overwhelming bacterial infection is generalized organ failure due to septic shock. In the case of Gram-negative bacterial infection, this event is thought to be mediated by LPS, a major glycolipid component found in the outer membrane (1). LPS-induced stimulation of cells of the innate immune system subsequently activates numerous signal transduction cascades, including NF-κB-dependent production of inflammatory cytokines (2). Although CD14 has been recognized as a nonsignaling coreceptor for LPS (1), members of the Toll-like receptor (TLR)3 family have recently emerged as candidate receptors capable of transmitting LPS signaling across the cell membrane.

Currently, there are at least six TLR family members (TLR1–6) (3–6), and two of these, TLR2 and TLR4, have been associated with LPS signaling (7–12). A point mutation within tlr4 underlies the LPS hyporesponsiveness of C3H/HeJ mice (7–9), while overexpression of either TLR2 or TLR4 has been reported to confer responsiveness to LPS in cell lines (10–12). More recent data examining LPS responses in TLR2-deficient mice and hamsters indicate that TLR2 is not required for LPS signaling when TLR4 is present (13–15). TLR2 also has numerous non-LPS ligands (15–26), and a possible explanation for the discrepancy concerning whether TLR2 and/or TLR4 mediate(s) LPS signaling is that the commercial LPS preparations used in the transfection experiments were contaminated with one or more of these ligands. Historically, investigators have documented that established protocols for isolating LPS result in the copurification of varying amounts of endotoxin protein(s) (27–32). These contaminants are known to possess extremely potent bioactivity (28–34). Thus, assigning cellular responses to the LPS component of a particular preparation may be confounded by the presence of these contaminants. Using a protocol shown previously to remove endotoxin proteins from commercial LPS preparations (28), we investigated whether TLR2 mediates LPS responses in the absence of protein in vitro. Our results demonstrate that overexpressed TLR2 is extremely sensitive to minor contaminants in commercial LPS preparations.

Materials and Methods

Cell lines and reagents

The human astrocytoma cell line U87 was obtained from the American Type Culture Collection (Manassas, VA). Bone marrow-derived macrophages were prepared from C3H/HeN and C3H/HeJ mice (National Cancer Institute, Frederick, MD) as described (35). The subclone of the human embryonic kidney epithelial cell line 293 and the constructs for human TLR, endothelial cell-leukocyte adhesion molecule (ELAM-1) luciferase, and respiratory syncytial virus (RSV)-β-galactosidase were provided by Tularik (South San Francisco, CA) (10). LPS from Escherichia coli O111:B4 (smooth), J5 (Rc), and K12, D31 m4 (Re) were obtained from List Biological Laboratories (Campbell, CA). Recombinant OspA was provided by John Dunn (Brookhaven National Laboratories) (36). Synthetic lipid A was obtained from ICN Pharmaceuticals (Costa Mesa, CA). All other reagents were obtained from Sigma (St. Louis, MO). The coding

*Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT 84132; and †Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814

Received for publication March 23, 2000. Accepted for publication May 11, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by Public Health Service Grants AI-32223 and AI-43521 to J.J.W., AI-24158 to J.H.W., AI-18797 to S.N.V., and 5P30-CA-42014 to the University of the Health Sciences. The project described was also supported in part by an award from the American Lung Association (to J.H.W.).

Address correspondence and reprint requests to Dr. Janis J. Weis, Department of Pathology, University of Utah School of Medicine, 50 North Medical Drive, Salt Lake City, UT 84132. E-mail address: janis.weis@path.med.utah.edu

Copyright © 2000 by The American Association of Immunologists

0022-1767/00/$02.00

3 Abbreviations used in this paper: TLR, Toll-like receptor; ELAM-1, endothelial cell-leukocyte adhesion molecule (E-selectin); TEA, triethylamine; DOC, deoxycholate; RSV, respiratory syncytial virus.
sequence of TLR2 from C3H/HeN mice was amplified from genomic DNA and cloned into the mammalian expression vector pFLAG-CMV-1 (Sigma).

**Removal of endotoxin protein from LPS**

At room temperature, 5 mg of smooth, Re, and Re LPS were individually resuspended in 1 ml of endotoxin-free water containing 0.2% triethylamine (TEA). Each sample was split into two 500-μl aliquots, and one aliquot was stored at 4°C without further manipulation (“unextracted LPS”). Deoxycholate (DOC) was added to the remaining aliquot to a final concentration of 0.5%, followed by the addition of 500 μl of water-saturated phenol. The samples were vortexed intermittently for 5 min, and the phases were allowed to separate at room temperature for 5 min. Samples were placed on ice for 5 min, followed by centrifugation at 4°C for 2 min at 10,000 × g. The top aqueous layer was transferred to a new tube, and the phenol phase was subjected to re-extraction with 500 μl of 0.2% TEA/0.5% DOC. The aqueous phases were pooled and re-extracted with 1 ml of water-saturated phenol. The pooled aqueous phases were adjusted to 75% ethanol and 30 mM sodium acetate and were allowed to precipitate at −20°C for 1 h. The precipitates were centrifuged at 4°C for 10 min at 10,000 × g, washed in 1 ml of cold 100% ethanol, and air-dried. The precipitates were resuspended in the original volume (500 μl) of 0.2% TEA. One hundred percent recovery was assumed for the purified LPS samples (28), which will be referred to as “phenol re-extracted LPS.” This method was previously reported by Manthey et al. to eliminate the stimulatory activity of various LPS preparations on C3H/HeJ macrophage gene expression by removal of protein contaminants (28, 31).

**Transfections**

U87 cells were transfected in 12-well plates using pFx-2 (Invitrogen, Carlsbad, CA) with 2 μg of either TLR2 or TLR4 expression construct. Cells were then grown for 24 h in DMEM with Nutridoma-HU (Boehringer Mannheim, Indianapolis, IN) followed by stimulation with agonist for an additional 24 h in DMEM containing 2% human serum. 293 cells were cotransfected in six-well plates using a calcium phosphate kit (Clontech, Palo Alto, CA) at a ratio of 2:0.5:0.5 μg for the TLR2 expression construct, the ELAM-1 luciferase reporter construct, and the RSV β-galactosidase construct to normalize for transfection efficiency. Cells were grown for 36 h and stimulated with the indicated agonist for an additional 6 h.

**Luciferase and cytokine assays**

IL-6 (U87) and IL-8 (293) production were measured by ELISA (Endogen, Woburn, MA). Transfected 293 cells were lysed using reporter lysis buffer (Promega, Madison, WI), and 20 μl of lysis was assayed for luciferase and β-galactosidase activity using a Dynex MLX luminometer after incubation in luciferase assay reagent (Promega) or Galacto-Light with light emission accelerator (Tropix, Bedford, MA), respectively.

**Results**

To assess whether TLR2-mediated signaling is due to LPS and/or to contaminating endotoxin protein, one smooth and two rough (Re and Re) commercial E. coli LPS preparations were repurified as described by Manthey and Vogel (28). This protocol employs a modified phenol re-extraction of LPS to eliminate trace endotoxin protein contamination and was demonstrated to be without significant loss of either LPS concentration or bioactivity (28). The bioactivities of the phenol re-extracted LPS preparations were first compared in bone marrow-derived macrophages from C3H/HeJ (Lps<sup>R</sup>) and C3H/HeN (Lps<sup>+</sup>) mice. C3H/HeN macrophages responded to increasing doses of both unextracted and phenol re-extracted Re LPS similarly, as assayed by IL-6 production, until LPS levels equaled 100 ng/ml (Fig. 1A). At this and higher doses, unextracted Re LPS caused an increase in IL-6 production relative to phenol re-extracted Re LPS. We hypothesize that this result is due to a synergistic stimulation of macrophages by LPS and contaminating endotoxin protein, an effect that has been described previously (31, 37). In contrast, C3H/HeJ macrophages produced significant quantities of IL-6 only upon stimulation with unextracted Re LPS (Fig. 1A), suggesting that this LPS preparation was contaminated with endotoxin protein. Phenol re-extracted LPS did not stimulate IL-6 production at doses up to 10 μg/ml (Fig. 1A).

![FIGURE 1](http://www.jimmunol.org/)
OspA was used at 500 ng/ml in the presence of 5 unextracted (○) Rc LPS or phenol re-extracted (□) Rc LPS; recombinant luciferase reporter construct. Cells were stimulated for 6 h with either unextracted (○) or phenol re-extracted (□) Rc LPS. NF-κB nuclear translocation is indicated by luciferase units. B, NF-κB nuclear translocation in murine TLR2-expressing cells. 293 cells were transiently transfected with TLR2 expression construct derived from C3H/HeN mice in the presence of the ELAM-1 luciferase reporter construct. Cells were stimulated for 6 h with either unextracted (○) Rc LPS or phenol re-extracted (□) Rc LPS; recombinant OspA was used at 500 ng/ml in the presence of 5 μg/ml of polymyxin B (■). NF-κB nuclear translocation is indicated by luciferase units.

either response at doses up to 10 μg/ml (Fig. 2A). A similar response profile was seen using either unextracted and phenol re-extracted Rc LPS (data not shown), and neither unextracted nor phenol re-extracted smooth LPS stimulated TLR2-transfected 293 cells (data not shown). Thus, human TLR2 does not mediate a LPS response in vitro when contaminating endotoxin proteins have been removed.

To investigate whether phenol re-extracted LPS was able to initiate signaling by TLR2 from other species, TLR2 was cloned from C3H/HeN mice into the same expression plasmid used for the murine TLR2 (18, 20, 22). In contrast, phenol re-extracted bacterial lipoproteins (Fig. 3, A and B), suggesting that the protein contaminants in unextracted LPS may be signaling through a similar pathway. These results again provide evidence that TLR4, not TLR2, mediates signaling by LPS in the absence of endotoxin protein.

The lipid A portion of LPS is responsible for its biological activity (38), and a synthetic preparation, free of contaminating endotoxin proteins, was used to treat human TLR2-transfected 293 and U87 cells. In contrast to unextracted Rc LPS, synthetic lipid A was unable to elicit either NF-κB translocation in 293 cells or IL-6 production in U87 cells (Fig. 4). However, synthetic lipid A did stimulate release of IL-6 in U87 cells transfected with human TLR4 (Fig. 4). These results strengthen the hypothesis that TLR4, not TLR2, mediates signaling by LPS.

In a previous report, we observed that polymyxin B could inhibit TLR2-mediated signaling upon stimulation with unextracted LPS (18). TLR2-transfected 293 cells were treated with increasing doses of both unextracted and phenol re-extracted LPS than transfection of TLR2. U87 cells are also naturally responsive to purified bacterial lipopolysaccharides (Fig. 3, A and B), suggesting that the protein contaminants in unextracted LPS may be signaling through a similar pathway. These results again provide evidence that TLR4, not TLR2, mediates signaling by LPS in the absence of endotoxin protein.

The lipid A portion of LPS is responsible for its biological activity (38), and a synthetic preparation, free of contaminating endotoxin proteins, was used to treat human TLR2-transfected 293 and U87 cells. In contrast to unextracted Rc LPS, synthetic lipid A was unable to elicit either NF-κB translocation in 293 cells or IL-6 production in U87 cells. In contrast, phenol re-extracted Rc LPS did not elicit responses at doses up to 10 μg/ml in cells transfected with murine TLR2. These data support findings that suggest murine TLR2 does not mediate LPS signaling (13, 15, 24).

The ability of TLR4 to mediate signaling by phenol re-extracted LPS was tested in another LPS-unresponsive cell line, U87. When TLR4 was transfected into U87 cells, both unextracted and phenol re-extracted Rc LPS caused secretion of IL-6 (Fig. 3A). This effect was not seen with either untransfected (data not shown) or TLR2-transfected (Fig. 3B) U87 cells, in which secretion of IL-6 was only increased when stimulated with unextracted LPS. In fact, expression of TLR4 enabled U87 cells to respond to 100-fold lower
in the presence or absence of nuclear translocation is indicated by luciferase units.

with LPS. Requires a close physical association of protein contaminants B to inhibit endotoxin protein stimulation of TLR2 signaling re-

OspA (Fig. 5 and Ref. 18), suggesting that the ability of polymyxin B to inhibit endotoxins protein stimulation of TLR2 signaling requires a close physical association of protein contaminants with LPS.

**Discussion**

The role of TLR2 in LPS signaling has been very controversial. Certainly, the original reports of TLR2-mediated signaling in transfected 293 cells were quite convincing (10, 11), and similar results have been reported by numerous laboratories (14, 16, 18, 20, 22, 25, 39). However, once it was demonstrated that TLR2-deficient mice responded normally to LPS, while TLR4-deficient mice were refractory (7–9, 13), the physiological role of TLR2 in LPS signaling came under more careful scrutiny. Several groups, in fact, have demonstrated LPS signaling in the absence of TLR2 in various primary cells and cell lines (14, 15, 20, 21, 24, 25, 40). The data presented in this report attempt to clarify the putative contribution of TLR2 to LPS signaling.

Our results suggest that the overexpression of either human or murine TLR2 causes cell lines to become extremely sensitive to the potent ‘endotoxin protein’ contaminants present in many commercial LPS preparations. Our data clearly point to non-LPS ligands as the active agent(s) in previous papers that describe LPS-

mediated TLR2 signaling and resolve the discrepancy between results from transfection studies and TLR2-deficient mice. However, the biology of TLR signaling is likely to be more complex.

It is certainly possible that interactions among different TLRs to the pathogenesis of inflammatory events.

**Acknowledgments**

We thank Carsten J. Kirschning, Ralf Schwandner, and Holger Wescbe for providing 293 cells and the expression constructs of human TLR2, TLR4, ELAM-1 luciferase, and RSV β-galactosidase, John Dunn for providing recombinant OspA, R. Mark Wooten for providing murine genomic DNA, and Carl L. Manthey for technical advice.

**References**


negative and Gram-positive bacterial cell wall components. Immunity 11:443.


