

Toll-like Receptor 2 Functions as a Pattern Recognition Receptor for Diverse Bacterial Products*

(Received for publication, July 6, 1999)

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Toll-like receptors (TLRs) 2 and 4 are signal transducers for lipopolysaccharide, the major proinflammatory constituent in the outer membrane of Gram-negative bacteria. We observed that membrane lipoproteins/lipopeptides from *Borrelia burgdorferi*, *Treponema pallidum*, and *Mycoplasma fermentans* activated cells heterologously expressing TLR2 but not those expressing TLR1 or TLR4. These TLR2-expressing cells were also stimulated by living motile *B. burgdorferi*, suggesting that TLR2 recognition of lipoproteins is relevant to natural *Borrelia* infection. Importantly, a TLR2 antibody inhibited bacterial lipoprotein/lipopeptide-induced tumor necrosis factor release from human peripheral blood mononuclear cells, and TLR2-null Chinese hamster macrophages were insensitive to lipoprotein/lipopeptide challenge. The data suggest a role for the native protein in cellular activation by these ligands. In addition, TLR2-dependent responses were seen using whole *Mycobacterium avium* and *Staphylococcus aureus*, demonstrating that this receptor can function as a signal transducer for a wide spectrum of bacterial products. We conclude that diverse pathogens activate cells through TLR2 and propose that this molecule is a central pattern recognition receptor in host immune responses to microbial invasion.

Microbial invasion of the host is followed by a series of events designed to control and eventually resolve the infection. The immediate response to the invading organism is coordinated by the innate immune system. The cells of this system are responsible for first-line bacterial clearance and modulation of the adaptive immune response through soluble factors or co-stimulatory signals provided by antigen-presenting cells (1). Jane-

way and co-workers (2, 3) have hypothesized that the innate immune system can sense invading pathogens by virtue of nonclonal pattern recognition receptors that interact with microbial structures and deliver a danger signal to the host cell.

Toll is a type I transmembrane receptor, first described in *Drosophila*, that shares homology to components of the interleukin-1 (IL-1)¹ signaling pathway (4). Toll, and the related molecule 18-Wheeler, appear to control important antimicrobial responses against both fungi and bacteria in the fruit fly (5, 6). In evolutionary terms, these proteins are primordial pattern recognition receptors for animals that totally lack acquired immunity. Recently, mammalian homologues of Toll have been cloned and designated Toll-like receptors (TLRs) (7–9). At least 10 such receptors have been identified, but only 2 TLRs have any known function. TLR2 and TLR4 have been implicated in cellular responses to lipopolysaccharide (LPS), the major constituent of the Gram-negative bacterial outer membrane (10–12). However, the mechanism behind TLR-mediated recognition of LPS, the interactions with other receptor molecules, such as CD14 (13, 14), and the details of the subsequent cellular activation pathway still require elucidation.

Lyme disease and syphilis are acute and chronic inflammatory disorders caused by the spirochetal pathogens *Borrelia burgdorferi* and *Treponema pallidum* subsp. *pallidum*, respectively (15, 16). Both spirochetes lack LPS (17, 18); however, they do possess abundant membrane lipoproteins (19). There now exists a large body of evidence that spirochetal lipoproteins and synthetic lipohexapeptide analogs are potent activators of monocytes/macrophages, neutrophils, lymphocytes, endothelial cells, and fibroblasts and that acyl modification of the peptides is essential for these proinflammatory activities (20–29). More recent observations suggest that the mechanisms underlying monocytic cell activation by motile *B. burgdorferi* and *T. pallidum* are identical to those employed by their purified membrane constituents (30). These results support the notion that lipoproteins are the principle component of intact spirochetes driving the host immune response during Lyme disease and syphilis. Similarly, lipoproteins and lipopeptides derived from the human pathogen *Mycoplasma fermentans* are also potent activators of monocytes/macrophages and may play

* This work was supported by National Institutes of Health Grants GM54060, AI38515, and AI01476 (to R. R. I. and D. T. G.), AI09973 (to T. J. S.), AI31628–08 (to R. W. F.), and AI29735 and AI38894 (to J. D. R.); the Research Council of Norway; the Norwegian Cancer Society (to E. L., T. H. F., and T. E.); and a grant from the Arthritis Foundation (to T. J. S.). 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.

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¹ The abbreviations used are: IL, interleukin; TLR, Toll-like receptor; LPS, lipopolysaccharide; Osp, outer surface protein; nOspA, native OspA; CHO, Chinese hamster ovary; sMALP-2, synthetic macrophage-activating lipopeptide-2; TNF, tumor necrosis factor; NF- κ B, nuclear factor- κ B; FBS, fetal bovine serum; PBMC, peripheral blood mononuclear cells.

an important role in the inflammatory response during infection (31–33).

The cellular activation induced by the lipoproteins or lipoprotein-derived lipopeptides from *B. burgdorferi* and *T. pallidum* resembles that of the LPS signaling pathway, as CD14 appears to facilitate cellular activation by both types of pathogenic membrane structures (21, 25). However, several differences have been observed between LPS and lipoprotein cellular activation, indicating the utilization of different signaling elements. For example, spirochetal and mycoplasma lipoproteins and lipopeptides activate macrophages from LPS hyporesponsive C3H/HeJ mice (23, 24, 27, 31). In addition, whereas Chinese hamster ovary (CHO)-K1 cells become remarkably sensitive to LPS after transfection with CD14 (34–36), they remain insensitive to the lipoproteins, lipopeptides, and motile *B. burgdorferi* (21, 30, 32). These observations led us to hypothesize that differences in main signaling components exist between lipoproteins and LPS.

We have recently found that CHO-K1 cells do not express an mRNA transcript for full-length and functional TLR2 (37). This observation raised the possibility that the lack of functional TLR2 might account for the failure of CHO/CD14 cells to respond to bacterial structures other than LPS. To test this hypothesis, we engineered stable CHO/CD14 fibroblast cell lines that express TLR2. The transfected cells were highly susceptible to activation by lipoproteins and lipopeptides from *B. burgdorferi*, *T. pallidum*, and *M. fermentans*, as well as to activation by live motile *B. burgdorferi*. In contrast, cells expressing TLR1 or TLR4 did not acquire responsiveness to bacterial lipoproteins/lipopeptides. Moreover, we observed a TLR2-mediated cell activation by *Mycobacterium avium*, an important pathogen in AIDS. Similar studies have documented inducible responses to other bacteria as well, including staphylococci, listeria, tuberculosis, and the pneumococcus, suggestive of wide-spread recognition of bacteria by TLR2 (10, 11, 38, 39).^{2,3} We propose that TLR2 mediates cellular responses to structures from numerous microbial cell wall constituents and may thus be central in host recognition of diverse bacterial pathogens. Therapies directed at the TLRs may be useful anti-inflammatory agents for a large variety of chronic and acute bacterial infections.

MATERIALS AND METHODS

Reagents—PBS, Ham's F-12 medium, RPMI 1640 medium, and trypsin-versene mixture (trypsin-EDTA) were from BioWhittaker (Walkersville, MD). Low endotoxin FBS was from Summit Biotechnologies (Greeley, CO), and ciprofloxacin was a gift from Miles Pharmaceuticals (West Haven, CT). Hygromycin B was purchased from Calbiochem (San Diego, CA), puromycin was from Sigma, and G418 was from Life Technologies, Inc. Protein-free LPS from *Salmonella minnesota* Re595 was a gift from N. Qureshi (Middleton Veterans Affairs Hospital, Madison, WI). Antibodies for flow cytometry were purchased from Becton Dickinson, and human IL-1 β and tumor necrosis factor α (TNF α) were from Genzyme (Cambridge, MA).

Lipoproteins and Lipopeptides—Native OspA (nOspA) was immunofluorescence purified from *B. burgdorferi* strain T11-EV (40). Hexapeptides similar to the N termini of *B. burgdorferi* OspA (CKQNVS), OspC (CNNSGK), and *T. pallidum* 47-kDa major lipoprotein (CGSSH) were synthesized on an Applied Biosystems (Foster City, CA) peptide synthesizer. Lipopeptides (OspAL, OspCL, and 47L) corresponding to the acylated N termini of natural OspA, OspC and 47-kDa lipoprotein were synthesized using tripalmitoyl-S-glyceryl-cysteine in a solid-phase pro-

cedure (41). A synthetic (s) lipopeptide based upon the full-length MALP-2 membrane lipopeptide from *M. fermentans* (sMALP-2; CGNNDENISFKKEK) was prepared using dipalmitoyl-S-glyceryl cysteine as described (32). An unlipidated version of sMALP-2 was also synthesized (32). Lipoproteins and lipopeptides were frozen at -20°C as 1 mg/ml or 200 μM (sMALP-2) stock solutions in PBS (OspAL, OspCL, and 47L) or in PBS, 25 mM octyl glucoside (nOspA and sMALP-2). Endotoxin levels were undetectable in all lipoprotein/lipopeptide stock solutions as measured by *Limulus* assay.

Bacterial Strains—*B. burgdorferi* strain B31 (42) (provided by R. Lathigra, MedImmune, Inc., Gaithersburg, MD) was grown *in vitro* at 34°C in Barbour-Stoenner-Kelly H medium (Sigma). Microorganisms were quantified by dark-field microscopy. Spirochetes were passaged five times or less prior to experimentation, and infectivity was assessed by intradermal infection of C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME), followed by culture of an ear biopsy. For flow cytometry experiments, *B. burgdorferi* were labeled with PKH2 green fluorescent dye (Sigma) according to the manufacturer's instructions. *M. avium* strain 969 A45, originally a clinical isolate, was grown in Middlebrook 7H9 medium with OADC supplementation (Baltimore Biological Laboratories, Baltimore, MD). The bacterial cells were harvested by centrifugation, washed twice and resuspended in PBS, passed through a 5 μm filter to remove cell clumps, and enumerated by plating. Heat-killed *Staphylococcus aureus* (ATCC 25923) was prepared as described (38). Bioparticles consisting of killed *Escherichia coli* K-12 strain were purchased from Molecular Probes (Eugene, OR) and resuspended in PBS.

Cell Lines—The CHO/CD14.ELAM.Tac reporter cell line (clone 3E10) expresses inducible membrane CD25 under control of a region from the human E-selectin promoter containing nuclear factor- κB (NF- κB) binding sites; this promoter element is absolutely dependent upon NF- κB (43). Control reporter cells (CHO/ELAM.Tac (clone EL1)) were similarly constructed to express surface CD25 upon IL-1 or TNF stimulation by transfecting cells with the reporter construct and the hygromycin vector pCEP4 alone (*i.e.* without CD14). CHO/CD14 cells expressing TLRs were engineered by stable transfection of the CHO/CD14 reporter cell line with the cDNA for human TLR1, TLR2, or TLR4 in the pFLAG-CMV-1 vector (a gift from C. Kirschning and M. Rothe, Tularik Inc., South San Francisco, CA (11)) as described (38). All CHO reporter cell lines were grown in Ham's F-12 medium containing 10% FBS, 10 $\mu\text{g}/\text{ml}$ ciprofloxacin, and 400 units/ml hygromycin B. The TLR expressing cell lines contained additional selection antibiotics (for CD14/TLR2, 0.5 mg/ml G418; for CD14/TLR1 and CD14/TLR4, 50 $\mu\text{g}/\text{ml}$ puromycin). A CHO/CD14 reporter cell line with defects in the LPS signaling pathway (clone 7.7 (43)) was stably transfected with TLR2 or TLR4 using calcium phosphate as described elsewhere⁴ and grown in the presence of hygromycin and G418.

Flow Cytometry Analysis—Cells were plated at a density of 1×10^5 cells/well in 24-well dishes. The following day, the cells were stimulated as indicated in Ham's F-12 medium containing 10% FBS (total volume of 0.25 ml/well). Subsequently, the cells were harvested with trypsin-EDTA and labeled with fluorescein isothiocyanate anti-CD25 in PBS, 1% FBS for 30 min on ice. After labeling, the cells were washed once and resuspended in PBS, 1% FBS containing propidium iodide to exclude dead cells. The cells were analyzed by flow cytometry using a FACScan microfluorometer (Becton Dickinson).

Peritoneal Macrophages—Ten-week-old Chinese hamsters (Cytogen Research and Development, West Roxbury, MA) and C3H/OuJ mice (The Jackson Laboratory) were injected intraperitoneally with 2 ml of 3% thioglycollate (Sigma). After 3 days, peritoneal exudate cells were harvested by lavage with 7 ml of RPMI 1640 medium containing 10% FBS and 10 $\mu\text{g}/\text{ml}$ ciprofloxacin. The cells were washed with medium, counted, and plated at a density at 1.2×10^6 cells/well in six-well dishes, followed by overnight incubation. The nonadherent cells were then removed by washing with medium. Two days after harvesting, the cells were washed twice with medium and stimulated for 1 h. Nuclear extracts were isolated and analyzed for binding to a ³²P-labeled NF- κB specific oligonucleotide by electrophoretic mobility shift assay, as described (35).

Isolation of Peripheral Blood Mononuclear Cells (PBMC) and Measurement of TNF—Human PBMC were isolated by gradient centrifugation of heparinized blood on Histopaque® 1077 (Sigma) according to the manufacturer's protocol. The cells were resuspended in RPMI 1640 medium containing 10% human serum and plated at a density of $7 \times$

² T. H. Flo, Ø. Halaas, E. Lien, L. Ryan, G. Teti, D. T. Golenbock, A. Sundan, and T. Espevik, submitted for publication.

³ Means, T. K., Wang, S., Lien E., Yoshimura, A., Golenbock, D. T. and Fenton, M. J. (1999) *J. Immunol.* **163**, 3920–3927 and Brightbill, H. D., Libraty, D. H., Krutzik, S. R., Yang, R. B., Belisle, J. T., Blehariski, J. R., Maitland, M., Norgard, M. V., Plevy, S. E., Smale, S. T., Brennan, P. J., Bloom, B. R., Godowski, P. J. and Modlin, R. L. (1999) *Science* **285**, 732–736

⁴ A. Yoshimura, H. Heine, and D. T. Golenbock, manuscript in preparation.

10⁵ cells/well in a 96-well dish. Immediately before stimulation with the indicated compounds, 1:5 (v/v) dilutions of a hybridoma supernatant containing the TLR2-specific antibody TL2.1² or a control antibody (mouse IgG, Sigma, diluted in hybridoma medium), to a final antibody concentration of 5 µg/ml, was added. The cells were stimulated for 12 h, and cell-free supernatants were harvested and analyzed for TNF α release by enzyme-linked immunosorbent assay (matching antibody pair from Roche Molecular Biochemicals).

RESULTS

TLR2, but Not TLR4 or TLR1, Imparts Cellular Activation by *B. burgdorferi*, *T. pallidum*, and *M. fermentans* Lipoproteins and Lipopeptides—In order to test the potential role of TLRs in *B. burgdorferi* and *T. pallidum* infections, we constructed several TLR-expressing reporter cell lines in a CHO fibroblast background that contained an inducible NF- κ B-dependent promoter driving the surface expression of membrane CD25 (43). Thus, the induction of proinflammatory activity could be quantified by flow cytometry. We exposed CHO/CD14, CHO/CD14/TLR1, CHO/CD14/TLR2, and CHO/CD14/TLR4 reporter cell lines to the purified native *B. burgdorferi* outer surface protein A (nOspA), a synthetic lipohexapeptide based upon the N terminus of the 47-kDa major *T. pallidum* lipoprotein (47L), and a synthesized version of the sMALP-2 full-length *M. fermentans* lipopeptide. All of the cell lines were engineered to express CD14, thereby conferring responsiveness to LPS, as indicated by increased membrane expression of the CD25 reporter transgene. Cells expressing TLR2 were activated by lipoprotein or lipopeptide structures (Fig. 1A). In contrast, CHO/CD14, CHO/CD14/TLR1, and CHO/CD14/TLR4 cells were not activated by any of the spirochetal molecules. These results also illustrate an important point concerning the purity of our preparations. Lack of stimulation of the highly LPS-sensitive CHO/CD14 line is strong evidence against the possibility that environmental endotoxin contaminated our preparations.

The N terminus of mature *B. burgdorferi* and *T. pallidum* lipoproteins consists of a diacylglycerol moiety in thioether linkage to a cysteine residue and a third fatty acid amide-linked to the α -amino group of the cysteine (19). In contrast, *M. fermentans* MALP-2 possesses an *N*-acyl-*S*-diacylglycerol cysteine with a free N terminus (31). Several reports demonstrate dependence on lipid modification for both *in vivo* and *in vitro* cellular activation by *B. burgdorferi*, *T. pallidum*, and *M. fermentans* lipoproteins and synthetic lipopeptides (22, 23, 29, 32). As shown in Fig. 1B, only lipidated peptides (*B. burgdorferi* OspCL, OspAL, *T. pallidum* 47L, and *M. fermentans* sMALP-2) activated the CHO/CD14/TLR2 reporter cell line, whereas the nonlipidated peptides completely lacked stimulatory activity. These data demonstrate that TLR2 mediates cellular activation by lipoproteins/lipopeptides and that the *N*-acyl-*S*-diacylglycerol moiety appears to be more important than the amide-linked fatty acid for their biological activity.

The Lack of TLR4 Activity after Lipoprotein / Lipopeptide Exposure Is Due to the Lack of Ligand-specific Recognition—Although the inability of the CHO/CD14/TLR4 cell line to respond to lipoproteins and lipopeptides may reflect the fact that TLR4 is not involved in lipoprotein recognition, it is possible that these cell lines expressed a nonfunctional TLR4. Control conditions were difficult to establish, because LPS already activates CHO/CD14 cells through the endogenous hamster TLR4. Therefore, an alternative approach was employed to confirm the functionality of the transfected TLR4 protein before concluding that bacterial lipoproteins and lipopeptides were not TLR4 ligands.

Our laboratory has recently described CHO/CD14 cells with a genetic defect in LPS, but not in IL-1- or TNF-induced signal transduction (43). These cells respond to LPS after transfection with TLR2 or TLR4, as these Toll proteins bypass their genetic

lesion.⁴ As shown in Fig. 2, transfection with TLR2 enabled the cells to respond to lipopeptides, lipoproteins, and LPS. In stark contrast, TLR4-transfected cells responded to LPS only, demonstrating that the transfected TLR4 is functional in CHO/CD14 cells but will not transduce a signal in response to lipoproteins/lipopeptides. These data suggest that TLR2 is able to serve as a receptor for a broad repertoire of bacterially derived ligands, whereas TLR4 appears to be a more specific receptor for LPS.

TLR2 Mediates Cellular Responses upon Exposure to Live *B. burgdorferi*—Similar to spirochetal lipoproteins/lipopeptides, live *B. burgdorferi* and *T. pallidum* activated monocytic cells but failed to stimulate CHO/CD14 cells (30). These findings are one of several pieces of evidence supporting the hypothesis that live spirochetes and their constituent lipoproteins activate cells by similar, if not identical, mechanisms. In light of these results and the above observations it was of interest to test whether motile spirochetes signal through TLR2. We found that only TLR2-transfected cells were activated upon exposure to *B. burgdorferi* (Fig. 3A), whereas CHO/CD14/TLR4 cells remained insensitive to spirochetal challenge (data not shown). Experiments with fluorescein isothiocyanate-labeled *B. burgdorferi* showed a similar high degree of binding of the spirochete to all cell lines (data not shown), indicating that membrane attachment was not sufficient to initiate cellular responses. Again, motile *B. burgdorferi* stimulated the TLR2-transfected LPS nonresponder mutant CHO/CD14 cells, whereas TLR4-transfected cells were enabled to respond to LPS, but not to the spirochetes (Fig. 3B). Thus, the recognition of lipopeptides and lipoproteins by TLR2 appears to be relevant to the responses observed during natural infection in man. These results demonstrate that TLR2 but not TLR4 mediates responses to whole *B. burgdorferi* and that TLR4 is unlikely to be involved in responses to spirochetes.

TLR2 Is a Pattern Recognition Receptor—Many microbial infections induce similar clinical symptoms, which may reflect similarities in host responses to invasion. Recent observations suggest that bacterial cell wall structures, such as peptidoglycan from Gram-positive organisms (38, 39), are able to signal through TLR2. *M. avium* is an opportunistic pathogen, which leads to serious complications in HIV-1 disease; patients with *M. avium* experience profound fevers, diffuse pains, and generalized wasting (44). Recent observations suggest that structures from *M. avium* activate the LPS signaling pathway by utilizing CD14 (45). We exposed the transfected fibroblasts to live *M. avium* and killed *S. aureus* and *E. coli* in order to determine whether there were similarities in utilization of TLR2 by bacteria containing different membrane constituents. The patterns of response demonstrated the following (Fig. 4): CHO cells required expression of CD14 in order to respond to Gram-negative cell wall products. However, cells that co-expressed CD14 with TLR2 were capable of responding to stimulation by all the bacteria tested, including the atypical mycobacterium *M. avium* and the Gram-positive bacterium *S. aureus*. Hence, although they are phylogenetically diverse and contain a variety of proinflammatory constituents, *M. avium*, *S. aureus*, *B. burgdorferi*, *T. pallidum*, and *M. fermentans* all appear to activate cells through the same receptor system.

TLR2-null Chinese Hamster Macrophages Fail to Respond to Lipoproteins / Lipopeptides—Chinese hamster macrophages respond to LPS, although they do not express mRNA for a full-length TLR2 (37). Sequence analysis of TLR2 from the Chinese hamster, compared with human and mouse TLR2, revealed a single base pair deletion that resulted in a frameshift mutation; this mutation encodes a protein fragment devoid of transmembrane and intracellular domains. In contrast, CHO/CD14

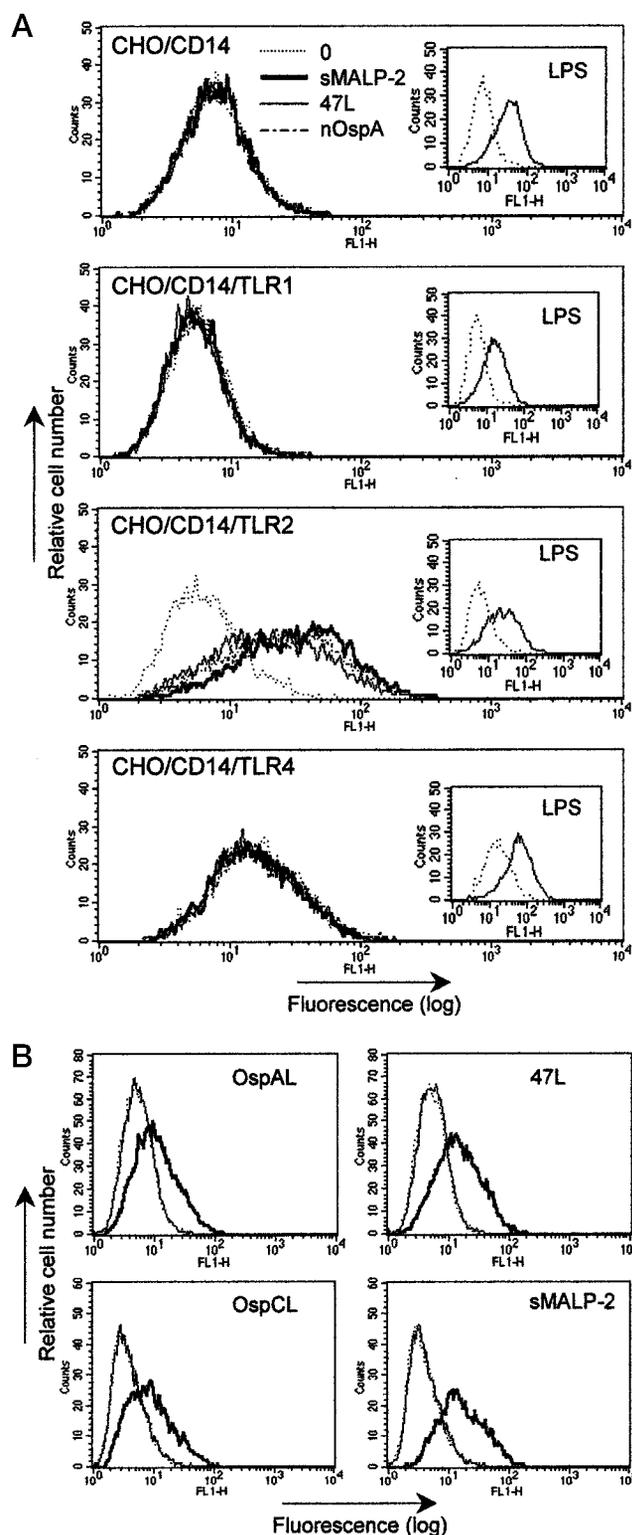


FIG. 1. Expression of TLR2 in CHO cells confers responsiveness to *B. burgdorferi* natural lipoprotein nOspA, *T. pallidum* synthetic lipopeptide 47L, and *M. fermentans* synthetic lipopeptide sMALP-2. A, CHO/CD14, CHO/CD14/TLR1, CHO/CD14/TLR2 and CHO/CD14/TLR4 reporter cells were incubated with medium alone (dotted lines), *B. burgdorferi* nOspA (broken lines) (300 ng/ml), *T. pallidum* 47L (thin lines) (10 μ g/ml), *M. fermentans* sMALP-2 (thick lines) (25 nM) and LPS (insets, thin lines) (100 ng/ml) for 20 h. Activation of NF- κ B was measured by the appearance of reporter transgene (surface CD25) by flow cytometry. Relative cell number is given on the y axis and fluorescence on the x axis. B, the CHO/CD14/TLR2 reporter cell line was exposed to 3.4 μ M synthetic lipopeptides OspAL and 47L, 6.8 μ M OspCL, and 100 nM sMALP-2 or similar amounts of the corresponding unlipidated hexapeptides as described in A. Cells stimulated with lipopeptides are indicated by the thick lines; the corresponding unlipi-

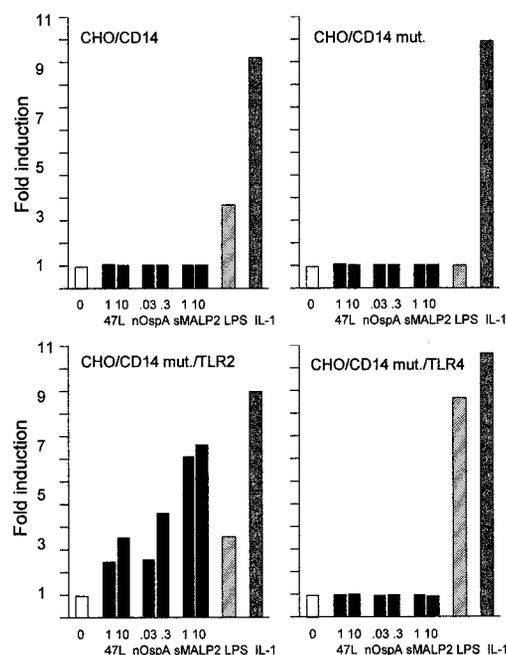


FIG. 2. Transfection of a mutant LPS nonresponder CHO/CD14 cell line with TLR2, but not TLR4, renders the cells responsive to bacterial lipoproteins and lipopeptides. LPS nonresponder mutant CHO/CD14 cells (CHO/CD14 mut.) (43) were transfected with TLR2 or TLR4. Clonal derivatives were analyzed in comparison to LPS responsive CHO/CD14 cells, and the untransfected mutant cell line for responses to IL-1 (gray bars), LPS (hatched bars), or lipoprotein/lipopeptides (black bars). 0 signifies no treatment (open bars); concentrations for 47L and nOspA are given in μ g of ligand per ml; sMALP-2 in nM, LPS was used at 100 ng/ml, and IL-1 was at 1 ng/ml. Cells were stimulated for 20 h. The cells were then stained for reporter transgene expression and analyzed by flow cytometry. Activation is expressed as the fold induction of median channel fluorescence in comparison to unstimulated cells. Shown is one representative experiment out of two performed.

cells and macrophages from Chinese hamsters appear to have a full-length and functional TLR4.⁵ We isolated peritoneal macrophages from Chinese hamsters in order to test the action of lipoproteins/lipopeptides toward TLR2-null primary phagocytes. We found that the hamster macrophages responded to LPS, but not to nOspA or 47L, as measured by nuclear translocation of NF- κ B (Fig. 5A). In contrast, macrophages from C3H/OuJ mice responded to LPS, nOspA, and 47L. These results suggested that the lack of TLR2 in primary Chinese hamster macrophages made them unable to recognize bacterial lipoproteins and lipopeptides.

The anti-TLR2 mAb TL2.1 Inhibits Lipoprotein/Lipopeptide and M. avium-induced Release of TNF from Human Peripheral Blood Mononuclear Cells—In order to determine whether our findings in transfected cell lines reflect the signal transduction systems used by native phagocytes, we stimulated freshly isolated human PBMC with nOspA, 47L, and *M. avium* in the presence of the TLR2 antibody TL2.1. As shown in Fig. 5B, TL2.1 inhibited TNF production from PBMC after exposure to nOspA, 47L, and live *M. avium* by 40–70%. These data support the hypothesis that TLR2 may play an important role in *in vivo* responses to various bacterial structures. In the presence of TL2.1, LPS-induced responses in primary cells were only min-

⁵ H. Heine, E. Lien, B. Monks, and D. T. Golenbock, unpublished observations.

dated peptides are represented by thin lines, and unstimulated cells are represented by dotted lines. Shown is one representative experiment out of four performed.

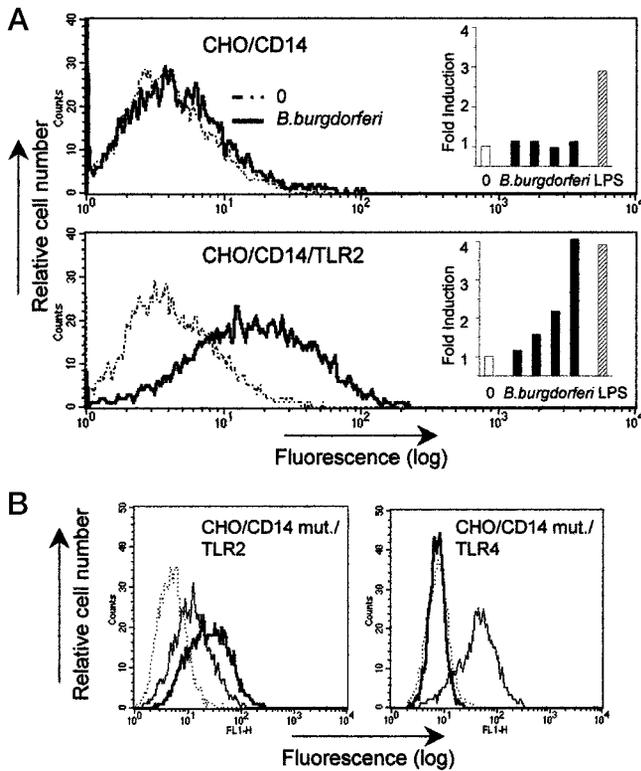


FIG. 3. TLR2 mediates cellular activation upon exposure to live *B. burgdorferi*. A, CHO/CD14 or CHO/CD14/TLR2 cells were left untreated (dotted lines) or exposed to motile *B. burgdorferi* (thick lines) (1000 spirochetes/cell = 2.5×10^7 spirochetes/ml) for 8 h. The cells were harvested, stained for reporter gene expression, and analyzed by flow cytometry, as in Fig. 1. Indicated in insets is the fold increase of median fluorescence relative to unstimulated cells. From left to right: untreated cells (0, open bars), cells exposed to different doses of live *B. burgdorferi* (black bars) (1, 10, 100, and 1000 spirochetes/cell, respectively) and LPS (hatched bars) (100 ng/ml). B, LPS nonresponder mutant CHO/CD14 cells transfected with TLR2 or TLR4 were exposed to medium (dotted lines), 1000 spirochetes/ml (thick lines), or LPS (thin lines) (100 ng/ml). Shown is one representative experiment out of three performed.

imally reduced (results not shown). Although the relative importance of TLR2 in LPS signaling remains unclear, expression of TLR2 (unlike TLR4) does not appear to be required for cell responses to low concentrations of LPS (37).

DISCUSSION

The severity of clinical symptoms associated with bacterial diseases varies according to the type of infectious agent, bacterial burden, affected tissue, and co-existing illness. Nevertheless, in many aspects, similar host responses are observed. For example, several clinical and immunological similarities can be seen between therapy-induced Jarisch-Herxheimer reaction during infection with *Treponema* and *Borrelia* spp. (46, 47) and Gram-negative and Gram-positive sepsis (48). Hence, one is tempted to speculate that the pathophysiological similarities observed with these diverse infections are due to the activation of analogous signaling pathways in response to bacterial exposure. The present study implicates TLR2 in host interactions with *B. burgdorferi*, *T. pallidum*, *M. fermentans*, and *M. avium*, as well as components of Gram-negative and Gram-positive bacteria. Thus, this receptor can mediate host inflammatory reactions to a variety of microbial pathogens, indicating a remarkable spectrum of bacterial recognition.

Previous reports have identified mechanisms of cellular activation by many microbial structures that are similar, yet never identical, to the LPS signaling pathway. In most cases, the reported observations concerned the ability of the microbes

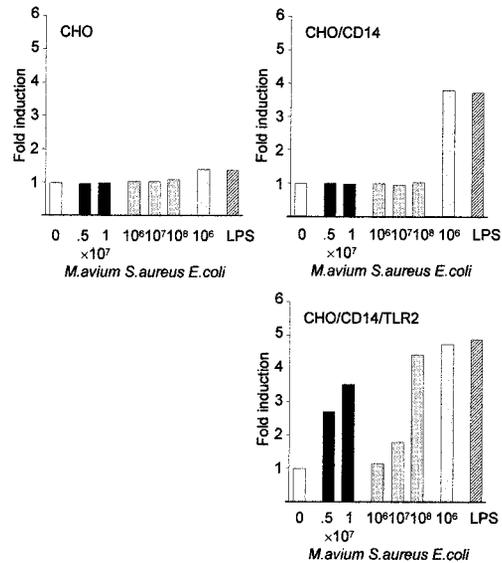


FIG. 4. Microbial pattern recognition via CD14 and TLR2. CHO control, CHO/CD14, or CHO/CD14/TLR2 reporter cell lines were exposed to the following stimuli (from left to right): medium (0, open bars), live *M. avium* (black bars), heat-killed *S. aureus* (dark gray bars), *E. coli* bioparticles (light gray bars), or LPS (hatched bars) (100 ng/ml). Numbers indicate the density of the bacteria per ml. After 20 h, the cells were harvested, stained for reporter gene expression, and analyzed by flow cytometry, as in Fig. 1. The y axis indicates fold increase of median fluorescence compared with unstimulated cells. Shown is one representative experiment out of three performed.

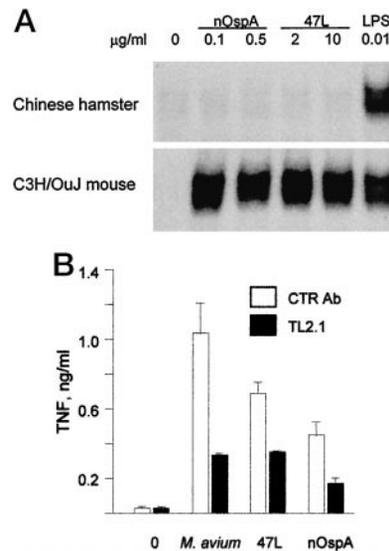


FIG. 5. TLR2 mediates responses to lipoproteins/lipopeptides in primary cells. A, TLR2-null peritoneal macrophages from Chinese hamsters and C3H/OuJ mice were stimulated with nOspA, 47L, and LPS for 1 h in RPMI 1640 medium containing 10% FBS. Nuclear extracts were isolated and analyzed for binding to a NF- κ B specific probe by electrophoretic mobility shift assay. Shown is the NF- κ B band, in one representative experiment out of two performed. B, human PBMC were isolated by gradient centrifugation, resuspended in RPMI 1640 medium containing 10% human serum, and plated at a density of 7×10^5 cells/well in a 96-well dish. The mouse anti-human TLR2 antibody TL2.1 or control antibodies (mouse IgG) were added to a final concentration of 5 $\mu\text{g/ml}$, and the cells were exposed to *M. avium* (5×10^6 bacteria/ml), 47L (1 $\mu\text{g/ml}$), or nOspA (300 ng/ml) in a total volume of 0.2 ml for 12 h. The supernatants were harvested and assayed for TNF by immunoassay. The antibody did not block activation induced by phorbol ester (not shown). Data are from one representative experiment out of three performed. Shown is the mean of duplicate wells \pm S.D.

to utilize CD14. In addition to being a high affinity receptor for LPS, CD14 has been implicated in the responses to several bacteria and their microbial products, including *Borrelia* and

TABLE I
Bacterial strains and compounds reported to activate cells via TLR2

Organisms	Reference	Stimulus tested
Gram-negative bacteria	Yang <i>et al.</i> (10)	LPS (various sources), lipid A
	Kirschning <i>et al.</i> (11)	LPS (various sources), lipid A
Gram-positive bacteria	Yoshimura <i>et al.</i> (38)	<i>S. aureus</i> , <i>S. pneumoniae</i> ; peptidoglycan
	Schwandner <i>et al.</i> (39)	<i>S. aureus</i> , <i>B. subtilis</i> , <i>Streptococcus</i> sp.; peptidoglycan, lipoteichoic acid
	Footnote 2	<i>Listeria monocytogenes</i>
	Present study	<i>S. aureus</i>
Mycobacteria	Footnote 3	<i>Mycobacterium tuberculosis</i> ; ara-lipoarabinomannan
	Present study	<i>M. avium</i>
Spirochetes	Aliprantis <i>et al.</i> (60) Hirschfeld <i>et al.</i> (61) Present study	<i>B. burgdorferi</i> ; lipoproteins, lipopeptides from <i>T. pallidum</i> and <i>Borrelia</i>
Mycoplasmas	Present study	Lipopeptide from <i>M. fermentans</i>

Treponema sp. (21, 25), peptidoglycan, and other cell wall components of *S. aureus* (14, 49), group B streptococci (50), structures from mycobacteria (14, 45, 51, 52), and mannuronic acid polymers from *Pseudomonas aeruginosa* (53). Because it can facilitate responses to all of these bacterial structures listed, CD14 has been termed a pattern recognition receptor by Pugin *et al.* (14). Yet CD14 lacks specificity in bacterial product recognition, and some controversy exists about whether CD14 is a true pattern recognition receptor (54). The identification of TLR2 in the recognition of most of these pathogens adds another layer of complexity to our understanding of the mammalian response to microbes. In contrast to CD14, TLR2 contains all of the characteristics that one would expect from a true pattern recognition receptor, including the presence of a true signal-transducing intracellular domain. Although only recently described, the list of putative ligands for TLR2 is already impressively large (Table I). Of particular interest is the observation that despite the apparent interactions of TLR2 with many Gram-positive bacteria, group B streptococci do not seem to stimulate cells through this receptor.² This highlights the fact that we cannot exclude the involvement of additional receptors, functioning either alone or as part of a receptor complex, in host responses to the microbial structures described.

Although TLR2 has the features of a pattern recognition receptor, it is difficult to define a common microbial pattern among all of these putative ligands. The list of TLR2 ligands is still not complete, and there is no evidence yet that TLR2 directly binds these microbial products. Thus, attempting to define the biophysical properties responsible for TLR2/ligand interactions may be premature. Nevertheless, we hypothesize that elements of amphipathicity may prove to be the most important for the stimulation of cells through TLR2. All of the lipoproteins/lipopeptides tested in this study activated TLR2-expressing cells, and acylation of the spirochetal proteins was the critical modification that enabled their activation of TLR2 (Fig. 1B). Other putative TLR2 ligands, including peptidoglycan, may also have amphipathic characteristics that are not yet appreciated.

Both TLR2 (10, 11, 37) and TLR4 (12, 55) have been reported to function as LPS signal transducers. Our data support these conclusions, although they suggest that the two related proteins clearly have different roles in pathogen recognition: TLR4 is required for sensitive responses to LPS, whereas TLR2 is not. For example, cells from Chinese hamsters, which express a truncated and nonfunctional TLR2 (37) but a full-length TLR4,⁵ respond to LPS but not to lipoproteins/lipopeptides. This contrasts with the finding that TLR4 is responsible for the LPS nonresponder phenotype of the C3H/HeJ mouse (12). Although these mice fail to respond to low concentrations of LPS,

the ability of *Borrelia* spirochetes and lipoproteins to activate the C3H/HeJ mice (23, 24, 27, 56) demonstrates that these ligands do not require TLR4 expression to elicit productive responses and strongly suggests a functional TLR2 in these animals. In a broad sense, the accumulated data indicate that the preferential utilization of TLRs underlies both the observed similarities, as well as the differences, in specific pathogen recognition.

What remains unclear is why, if TLR2 is expressed in phagocytic cells under resting conditions, TLR4 mutant mice (C3H/HeJ and C57BL10/ScCr (12)) are not still sensitive to LPS via the signal transduction capabilities of TLR2. It may be that the levels of TLR2 expression in native phagocytes, in contrast to transfected cells, are insufficient to enable LPS responses in the mice. We note that chronically stimulated C3H/HeJ mice have been reported to exhibit immune activation in response to LPS challenge (57), an effect that may be due to the up-regulation of TLR2. Furthermore, the present data do not rule out the possibility that TLR2 may have a more important function in LPS recognition by nonphagocytic cells.

The downstream signaling molecules involved in TLR-mediated cellular activation have not been definitively defined. However, both TLR2 and TLR4 have a cytoplasmic domain that is homologous to the IL-1 receptor. Thus, it is likely that both TLRs activate the NF- κ B pathway, and perhaps other proinflammatory pathways as well, via their interactions with IL-1 receptor signaling genes, including MyD88, TRAF6, and IRAK (11, 58, 59). The similarities in the signal transduction process that appear to constitute the inflammatory response to invasion by a variety of bacteria suggest the exciting possibility that novel therapies directed against the harmful proinflammatory response to nearly all forms of infectious illnesses can one day be developed.

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and Douglas T. Golenbock
J. Biol. Chem. 1999, 274:33419-33425.
doi: 10.1074/jbc.274.47.33419

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