

Cutting Edge: Repurification of Lipopolysaccharide Eliminates Signaling Through Both Human and Murine Toll-Like Receptor 2¹

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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 (*Lps*^d) 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- κ B-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

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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, Rc, 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- μ l 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 μ l 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 \times g. The top aqueous layer was transferred to a new tube, and the phenol phase was subjected to re-extraction with 500 μ l 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 \times g, washed in 1 ml of cold 100% ethanol, and air-dried. The precipitates were resuspended in the original volume (500 μ l) 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 pF_x-2 (Invitrogen, Carlsbad, CA) with 2 μ g 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 μ g 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 μ l of lysate 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 (Rc 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 (*Lpsrd*) and C3H/HeN (*Lps^{rn}*) mice. C3H/HeN macrophages responded to increasing doses of both unextracted and phenol re-extracted Rc LPS similarly, as assayed by IL-6 production, until LPS levels equaled 100 ng/ml (Fig. 1A). At this and higher doses, unextracted Rc LPS caused an increase in IL-6 production relative to phenol re-extracted Rc 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 Rc 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 μ g/ml (Fig. 1A).

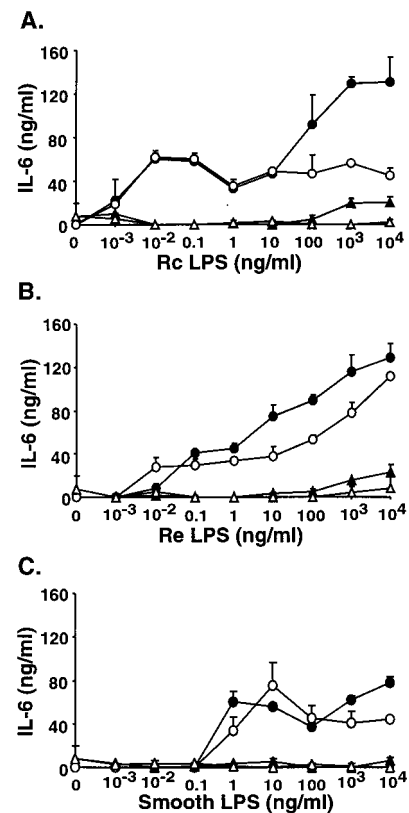


FIGURE 1. Phenol re-extraction of commercial LPS preparations eliminates stimulation of C3H/HeJ macrophages without loss of bioactivity on C3H/HeN macrophages. Bone marrow-derived macrophages from C3H/HeN (circles) and C3H/HeJ (triangles) mice were isolated and treated with both unextracted (filled symbols) and phenol re-extracted (open symbols) *E. coli* J5 (Rc) LPS (A), *E. coli* K12, D31 m4 (Re) LPS (B), or *E. coli* 0111: B4 (smooth) LPS (C).

Thus, phenol re-extraction of Rc LPS appears to have removed the non-LPS (protein) component while retaining LPS bioactivity, similar to the observations seen by Manthey et al. (28, 31). A similar IL-6 secretion profile was observed in C3H/HeN and C3H/HeJ macrophages stimulated with unextracted and phenol re-extracted Re LPS (Fig. 1B). In contrast, neither unextracted nor phenol re-extracted smooth LPS stimulated IL-6 secretion in C3H/HeJ macrophages (Fig. 1C), suggesting the level of endotoxin protein contamination in this particular commercial LPS preparation was relatively low. Both unextracted and phenol re-extracted smooth LPS stimulated macrophages from C3H/HeN mice in a similar manner (Fig. 1C).

To test more directly whether the removal of endotoxin protein influenced LPS signaling through TLR2, a human TLR2 expression construct was transiently transfected into a subclone of the cell line 293 along with a NF- κ B-dependent luciferase reporter plasmid that contains the E-selectin (ELAM-1) promoter. This particular 293 subclone was previously described as LPS-unresponsive unless transfected with TLR2, and this acquired LPS responsiveness was augmented either by cotransfection with the CD14 gene or the presence of soluble CD14 in serum (10). In this and following experiments, soluble CD14 was provided in serum. To normalize for transfection efficiency, an RSV β -galactosidase control plasmid was also cotransfected. Only unextracted Rc LPS was able to elicit a potent response in TLR2-transfected 293 cells and was reflected in both luciferase (Fig. 2A) and endogenous IL-8 (data not shown) production. Phenol re-extracted LPS did not elicit

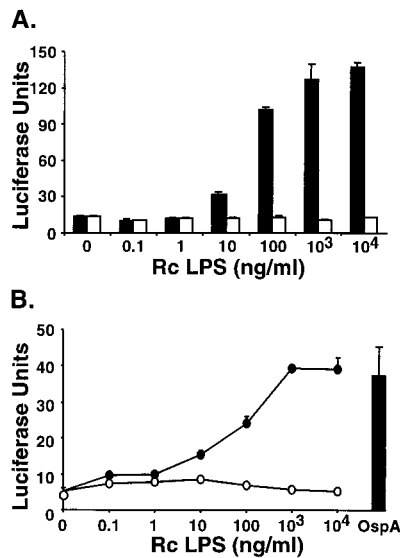


FIGURE 2. Phenol re-extraction of LPS eliminates both human and murine TLR2-mediated signaling in 293 cells. *A*, NF- κ B nuclear translocation in human TLR2-expressing cells. 293 cells were transiently transfected with human TLR2 expression construct plus the ELAM-1 luciferase reporter construct. Cells were stimulated for 6 h with either unextracted (■) or phenol re-extracted (□) Rc LPS. NF- κ B nuclear translocation is indicated by luciferase units. *B*, NF- κ B 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 μ g/ml of polymyxin B (■). NF- κ B nuclear translocation is indicated by luciferase units.

either response at doses up to 10 μ g/ml (Fig. 2A). A similar response profile was seen using either unextracted and phenol re-extracted Re 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 human construct described above. This construct was cotransfected into 293 cells with both the ELAM-1-luciferase reporter plasmid and the RSV β -galactosidase plasmid to normalize for transfection efficiency. As with human TLR2, only unextracted Rc LPS stimulated NF- κ B translocation (Fig. 2B) and endogenous IL-8 production (data not shown) in murine TLR2-transfected 293 cells. Murine TLR2 also displayed strong reactivity to the purified bacterial lipoprotein, OspA (Fig. 2B), as previously reported for human TLR2 (18, 20, 22). In contrast, phenol re-extracted Rc LPS did not elicit responses at doses up to 10 μ g/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

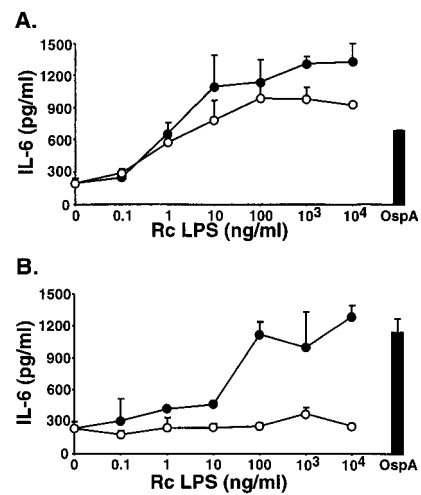


FIGURE 3. Transfection of TLR4, but not TLR2, confers responsiveness to phenol re-extracted LPS in U87 cells. U87 cells were transiently transfected with either human TLR4 (*A*) or human TLR2 (*B*). Cells were stimulated for 24 h with either unextracted (●) or phenol re-extracted (○) Rc LPS; recombinant OspA was used at 500 ng/ml in the presence of 5 μ g/ml of polymyxin B (■). Supernatants were collected and were assayed for IL-6 production by ELISA.

doses of both unextracted and phenol re-extracted LPS than transfection of TLR2. U87 cells are also naturally responsive to purified 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- κ B 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

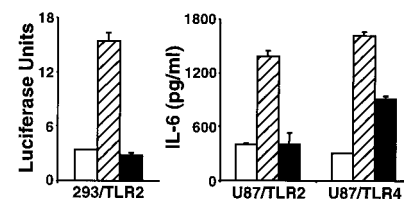


FIGURE 4. Transfection of TLR4, but not TLR2, confers responsiveness to synthetic lipid A. 293 and U87 cells were transiently transfected with either human TLR2 (293 and U87 cells) or human TLR4 (U87 cells). 293 cells were also transiently transfected with the ELAM-1 luciferase reporter construct. Cells were stimulated for 6 h (293 cells) or 24 h (U87 cells) with media alone (□), 10 μ g/ml unextracted Rc LPS (▨), or 10 μ g/ml synthetic lipid A (■). NF- κ B nuclear translocation is indicated by luciferase units (293 cells), and IL-6 production was assayed by ELISA (U87 cells).

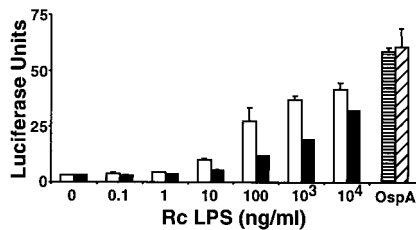


FIGURE 5. Polymyxin B inhibits TLR2-mediated signaling by unpurified LPS. 293 cells were transiently transfected with human TLR2 expression construct plus the ELAM-1 luciferase reporter construct. Cells were stimulated for 6 h with unextracted Rc LPS in the presence (■) or absence (□) of 5 μ g/ml of polymyxin B; recombinant OspA was used at 500 ng/ml in the presence (▨) or absence (▩) of 5 μ g/ml of polymyxin B. NF- κ B nuclear translocation is indicated by luciferase units.

doses of unextracted Rc LPS in the presence and absence of polymyxin B (Fig. 5). Unextracted Rc LPS stimulation was partially inhibited at doses up to 10 μ g/ml in the presence of polymyxin B, with greatest inhibition seen at 100 ng/ml, the dose used in the previous report (18). In contrast, polymyxin B did not inhibit TLR2-mediated signaling by the purified bacterial lipoprotein, OspA (Fig. 5 and Ref. 18), suggesting that the ability of polymyxin B to inhibit endotoxin 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 could confer unique specificities capable of mediating LPS signaling in some cell types (41). Additionally, other, less well-characterized LPS signaling pathways may exist and may depend on the cell line (42) or the genetic background of the mouse strain (43). It is also possible that certain nonenterobacterial lipid A structures bind and/or signal through TLRs apart from TLR4 (42). However, these pathways do not seem to be active to any significant extent in TLR4-deficient mice (9, 13) or in the naturally occurring mutant strains C3H/HeJ and C57BL/10ScCr (7, 8, 28, 31); nor do they appear to occur to any measurable extent in the 293 or U87 human cells used in our study.

Although we have not characterized the biochemical nature of the contaminants responsible for TLR2-mediated signaling, it

seems likely that bacterial lipoproteins could be at least partially responsible, given past reports demonstrating lipoprotein signaling mediated by TLR2 (18, 20, 22, 23, 26). In addition, lipoproteins possess extremely potent bioactivity, with some variants exhibiting half-maximal stimulation at levels as low as 3 pM in vitro (44). Similar concentrations of lipoproteins should easily be attainable in commercial preparations of LPS, which may be contaminated with up to 10% endotoxin protein (32). Undoubtedly, both TLR2 and TLR4 are important in the inflammatory response to Gram-negative bacterial infection because both endotoxin proteins and LPS are present in the context of whole bacteria. Thus, investigators should be aware of the contribution of combinations of microbial components and their subsequent activation of various TLRs to the pathogenesis of inflammatory events.

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References

- Ulevitch, R. J., and P. S. Tobias. 1999. Recognition of Gram-negative bacteria and endotoxin by the innate immune system. *Curr. Opin. Immunol.* 11:19.
- Sweet, M. J., and D. A. Hume. 1996. Endotoxin signal transduction in macrophages. *J. Leukocyte Biol.* 60:8.
- Takeuchi, O., T. Kawai, H. Sanjo, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, K. Takeda, and S. Akira. 1999. TLR6: A novel member of an expanding Toll-like receptor family. *Gene* 231:59.
- Rock, F. L., G. Hardiman, J. C. Timans, R. A. Kastelein, and J. F. Bazan. 1998. A family of human receptors structurally related to *Drosophila* Toll. *Proc. Natl. Acad. Sci. USA* 95:588.
- Chaudhary, P. M., C. Ferguson, V. Nguyen, O. Nguyen, H. F. Massa, M. Eby, A. Jasmin, B. J. Trask, L. Hood, and P. S. Nelson. 1998. Cloning and characterization of two Toll/interleukin-1 receptor-like genes TIL3 and TIL4: evidence for a multi-gene receptor family in humans. *Blood* 91:4020.
- Medzhitov, R., P. Preston-Hurlburt, and C. A. Janeway, Jr. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 388:394.
- Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. V. Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, et al. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282:2085.
- Qureshi, S. T., L. Lariviere, G. Leveque, S. Clermont, K. J. Moore, P. Gros, and D. Malo. 1999. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J. Exp. Med.* 189:615.
- Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the *Lps* gene product. *J. Immunol.* 162:3749.
- Kirschning, C. J., H. Wesche, T. M. Ayres, and M. Rothe. 1998. Human Toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. *J. Exp. Med.* 188:2091.
- Yang, R. B., M. R. Mark, A. Gray, A. Huang, M. H. Xie, M. Zhang, A. Goddard, W. I. Wood, A. L. Gurney, and P. J. Godowski. 1998. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature* 395:284.
- Chow, J. C., D. W. Young, D. T. Golenbock, W. J. Christ, and F. Gusovsky. 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J. Biol. Chem.* 274:10689.
- Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. *Immunity* 11:443.
- Heine, H., C. J. Kirschning, E. Lien, B. G. Monks, M. Rothe, and D. T. Golenbock. 1999. Cutting edge: cells that carry a null allele for Toll-like receptor 2 are capable of responding to endotoxin. *J. Immunol.* 162:6971.
- Underhill, D. M., A. Ozinsky, A. M. Hajjar, A. Stevens, C. B. Wilson, M. Bassetti, and A. Aderem. 1999. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature* 401:811.
- Schwandner, R., R. Dziarski, H. Wesche, M. Rothe, and C. J. Kirschning. 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. *J. Biol. Chem.* 274:17406.
- Yoshimura, A., E. Lien, R. R. Ingalls, E. Tuomanen, R. Dziarski, and D. Golenbock. 1999. Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J. Immunol.* 163:1.
- Hirschfeld, M., C. J. Kirschning, R. Schwandner, H. Wesche, J. H. Weis, R. M. Wooten, and J. J. Weis. 1999. Cutting edge: inflammatory signaling by *Borrelia burgdorferi* lipoproteins is mediated by Toll-like receptor 2. *J. Immunol.* 163:2382.

19. Means, T. K., S. Wang, E. Lien, A. Yoshimura, D. T. Golenbock, and M. J. Fenton. 1999. Human Toll-like receptors mediate cellular activation by *Mycobacterium tuberculosis*. *J. Immunol.* 163:3920.
20. Lien, E., T. J. Sellati, A. Yoshimura, T. H. Flo, G. Rawadi, R. W. Finberg, J. D. Carroll, T. Espevik, R. R. Ingalls, J. D. Radolf, and D. T. Golenbock. 1999. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. *J. Biol. Chem.* 274:33419.
21. Means, T. K., E. Lien, A. Yoshimura, S. Wang, D. T. Golenbock, and M. J. Fenton. 1999. The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors. *J. Immunol.* 163:6748.
22. Brightbill, H. D., D. H. Libraty, S. R. Krutzik, R. B. Yang, J. T. Belisle, J. R. Bleharski, M. Maitland, M. V. Norgard, S. E. Plevy, S. T. Smale, et al. 1999. Host defense mechanisms triggered by microbial lipoproteins through Toll-like receptors. *Science* 285:732.
23. Aliprantis, A. O., R. B. Yang, M. R. Mark, S. Suggett, B. Devaux, J. D. Radolf, G. R. Klimpel, P. Godowski, and A. Zychlinsky. 1999. Cell activation and apoptosis by bacterial lipoproteins through Toll-like receptor-2. *Science* 285:736.
24. Underhill, D. M., A. Ozinsky, K. D. Smith, and A. Aderem. 1999. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. *Proc. Natl. Acad. Sci. USA* 96:14459.
25. Flo, T. H., O. Halaas, E. Lien, L. Ryan, G. Teti, D. T. Golenbock, A. Sundan, and T. Espevik. 2000. Human Toll-like receptor 2 mediates monocyte activation by *Listeria monocytogenes*, but not by group B streptococci or lipopolysaccharide. *J. Immunol.* 164:2064.
26. Takeuchi, O., A. Kaufmann, K. Grote, T. Kawai, K. Hoshino, M. Morr, P. F. Muhlrad, and S. Akira. 2000. Cutting edge: preferentially the *R*-stereoisomer of the mycoplasma lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a Toll-like receptor 2- and MyD88-dependent signaling pathway. *J. Immunol.* 164:554.
27. Skidmore, B. J., D. C. Morrison, J. M. Chiller, and W. O. Weigle. 1975. Immunologic properties of bacterial lipopolysaccharide (LPS). II. The unresponsiveness of C3H/HeJ Mouse spleen cells to LPS-induced mitogenesis is dependent on the method used to extract LPS. *J. Exp. Med.* 142:1488.
28. Manthey, C. L., and S. N. Vogel. 1994. Elimination of trace endotoxin protein from rough chemotype LPS. *J. Endotoxin Res* 1:84.
29. Sultz, B. M., and G. W. Goodman. 1976. Endotoxin protein: a B-cell mitogen and polyclonal activator of C3H/HeJ lymphocytes. *J. Exp. Med.* 144:821.
30. Morrison, D. C., S. J. Betz, and D. M. Jacobs. 1976. Isolation of a lipid A bound polypeptide responsible for "LPS-initiated" mitogenesis of C3H/HeJ spleen cells. *J. Exp. Med.* 144:840.
31. Manthey, C. L., P. Y. Perera, B. E. Henricson, T. A. Hamilton, N. Qureshi, and S. N. Vogel. 1994. Endotoxin-induced early gene expression in C3H/HeJ (*Lps*^d) macrophages. *J. Immunol.* 153:2653.
32. Luderitz, O., C. Galanos, E. T. Rietschel, and O. Westphal. 1986. Lipid A: relationships of chemical structure and biological activity. In *Immunobiology and Immunopharmacology of Bacterial Endotoxins*. A. Szentivanyi, H. Friedman, and A. Nowotny, eds. Plenum Press, New York, p. 65.
33. Hogan, M. M., and S. N. Vogel. 1987. Lipid A-associated proteins provide an alternate "second signal" in the activation of recombinant interferon- γ -primed, C3H/HeJ macrophages to a fully tumoricidal state. *J. Immunol.* 139:3697.
34. Hogan, M. M., and S. N. Vogel. 1988. Production of tumor necrosis factor by rIFN- γ -primed C3H/HeJ (*Lps*^d) macrophages requires the presence of lipid A-associated proteins. *J. Immunol.* 141:4196.
35. Ma, Y., K. P. Seiler, K. F. Tai, L. Yang, M. Woods, and J. J. Weis. 1994. Outer surface lipoproteins of *Borrelia burgdorferi* stimulate nitric oxide production by the cytokine-inducible pathway. *Infect Immun* 62:3663.
36. Dunn, J. J., B. N. Lade, and A. G. Barbour. 1990. Outer surface protein A (OspA) from the Lyme disease spirochete, *Borrelia burgdorferi*: high level expression and purification of a soluble recombinant form of OspA. *Protein Expr. Purif.* 1:159.
37. Zhang, H., J. W. Peterson, D. W. Niesel, and G. R. Klimpel. 1997. Bacterial lipoprotein and lipopolysaccharide act synergistically to induce lethal shock and proinflammatory cytokine production. *J. Immunol.* 159:4868.
38. Rietschel, E. T., T. Kirikae, F. U. Schade, U. Mamat, G. Schmidt, H. Loppnow, A. J. Ulmer, U. Zahringer, U. Seydel, F. Di Padova, M. Schreier, and H. Brade. 1994. Bacterial endotoxin: molecular relationships of structure to activity and function. *FASEB J.* 8:217.
39. Yang, R. B., M. R. Mark, A. L. Gurney, and P. J. Godowski. 1999. Signaling events induced by lipopolysaccharide-activated Toll-like receptor 2. *J. Immunol.* 163:639.
40. Cario, E., I. M. Rosenberg, S. L. Brandwein, P. L. Beck, H. C. Reinecker, and D. K. Podolsky. 2000. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. *J. Immunol.* 164:966.
41. Wyllie, D. H., E. Kiss-Toth, A. Visintin, D. Segal, G. W. Duff, and S. K. Dower. 2000. Evidence for an accessory protein function for the Toll-like receptor TLR1 in lipopolysaccharide responses. *FASEB J.* 14:91.30 (Abstr.).
42. Savedra, R., Jr., R. L. Delude, R. R. Ingalls, M. J. Fenton, and D. T. Golenbock. 1996. Mycobacterial lipoarabinomannan recognition requires a receptor that shares components of the endotoxin signaling system. *J. Immunol.* 157:2549.
43. Vogel, S. N., D. Johnson, P. Y. Perera, A. Medvedev, L. Lariviere, S. T. Qureshi, and D. Malo. 1999. Cutting edge: functional characterization of the effect of the C3H/HeJ defect in mice that lack an *Lps*^d gene: in vivo evidence for a dominant negative mutation. *J. Immunol.* 162:5666.
44. Muhlrad, P. F., M. Kiess, H. Meyer, R. Sussmuth, and G. Jung. 1998. Structure and specific activity of macrophage-stimulating lipopeptides from *Mycoplasma hyorhinis*. *Infect. Immun.* 66:4804.