The Journal of Immunology
RESEARCH ARTICLE | NOVEMBER 01 2005

# Differential TLR Recognition of Leptospiral Lipid A and Lipopolysaccharide in Murine and Human Cells[1] FREE

Marie-Anne Nahori; ... et. al

*J Immunol* (2005) 175 (9): 6022–6031.
https://doi.org/10.4049/jimmunol.175.9.6022

# Differential TLR Recognition of Leptospiral Lipid A and Lipopolysaccharide in Murine and Human Cells[1]

**Marie-Anne Nahori,* Edith Fournié-Amazouz,* Nanette S. Que-Gewirth,† Viviane Balloy,‡ Michel Chignard,‡ Christian R. H. Raetz,† Isabelle Saint Girons,* and Catherine Werts[2]*§**

***Leptospira interrogans*** **is a spirochete that is responsible for leptospirosis, a zoonotic disease. This bacterium possesses an unusual LPS that has been shown to use TLR2 instead of TLR4 for signaling in human cells. The structure of its lipid A was recently deciphered. Although its overall hexa-acylated disaccharide backbone is a classical feature of all lipid A forms, the lipid A of *L. interrogans* is peculiar. In this article, the functional characterization of this lipid A was studied in comparison to whole parental leptospiral LPS in terms of cell activation and use of TLR in murine and human cells. Lipid A from *L. interrogans* did not coagulate the *Limulus* hemolymph. Although leptospiral lipid A activated strongly murine RAW cells, it did not activate human monocytic cells. Results obtained from stimulation of peritoneal-elicited macrophages from genetically deficient mice for TLR2 or TLR4 clearly showed that lipid A stimulated the cells through TLR4 recognition, whereas highly purified leptospiral LPS utilized TLR2 as well as TLR4. In vitro experiments with transfected human HEK293 cells confirmed that activation by lipid A occurred only through murine TLR4-MD2 but not through human TLR4-MD2, nor murine or human TLR2. Similar studies with parental leptospiral LPS showed that TLR2/TLR1 were the predominant receptors in human cells, whereas TLR2 but also TLR4 contributed to activation in murine cells. Altogether these results highlight important differences between human and mouse specificity in terms of TLR4-MD2 recognition that may have important consequences for leptospiral LPS sensing and subsequent susceptibility to leptospirosis.** ***The Journal of Immunology,*** **2005, 175: 6022–6031.**

*Leptospira interrogans* is a spirochete responsible for a zoonotic disease, leptospirosis. This increasingly common disease occurs in highly populated, poor urban centers where flooding frequently occurs (1). Rodents constitute the main reservoir and excrete asymptomatically the bacteria in their urine. Humans get infected through contaminated water. Human leptospirosis reflects many different forms, from a flu-like syndrome to multiorgan failure leading to death. *Leptospira* species are peculiar because they possess LPS, which is a cell surface component, missing in other spirochetes such as the strict parasitic species *Borrelia* or *Treponema* (2).

Innate immune responses against microbes occur through stimulation of germline-encoded proteins called pattern recognition receptors that have evolved to recognize conserved microbial motifs called pathogen-associated molecular patterns such as peptidoglycan, LPS, or bacterial DNA CpG sequences. Among pattern recognition receptors, two families play a critical role in innate immune defense. First to be discovered were the TLRs, a membrane-bound protein family characterized by an extracellular leucine-rich repeat domain and a cytoplasmic Toll/IL-1R homology domain. More recently, the cytosolic nucleotide-binding oligomerization domain proteins of the NLR family have been shown to be intracellular sensors of peptidoglycan motifs (for review, see Ref. 3). Among the 10 TLRs present in humans, TLR4 has been the best characterized. At the cell surface, LPS from classical enterobacteria first binds to CD14, a GPI anchor or soluble protein that delivers the LPS to TLR4 associated with an accessory molecule MD2 (4). Subsequent recruitment of the intracellular adaptor protein MyD88 through homotypic Toll/IL-1R domains leads to intracellular signaling through NF-κB and MAPKs activation. The ultimate result of this LPS recognition is a proinflammatory cytokine and chemokine response.

Genetic data from mouse studies demonstrate that the sole receptor for classical enterobacterial LPS is indeed TLR4 (5). Enterobacterial LPS preparations have been shown to be frequently contaminated with lipoproteins, which are agonists responsible for TLR2 stimulation (6). An efficient method of repurification of LPS has been designed to clean LPS from such lipoprotein contaminants (7). We previously showed that LPS from *Leptospira* cleaned according to the above-cited method was atypical and activated human cells through CD14 and TLR2 recognition (8). Other examples of atypical LPS prepared from *Rhizobium*, *Legionella* (9), or *Porphyromonas gingivalis* (10) have also been reported to signal through TLR2.

Lipid A is the anchor moiety of LPS in the bacterial membrane and is the active component of LPS responsible for its toxic activity and functions. Lipid A from *Escherichia coli* or *Salmonella* species stimulates the TLR4 receptor complex. However, lipid A structures show some variability between bacterial species; the degree of acylation, phosphorylation, or the length of acyl chains of lipid A can be responsible for modified proinflammatory capacities of LPS (for review, see Ref. 11). To understand the structure/activity relationship of leptospiral LPS, we recently deciphered the structure of leptospiral lipid A that exhibits unique features (12). In the present work, we aimed to examine the proinflammatory properties of leptospiral lipid A, as compared with the whole LPS, and to determine which TLR was responsible for cell activation.

*Laboratoire des Spirochètes, Institut Pasteur, Paris, France; †Department of Biochemistry, Duke University Medical Center, Durham, NC 27710; ‡Unité de Défense Innée et Inflammation, Institut National de la Santé et de la Recherche Médicale E336, Institut Pasteur, Paris, France; and §G5 Immunité Innée & Signalisation, Institut Pasteur, Paris, France

Received for publication March 21, 2005. Accepted for publication August 11, 2005.

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.

[1] This work was supported by Grant PTR94 from the Institut Pasteur. C.R.H.R. and N.S.Q.G. are supported by National Institutes of Health Grant R37-GM-51796.

[2] Address correspondence and reprint requests to Dr. Catherine Werts, G5 Immunité Innée & Signalisation. Institut Pasteur, 28 rue du Dr Roux. 75015 Paris, France. E-mail address: cwerts@pasteur.fr



0022-1767/05/$02.00

## Materials and Methods

### *Mice*

Female mice 6- to 10-wk old were used for this study. C57BL/6J were purchased from Janvier. Mice deficient for TLR2 or TLR4 were initially provided by S. Akira (Osaka University, Osaka, Japan) and have been further backcrossed eight times into C57BL/6J. Mice were housed in the same animal facility in the Institut Pasteur for at least 2 wk before experiments. Double-deficient mice for TLR2 and TLR4 were generated and confirmed by genotyping. Mice were submitted to sanitary control tests at the CDTA (Orléans, France) to ensure proper pathogen-free status. All protocols were reviewed by the Institut Pasteur competent authority for compliance with the French and European regulations on Animal Welfare and with Public Health Service Recommendations.

### *Reagents*

Endotoxin-free FCS was from purchased HyClone and used after heat inactivation at 56°C for 30 min. All cell culture reagents and antibiotics were obtained from Invitrogen Life Technologies. Abs against MAPKs (p38 and ERK1/2) and their phosphorylated forms and anti-rabbit IgG HRP-linked Ab were purchased from Cell Signaling Technology and used as recommended by the manufacturer. TLR2 agonist $Pam_3cysSK_4$ was obtained from EMC microcollections. *E. coli* LPS 0111:B4 was purchased from InvivoGen and repurified according to Hirschfeld's procedure (7). LPS Re595 from *Salmonella minnesota*, already ultra-purified, was purchased from Alexis. Crude preparations of LPS and lipid A from *E. coli* 0119 were a gift from M. Caroff (Orsay University, Orsay, France). Lipid A from *L. interrogans* was purified as described previously (12). All lipids A and LPS were kept frozen at 1 mg/ml at −20°C and thoroughly vortexed after thawing and sonicated before use. *Staphylococcus aureus* strain RN4220 was from the collection of the Institut Pasteur. This strain was grown overnight at 37°C in complete brain-heart infusion medium (Invitrogen Life Technologies) to a density of $5 \times 10^9$ bacteria/ml, then washed twice in endotoxin-free PBS, resuspended to $10^{10}$ bacteria/ml, and then heat killed by boiling for 10 min. Heat-killed *S. aureus* (HKSA)[3] was kept frozen at −20°C.

### *Leptospiral LPS and lipid A*

*Leptospira interrogans* icterohemorragiae strain Verdun was obtained from the Institut Pasteur collection. Two bacterial isolates were used to prepare leptospiral LPS as described previously (8). Leptospiral LPS, termed Lv and Lav, were obtained, respectively, from the virulent strain, pathogenic for the young guinea pig, and from the avirulent strain. Leptospiral LPS was repurified according to Hirschfeld's method using phenol re-extraction in the presence of deoxycholate to eliminate lipoprotein contaminants (7). We recovered the leptospiral LPS from the phenol phase (instead of the aqueous phase) and dialyzed it extensively. To be sure that this procedure led to highly purified leptospiral LPS without residual active contaminant, we repurified in parallel a lipid A from *E. coli* (0119) that we found initially to activate through TLR2 and checked its phenol phase after dialysis on TLR-transfected HEK293 cells (data not shown). As expected, the repurified lipid A from *E. coli*, recovered from the aqueous phase, activated through TLR4 and no longer through TLR2, whereas the phenol phase was not active toward TLR2. This suggested that if a contaminant lipoprotein was still present in the phenol phase containing the leptospiral LPS, it should be inactive toward TLR2.

### *Cells*

RAW264.7, a murine macrophage cell line, was purchased from American Type Culture Collection (TIB-71) and was grown in DMEM/glutamax supplemented with 10% FCS. THP1-hCD14, a monocytic cell line stably expressing human CD14, was provided by R. Ulevitch (Scripps, San Diego, CA) and maintained in RPMI 1640 with 10 mM HEPES supplemented with 2 mM glutamine and 10% FCS. Human embryonic kidney cells (HEK293T) were provided by D. Philpott (Institut Pasteur, Paris, France) and grown in DMEM with 10 mM HEPES 10 mM supplemented with 2 mM glutamine and 10% FCS.

Human PBMC from healthy volunteers were obtained and provided by J. Fritz (13) as described previously. Human monocytes were obtained by adherence for 90 min of $3 \times 10^6$ PBMC in 24-well plates in RPMI 1640 without serum supplemented with antibiotics. Nonadherent cells were removed by washing and monocytes were stimulated for 24 h in 500 $\mu$l of RPMI 1640 medium supplemented with 10% human serum from BioWhittaker.

Mouse peritoneal macrophages were elicited by injection of 1.5 ml of thioglycolate medium (Bio-Rad) in the peritoneal cavity 4 days before peritoneal lavage with 5 ml of PBS complemented with heparin choay (10 U/ml; Sanofi). Cells were pooled from five to six mice then centrifuged ($256 \times g$, 5 min, room temperature). Cells were suspended to $10^6$ cells/ml in RPMI 1640/3% FCS and seeded in 24-well plates. After 90 min of incubation (37°C, 5% CO2), cells were thoroughly washed with PBS to remove nonadherent cells and 500 $\mu$l of RPMI 1640/0.2% FCS/penicillin 100 U/ml/streptomycin 100 $\mu$g/ml/260 ng/ml amphotericin B were added. Cells were left at least 2 h before being stimulated in duplicates or triplicates. After 18 h of stimulation, supernatants were aliquoted and frozen at −20°C for subsequent cytokine dosage.

### *Cytokine dosage*

The concentration of specific cytokines released into the medium of cells were measured by commercially available ELISA kits. Murine cytokines (IL-10, TNF, IL-6) were measured using BD Pharmingen opt EIA kits. Human cytokines (TNF, IL-8, IL-10) were measured using R&D Systems duosets.

Standards were included in every plate and the samples were tested at least in duplicates. Peroxidase activity was revealed using tetramethylbenzidine substrate from Kirkegaard and Perry Laboratories, and reaction was stopped with 1 N HCl according to the manufacturer's recommendations.

### *Immunobloting*

After stimulation (15–60 min), $2 \times 10^6$ peritoneal macrophages plated in 12-well plates were washed twice with cold PBS, scraped, transferred into microtubes, and centrifuged ($3000 \times g$, 10 min, 4°C). Supernatants were discarded and cells were lysed on ice with 50 $\mu$l of lysis buffer (62.5 mM Tris-HCl (pH 6.8), 1% SDS, 12.5% glycerol, 50 mM DTT, 2-ME, and 0.01% bromphenol blue). Samples were boiled for 10 min and 5 $\mu$l was electrophoresed on 10% SDS-PAGE gels. Fractionated proteins were then electrotransferred onto nitrocellulose membrane from Schleicher and Schüll Microscience and probed by immunoblotting using specific Abs. Bound Abs were detected using an ECL immunoblotting detection system from Amersham.

### *Transfection of cells and luciferase reporter assays*

HEK293 cells were seeded in 24-well plates ($10^5$ cells/well). The day after, cells (reaching 30–50% of confluency) were transiently transfected with a NF-$\kappa$B reporter construct pNF-$\kappa$B-luc from Stratagene along with constructs expressing various TLRs using the FuGene 6 reagent from Roche Diagnostics according to the manufacturer's recommendations. All plasmids were prepared with the Endofree Maxiprep Plasmid kit from Qiagen. Briefly, 75 ng of reporter construct was cotransfected with TLR constructs. For each transfection point, total DNA was adjusted to 300 ng by the empty vector pcDNA3.1. Transfections were performed with 100 ng of human (pUno hTLR1, hTLR2, or hTLR6) or mouse (pUno mTLR2) constructs or phTLR2 construct (provided by D. Philpott; Ref. 14). For TLR4 experiments, we transfected 10 ng of pUno hTLR4 along with 45 ng of pUno hMD2 and 45 ng of pUno hCD14 or 30 ng of pUno mTLR4 along with 30 ng of pUno mMD2 and 30 ng of pUno mCD14. To test heterodimerization, we used 10 ng of pDuo hTLR4-MD2 or pDuo mTLR4-MD2 constructs expressing both TLR4 and MD2 from human or mouse origin, Plasmids pUno and pDuo were obtained from InvivoGen. After 20 h of transfection, cells were stimulated for 6 h in triplicate. Then supernatants were discarded and cells were lysed in 100 $\mu$l of lysis buffer (25 mM Tris (pH 8), 8 mM, $MgCl_2$, 1% Triton X-100, 15% glycerol, and 1 mM DTT) for 5 min at room temperature. Lysed cells (10 $\mu$l) were analyzed for luciferase activity in lysis buffer complemented with 2 mM luciferin and 1 mM ATP from Sigma-Aldrich using a Microlumat plus from Berthold Technologies.

### *Injection of leptospiral LPS in mice*

Lethal effects of leptospiral LPS injection were tested in sensitized mice as described previously (8). Briefly, 5 $\mu$g of sonicated leptospiral LPS (Lv from virulent strain; $LD_{100}$ on C57BL/6J in this protocol) or 0.1 $\mu$g/mouse LPS Re595 ($LD_{100}$ on C57BL/6J in this protocol) were injected i.p. along with 20 mg of D-galactosamine (Accros Organics) in 200 $\mu$l of PBS 45 min after i.v. injection of IFN-$\gamma$ (1.25 $\mu$g/mouse; PeproTech). Lethality of mice was monitored over 24 h. Each experiment was conducted with age-matched mice from both wild-type (WT)- and TLR-deficient mice, with six to eight mice in each lot. At least three independent experiments were performed.

[3] Abbreviations used in this paper: HKSA, heat-killed *S. aureus*; WT, wild type; LAL, *Limulus* amebocyte gelation activity test; EU, endotoxin unit; PEM, peritoneal-elicited macrophage; h, human; m, murine.

### *EMSA*

EMSA were performed on nonadherent THP1-CD14 cells. Cells were stimulated for 60 min, after they reached a density of $2 \times 10^6$ cells/ml. Then cells were washed with cold PBS, harvested, and nuclear extracts prepared as already described (8). Briefly, nuclear extracts (2 $\mu$g of protein content) were incubated with a consensus double-strand NF-$\kappa$B oligonucleotide (5′-AGT TGAGGGGACTTTCCCAGG-3′) from Promega end-labeled with [$\gamma$-$^{33}$P]ATP from NEN. Products were separated by electrophoresis as described previously (8).

### Limulus *amebocyte gelation activity test (LAL)*

LAL was performed using the QCL-1000 from BioWhittaker according to the manufacturer's recommendations. Samples were tested in duplicates. Lipid A was thoroughly vortexed and sonicated before the test. The following concentrations of lipid A from *Leptospira*: 0.1 ng/ml; 10 ng/ml, 1000 ng/ml, and 10 $\mu$g/ml have been tested independently at least three times.

### *Flow cytometry*

HEK293 cells transfected with different TLR constructs for 48 h were harvested by washing with cold PBS. Cells ($3 \times 10^5$) in 50 $\mu$l of PBS/1% BSA were stained for 30 min with 2 $\mu$g/ml IgG2a mAbs TL2.1 from HyCult Biotechnology directed against hTLR2, or isotype control G4C17 directed against *Plasmodium falciparum* heat shock protein 70, provided by T. Blisnick (15). After washing, cells were incubated for 30 min with biotinylated anti-mouse IgG2a (dilution 1/500) from Southern Biotechnology Associates, then with streptavidin coupled to PE (1/500) from BD Pharmingen. Just before analysis using a FACSCalibur System (BD Biosciences), cells were labeled with propidium iodine (0,5 $\mu$g/ml) to gate out the dead cells. Results are expressed as the percentage of positive cells compared with cells transfected only with the NF-$\kappa$B reporter vector. Three independent experiments were performed, and to ascertain the functionality of TLRs, NF-$\kappa$B luciferase reporter assays were performed in parallel after stimulation of the cells.

### *Statistical analysis*

Survival of the different mouse strains was compared using the Kaplan-Meier analysis log rank test. In transfection experiments with luciferase reporter, data were expressed as the mean ± SD. Differences between groups were assessed for statistical significance using the ANOVA test followed by the Fisher test. A value of $p < 0.05$ was considered to be statistically significant.

## Results

### *Lipid A from* Leptospira *does not gelate the* Limulus *amebocyte lysate*

We tested the reactivity of lipid A from *Leptospira* in the LAL assay. The maximum concentration tested (10 $\mu$g/ml) of leptospiral lipid A gave only 0.055 endotoxin units (EU)/ml, which is equivalent to what we found for 1 ng/ml lipid A from *E. coli* 0119 (Table I). All of the other concentrations of leptospiral lipid A tested gave negative results. In our hands, reactivity of lipid A from *E. coli* was more than 100 times less than what we obtained with the parental whole LPS 0119, which, in terms of reactivity, is comparable to the endotoxin provided in the commercial test. We also tested the leptospiral LPS, which produced at 1 $\mu$g/ml less than 0.5 EU/ml. This represents ~20,000-fold less reactivity than LPS from *E. coli*. Therefore, the lack of reactivity of lipid A from *Leptospira* toward the LAL assay is in accordance with the very weak reactivity of whole leptospiral LPS.

Table I. *Lipid A from Leptospira does not react in the LAL assay*[a]

| LPS | EU/ml |
|---|---|
| LPS 0119, 0.1 ng/ml | 1.041 |
| Lipid A 0119, 1 ng/ml | 0.058 |
| LPS *Leptospira,* 1 $\mu$g/ml | 0.488 |
| Lipid A *Leptospira,* 10 $\mu$g/ml | 0.055 |

[a] Results of the LAL assay are expressed in EU/ml for LPS and lipids A from *E. coli* 0119 or *L. interrogans*.

### *Lipid A from* Leptospira *does not stimulate human monocyte cells but stimulates murine RAW cells*

To check that the purified lipid A from *Leptospira* was biologically active, we first tested it on the THP1-CD14 human monocytic cell line. In fact, despite a good NF-$\kappa$B translocation in response to lipid A from *E. coli* or LPS from *Leptospira*, no response was seen for up to 1 $\mu$g/ml lipid A from *Leptospira* (Fig. 1*A*). We next compared cytokine production (TNF) by THP1-CD14 and by the murine macrophage RAW264.7 cell line stimulated the same day using the same agonist dilutions (Fig. 1, *C* and *D*). A good response to lipid A from *Leptospira* was observed in murine RAW cells; however, no TNF production was observed in THP1-CD14 cells (Fig. 1*C*). Leptospiral lipid A also failed to stimulate IL-8 and IL-10 production in THP1-CD14 cells (data not shown). To check that the lack of responsiveness of human cells toward leptospiral lipid A was not peculiar to the THP1 cell line, we stimulated human monocytes from a healthy volunteer and measured TNF and IL-10 production in supernatants. Human monocytes did not respond to lipid A from *Leptospira*, although a good response was seen for lipid A from *E. coli* and leptospiral LPS (Fig. 1*B*). Then, to evaluate the reactivity of RAW cells toward leptospiral lipid A, we performed a dose-response curve from 0.1 to 1000 ng/ml lipid A as well as whole LPS from *Leptospira* or *E. coli* (Fig. 1*E*). In RAW cells, cytokine responses to lipid A from *E. coli* were ~100-fold weaker compared with whole *E. coli* LPS. In contrast, lipid A from *Leptospira* was a stronger inducer of cytokine production than whole LPS from *Leptospira*. The threshold for leptospiral lipid A to induce TNF production was between 10 and 100 ng/ml, although whole leptospiral LPS induced cytokine production between 100 and 1000 ng/ml. As a whole *L. interrogans* LPS structure is not deciphered, its molecular mass is unknown. Therefore, in these experiments we used identical weighted amounts of both LPS and lipid A, which could bias the results since lipid A accounts only for a part of the whole structure of LPS.

### *Cytokine production by peritoneal macrophages from TLR knockout mice upon stimulation by LPS or lipid A from* Leptospira

The differential response of human cells vs murine cells led us to determine the TLR specificity of leptospiral LPS and lipid A in murine peritoneal-elicited macrophages (PEM). We therefore stimulated PEM from WT, TLR2$^{-/-}$, TLR4$^{-/-}$, or double TLR2$^{-/-}$; TLR4$^{-/-}$ (DKO) mice with LPS and lipid A from *Leptospira*. We measured IL-6 production in supernatants of cells stimulated for 18 h with these bacterial products (Fig. 2). First, in accordance with our previous publication, response to whole leptospiral LPS was dramatically decreased in TLR2$^{-/-}$ mice, whereas the response in TLR4$^{-/-}$ was only partially diminished (Fig. 2, *A* and *B*). Unexpectedly, response to lipid A from *Leptospira* was not altered in TLR2$^{-/-}$ mice, whereas it was abolished in TLR4$^{-/-}$ mice (Fig. 2*A*). This contrasted pattern of recognition between leptospiral LPS and lipid A was quite surprising considering that lipid A was purified from whole leptospiral LPS. Furthermore, we can exclude a possible contamination with "classical" endotoxin because of the lack of reactivity in the LAL assay. In DKO mice, no residual responses were observed for the leptospiral LPS, neither for the different agonists, including $Pam_3Cys_3SK_4$ and LPS from *E. coli* (Fig. 2*C*). The exception was HKSA, which induced an IL-6 response in DKO mice. We measured, as well, IL-10 and TNF production with no qualitative differences in the results (data not shown). These findings demonstrate that no receptor other than TLR2

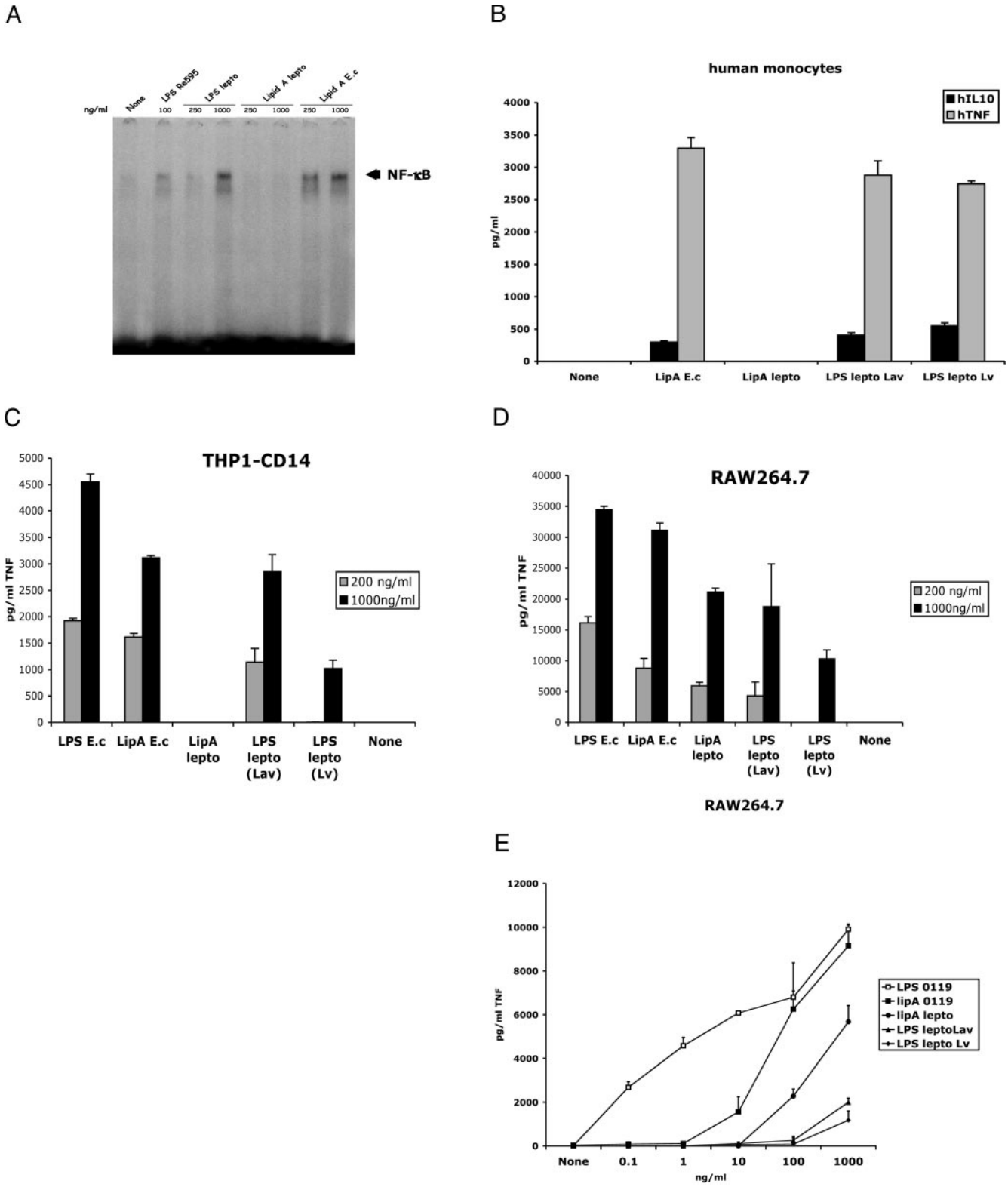


**FIGURE 1.** Lipid A from *Leptospira* is recognized by murine RAW264.7 cells but not by human monocytic cells. *A,* EMSA of transcription factor NF-κB of human THP1-CD14 cells stimulated by two concentrations (250 and 1000 ng/ml) of different LPS. LPS from *S. minnesota* Re595 or LPS from *L. interrogans* strain avirulent (LPS lepto) or lipids A from *L. interrogans* (lipA lepto) or *E. coli* 0119 (lipA E.c). *B–E,* Cytokine (TNF or IL-10) secretion was measured by ELISA in supernatants of human monocytes (*B*) stimulated for 24 h with 1 μg/ml indicated agonists. *C,* THP1-CD14 cells or *D*, RAW264.7 cells ($10^6$ cells/ml in 24-well plates) stimulated the same day with the same dilutions of LPS or lipids A from *E. coli* or *L. interrogans* virulent (LPS lepto Lv) or avirulent (LPS lepto Lav) strain for 18 h. Data are expressed as mean + SD of triplicate samples from one experiment and are representative of two independent experiments. *E,* RAW cells ($2 \times 10^5$ cells/250 μl seeded in 96-well plates) stimulated with increasing concentrations of LPS and lipids A. Data are expressed as mean + SD of quadruplicate samples and are representative of two independent experiments.

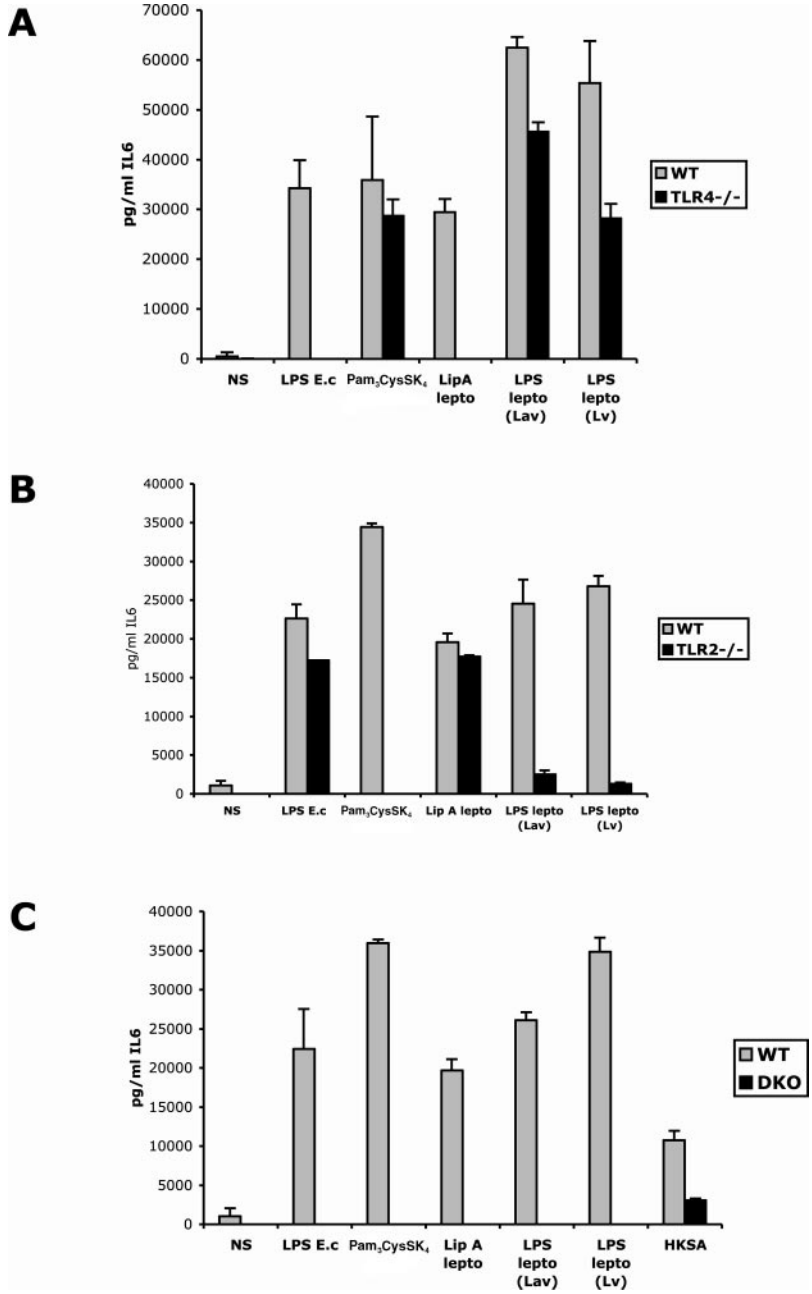


**FIGURE 2.** IL-6 production by thioglycolate-elicited peritoneal macrophages (PEM) from TLR-deficient mice vs parental C57BL/6J mice stimulated with different agonists. *A,* TLR4-deficient mice; *B,* TLR2-deficient mice; *C,* double TLR2/TLR4-deficient mice (DKO). PEM were stimulated for 18 h by LPS or lipids A from *Leptospira* (1 $\mu$g/ml); $Pam_3CysSK_4$ (100 ng/ml), LPS E.c (100 ng/ml), or HKSA ($10^7$ bacteria/ml). IL-6 was measured in supernatants by ELISA. Data are expressed as mean + SD of triplicate samples from one experiment and are representative of at least three independent experiments.

and TLR4 is required to mediate cytokine production upon leptospiral LPS stimulation.

### *MAPK phosphorylation of PEM stimulated by leptospiral LPS and lipid A*

To confirm that cytokine production obtained after 18 h of stimulation was a direct consequence of TLR signaling, we next stimulated the PEM from the different TLR backgrounds for 15 and 40 min with lipid A or LPS from *Leptospira* and examined the phosphorylation of MAPKs ERK1/2 and p38 by immunoblotting cell lysates (Fig. 3). We verified in parallel the expected phenotype of the PEM by using $Pam_3CysSK_4$ as a TLR2 agonist, LPS from *E. coli* as a TLR4 agonist, and HKSA as a positive control for DKO PEM. Stimulation with lipid A from *Leptospira* resulted in phosphorylation of the MAPKs in WT mice within 40 min of stimulation (Fig. 3). In $TLR4^{-/-}$, the phosphorylation was abolished although it was still strong in $TLR2^{-/-}$ PEM. Similar results were obtained after 15 min of stimulation. These results suggest that lipid A from *Leptospira* used only murine TLR4 for signaling.

Stimulation by LPS from *Leptospira* resulted in the phosphorylation of p38 in WT, $TLR4^{-/-}$, and $TLR2^{-/-}$, but not in DKO PEM after 15 and 40 min of stimulation (Fig. 3*A*). The phosphorylation of ERK1/2 by LPS from *Leptospira* was not observed in DKO PEM and appeared altered though still visible in $TLR2^{-/-}$ and in $TLR4^{-/-}$ PEM. Both results confirmed that LPS from *Leptospira* needs both murine TLR2 and TLR4 to signal.

### *Injection of LPS in TLR knockout mice*

To understand the respective contribution of murine TLR2 or TLR4 in the recognition of leptospiral LPS, we injected LPS into IFN-$\gamma$- and D-galactosamine- sensitized mice. In this model, injection of LPS is followed by a major release of proinflammatory cytokines such as TNF that contribute to lethal shock. Leptospiral

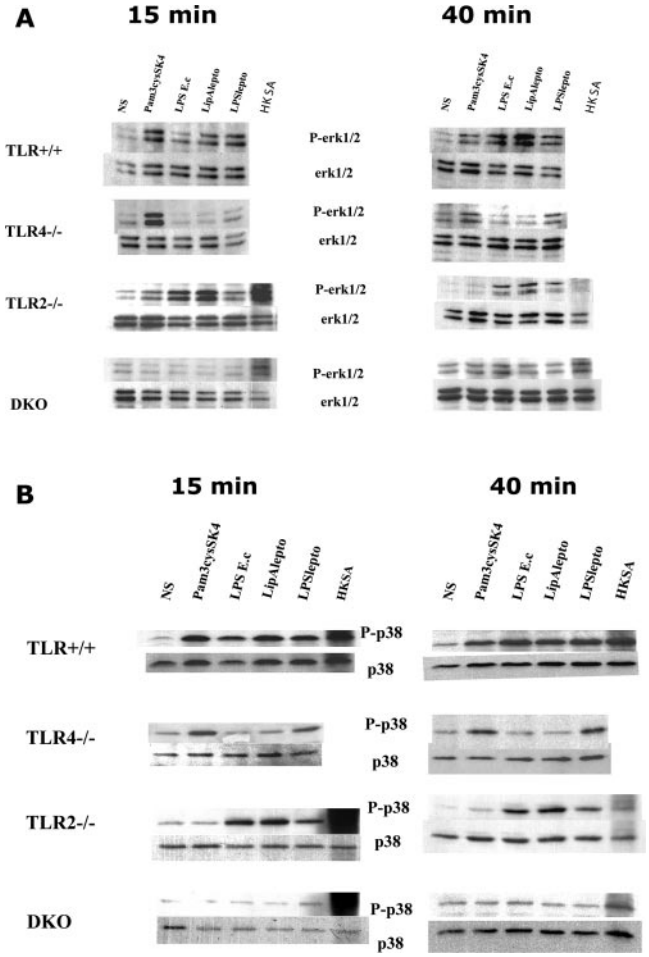


**FIGURE 3.** Phosphorylation of MAPKs of PEM from TLR-deficient or parental C57BL/6J ($TLR^{+/+}$) mice stimulated by LPS or lipid A from *Leptospira*. PEM were stimulated for 15 or 40 min by different TLR agonists ($Pam_3CysSK_4$, LPS E.c, leptospiral lipid A and LPS, 1 $\mu$g/ml, HKSA $10^8$ bacteria/ml) and then checked by Western blot with (*A*) anti-phospho-ERK1/2 Ab or (*B*) anti-phospho-p38 Ab (*upper lanes*). Membranes were stripped and reprobed with Abs detecting total ERK or p38 (*lower lanes*).

LPS (Lv) is not very toxic to mice but injected along with D-galactosamine, 5 $\mu$g of leptospiral LPS or 0.1 $\mu$g of LPS Re595 killed 75% of the WT C57BL/6J mice within 12 h after injection (data not shown). Using this protocol, we injected $TLR2^{-/-}$ and WT mice with leptospiral LPS. At 24 h after injection, lethality of $TLR2^{-/-}$ mice was statistically inferior to WT mice, with a lag of 4–6 h in lethality in $TLR2^{-/-}$ compared with WT mice (Fig. 4), thus confirming that TLR2 was indeed involved in the leptospiral LPS recognition. We also performed this experiment in $TLR4^{-/-}$ mice and found a statistically significant difference in lethality compared with WT mice, although it was less pronounced than that for $TLR2^{-/-}$ mice (Fig. 4). We then repeated this protocol using double $TLR2^{-/-}$/$TLR4^{-/-}$ mice (DKO) mice, showing a dramatic 100% survival of DKO mice compared with WT mice, who died as expected (Fig. 4). These in vivo data demonstrate that leptospiral LPS recognition is mediated through both murine TLR2 and TLR4.

### *TLR utilization by leptospiral LPS vs lipid A*

To understand the species specificity of the TLR responses toward whole LPS or lipid A from *Leptospira*, we sought to compare the TLR use of highly purified leptospiral LPS and lipid A in TLR-transfected human cells.

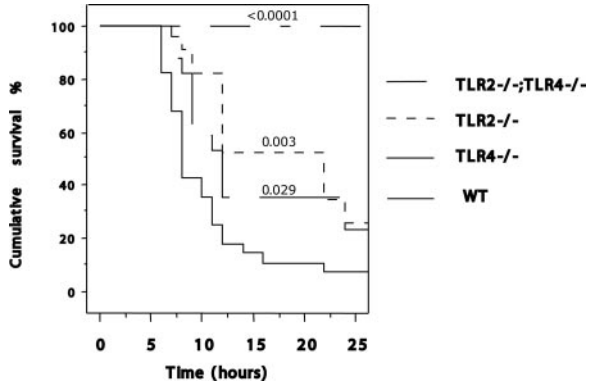


**FIGURE 4.** Kaplan-Meier plot of cumulative survival of mice injected with leptospiral LPS. TLR4 ($n = 17$), TLR2 ($n = 23$), double TLR4/TLR2-deficient mice (DKO) ($n = 23$), or parental C57BL/6J (WT; $n = 28$) were injected i.p. with 5 $\mu$g of leptospiral LPS (Lv) along with D-galactosamine 45 min after IFN-$\gamma$ priming. Survival was monitored over 24 h. Data represent cumulative results from at least three independent experiments comparing WT to TLR2, TLR4, or DKO-deficient mice, with five to eight mice in each group. Values of probability comparing TLR-deficient to WT mice are indicated.

### *TLR utilization in human cells*

We transiently transfected HEK293 cells with different constructs expressing TLR2 or TLR4/MD2/CD14 from murine or human origin, then stimulated these cells with lipid A or LPS from *Leptospira* and measured the luciferase activity driven by a cotransfected NF-$\kappa$B reporter construct (Fig. 5). LPS from *Leptospira* strongly activated cells transfected by either murine or human TLR2 (Fig. 5*A*) or murine TLR4-MD2-CD14 but did not stimulate cells transfected by human TLR4-MD2-CD14 (Fig. 5*B*). Lipid A from *Leptospira* activated cells transfected with TLR4-MD2-CD14 constructs from murine origin but failed to activate cells expressing human TLR4-MD2-CD14 or the TLR2 constructs from either human or murine origin (Fig. 5, *A* and *B*). We then performed control experiments (data not shown) to determine whether the species specificity of murine recognition of lipid A and LPS from *Leptospira* relied on TLR4, MD2, or CD14. Data showed that both mMD2 and mTLR4 were essential to the recognition, independent of the source of CD14, either provided by bovine serum or by coexpression of hCD14 or mCD14 in serum-free conditions. This result showing that CD14 was not responsible for the species specificity of recognition of the leptospiral lipid A is in accordance with previous lipid A studies (16). These results suggest that the lack of response of human cells upon leptospiral lipid A stimulation may be due to lack of human TLR4-MD2 recognition.

### *Role of TLR heterodimerization in leptospiral LPS recognition*

Heterodimerization between different TLRs has been reported to have either positive or inhibitory effects (17). Heterodimerization between TLR2 and TLR6 has been described to enhance TLR2 signaling by phenol-soluble modulin, a secreted factor of *S. aureus*, whereas heterodimerization between TLR2 and TLR1 had an inhibitory effect (18). Furthermore, heterodimerization of TLRs has been implicated in the differential recognition of pattern recognition receptors (17). Indeed, it has been reported that heterodimers of TLR2/TLR6 and TLR2/TLR1 could allow for the distinction between diacylated and triacylated lipopeptides, respectively (19). Heterodimers with TLR4 have also been reported. In endothelial cells, it has been demonstrated that TLR1 can form heterodimers with TLR4, resulting in an inhibition of TLR4 signaling induced by LPS (20).

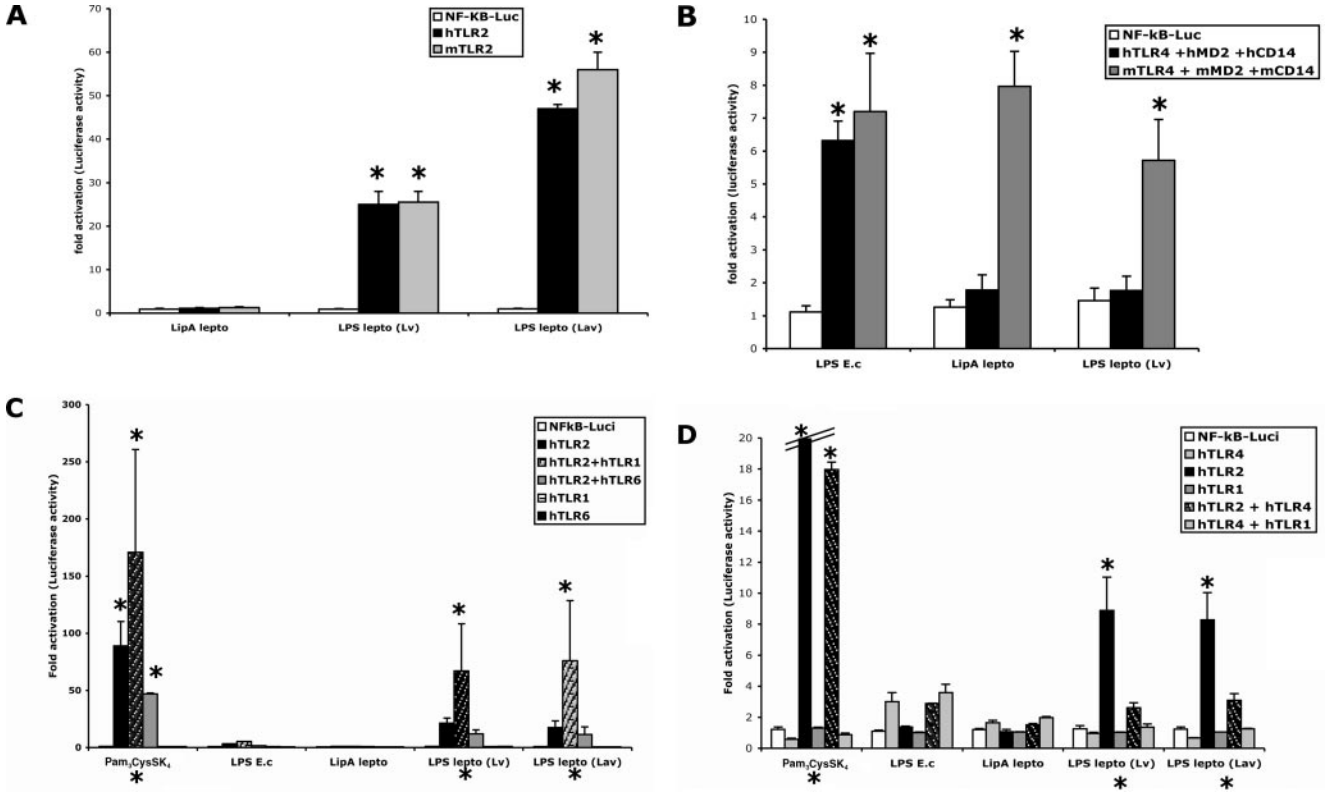


**FIGURE 5.** In vitro NF-κB-driven luciferase reporter assays of human and mouse TLR activation by leptospiral LPS and lipid A (1 μg/ml). *A* and *B,* Activation of transiently transfected human HEK293 cells. Data are expressed as" fold activation" (for each TLR transfection: luciferase activity upon agonist stimulation divided by luciferase activity of the nonstimulated cells) and expressed as mean +SD of fold activation from at least three independent experiments, each performed in duplicate. *C* and *D,* Activation of transiently transfected HEK293 cells with human TLR alone or in combination. Data are expressed as mean + SD of fold activation obtained from three independent experiments performed in duplicate. Asterisks above the bars represent a $p < 0.05$, comparing for one agonist a TLR transfection to the reporter NF-κB transfection. Asterisks below the histograms represent a $p < 0.05$, comparing transfection with TLR2 alone vs TLR2 in combination with TLR1 or TLR4.

Heterodimers between TLR4 and TLR5 have also been shown to mediate the NO production induced by flagellin signaling (21). The results we obtained so far suggested that murine TLR2 or TLR4 was required to initiate signaling by LPS from *Leptospira*. Therefore, we next tested whether potential heterodimerization between different human TLRs was required for proper activation by leptospiral LPS. We transiently transfected HEK cells with different combinations of hTLR1, hTLR2, hTLR6, and hTLR4-MD2 and stimulated these cells with leptospiral LPS and lipid A (Fig. 5, *C* and *D*). The coexpression of hTLR1 with hTLR2 led to a better stimulation by leptospiral LPS than hTLR2 alone (Fig. 5*E*). Coexpression of hTLR6 did not enhance hTLR2 stimulation by $Pam_3cysSK_4$ nor by LPS from *Leptospira*. These results suggested that LPS from *Leptospira* activated the human cells through heterodimers of hTLR2 and hTLR1. We did not observe any differences between virulent or avirulent leptospiral LPS preparations.

Transfection of HEK293 cells with TLR4 or TLR4-MD2 constructs from murine or human origin reproducibly led to a high luciferase expression background (data not shown) and low folds of activation compared with the other TLR constructs (Fig. 5*B*), making results of coexpression experiments difficult to interpret. Nonetheless, compared with hTLR2 alone, we obtained a statistically significant reduced relative luciferase activity when cells were transfected by hTLR2 along with hTLR4-MD2, although no response was seen with hTLR4-MD2 alone upon stimulation by leptospiral LPS. We verified in FACS experiments that the expression of hTLR2 was not affected upon cotransfection of other hTLRs (Table II). These results could either suggest that the formation of inactive heterodimers between TLR2 and TLR4 could titrate the TLR2 monomers and would impair the formation of active TLR2/TLR1 heterodimers or, most probably, that overexpression of TLR4 could titrate some specific signaling component of the TLR2 signaling pathway. We checked the functionality of constructs with LPS from *E. coli*, which as expected signaled through hTLR4 and was not inhibited when hTLR4 was coexpressed with hTLR1 or hTLR2 (Fig. 5*D*). This control shows that, at least, the expression of TLR2 does not impair the coexpression and functions of hTLR4. Interestingly we obtained a similar pattern of results with leptospiral LPS and $Pam_3cysSK_4$, a pure TLR2 agonist, suggesting that this negative interaction of TLR4 on TLR2 preexists to the stimulation. Additional experiments with TLR4 stably transfected HEK293 cells would be useful to validate this result showing an inhibitory effect of TLR4 expression on TLR2 signaling. Altogether, these results suggest that LPS from *Leptospira* used TLR2/TLR1 heterodimers but not TLR2/TLR4 to signal in human cells.

## Discussion

We previously showed that LPS from *Leptospira* activated human cells through TLR2 instead of TLR4 (8). The leptospiral lipid A moiety structure has been recently deciphered, revealing that it possesses some peculiar characteristics compared with lipid A from Gram-negative bacteria (12), such as a one phosphate residue that is capped with a methyl group, lack of a 4′ phosphate group,

Table II. *TLR2 expression is not modified upon coexpression of TLR4 or TLR1*[a]

| | NF-κB | hTLR2 | hTLR4 | hTLR1 | hTLR2 + hTLR4 | hTLR2 + hTLR1 |
|---|---|---|---|---|---|---|
| % TLR2-expressing cells | 1.3 | 44.3 | 1 | 2.05 | 41 | 39.06 |
| % Isotype control | 1.25 | 1.7 | 1.79 | 2.38 | 1.72 | 0.97 |
| Fold activation $Pam_3CysSK_4$ | 0.89 | 77 | 1.6 | 1.64 | 3.8 | 133 |

[a] HEK293T cells transfected with different TLR constructs alone or in combination along with a NF-κB reporter were checked for TLR2 expression by FACS after 48 h. Percentage of labeled cells with an isotype control is indicated. In parallel, the cells were stimulated for 6 h with $Pam_3cysSK_4$ (1 μg/ml) and a luciferase assay was performed. Fold luciferase activation compared to the nonstimulated cells is indicated. Data are representative of one of three independent experiments.

and four amide-linked acyl chains, longer than in enterobacterial lipids A, with two secondary unsaturated acyl chains. To understand the unusual use of TLR2 by whole leptospiral LPS, we therefore characterized the properties of leptospiral lipid A in relationship to cell signaling and TLR recognition.

LAL results showed that despite an overall structure very similar to lipid A from Gram-negative bacteria, lipid A from *Leptospira* showed an extremely weak reactivity in the LAL assay. This demonstrates a lack of recognition of this lipid A by the innate immune system of the horseshoe crab, which typically is very sensitive to detect the presence of minute amounts of lipid A or LPS. In contrast to other spirochetes, like *Borrelia*, which do not possess LPS and are strict parasites, *Leptospira* are able to survive in water or colonize different hosts, such as mammals, birds, and reptiles, demonstrating their ability to avoid immune defenses (2). It is tempting to speculate that the peculiar structure and properties of lipid A from *Leptospira* could reflect its ability to adapt to different growth conditions and animal hosts.

We showed previously that in human cells, most of the cell signaling initiated by heat-killed *Leptospira* occurs through TLR2, mostly signaling through its LPS and its lipoproteins (8). The fact that TLR4 plays little role in leptospiral recognition may represent an escape mechanism from human cell innate immune recognition. Other pathogenic bacteria are known to signal mainly through TLR2 and not through TLR4, including *Legionella pneumophila* or *Helicobacter pylori*. Interestingly both of these bacteria present an atypical lipid A that shows some similarities with the lipid A from *Leptospira*. Lipid A from *L. pneumophila* has a bisphosphorylated disaccharide backbone with only amide-linked acyl groups having 14–22 carbon atoms (9). Concerning *H. pylori*, the major lipid A species contain tetra-acylated forms with 16 or 18 carbons, with an ethanolamine substitution in position 1 and lack a phosphate in position 4′ (22). Such structural modifications of lipid A would be responsible for lowered endotoxic properties and lack of hTLR4 agonistic activity.

In this study, we showed that lipid A from *Leptospira* activated murine cells but not human cells, despite the fact that whole leptospiral LPS was able to stimulate human cells. Leptospiral lipid A stimulated cells through murine TLR4-MD2 but not through human TLR4-MD2, nor murine or human TLR2. Differences in ligand specificity between murine and human TLR4-MD2 have already been reported. Lipid IVA, a tetra-acylated precursor of LPS biosynthesis, is recognized by murine MD2-TLR4 but not by human MD2-TLR4. Genetic studies demonstrated that this species specificity of recognition is dependent on the presence of murine MD2 (23). Moreover, it has been shown that only human TLR4, and not murine TLR4, is able to discriminate between hexa- and penta-acylated forms of LPS that *Pseudomonas aeruginosa*, an opportunistic human pathogen, is able to synthesize during adaptation to the cystic fibrosis airway. This discrimination is mediated by an 82-aa region of human TLR4 that is hypervariable across species (23). We determined that both murine MD2 and TLR4 were required for the sensing of leptospiral lipid A. The critical regions of MD2 and TLR4 involved in the species specificity of leptospiral lipid A recognition remains to be studied.

Although unusual in its structure, the lipid A of *Leptospira* actually behaved as a classical lipid A, using mouse TLR4 to signal. This finding is comparable to what has been shown for lipid A from *Porphyromonas gingivalis*, which in opposition to the whole LPS, stimulates murine cells only through mTLR4-MD2 and not through mTLR2 (24).

Some questions remain to explain our results with whole LPS, which activated mainly through TLR2 in human cells. One possible explanation would be that the leptospiral LPS signals very weakly through TLR4 by its lipid A moiety and that a copurifying factor that signals through TLR2 would give a strong signal and possibly overcome the TLR4 response. However, we took care to avoid possible contaminants and purified LPS from *Leptospira* according to acknowledged procedures to remove lipoproteins (7). Recently, it was shown that enterobacterial LPS preparations contain LPS-mimetic TLR2 stimulatory substances that differ from bacterial lipopeptides (25). The activity of these compounds is enhanced by CD14, likewise for leptospiral LPS (8), whereas the activity of synthetic lipopeptide-like $Pam_3CysSK_4$ is not. In fact, these LPS-mimetic components are extracted from phenol-dialyzed extracts, a procedure we used to recover the LPS from *Leptospira*. We can speculate that these compounds are peculiar LPS species that partition into the phenol phase of the phenol-water emulsion according to differences in their relative acylation and glycosylation patterns. Unknown structural features of these phenol-extracted LPS species could allow for TLR2 rather than TLR4 recognition. To further understand this phenomenon, new protocols of LPS purification are currently being tested to obtain classical and leptospiral LPS, free of lipoprotein contaminants and without any loss in LPS fractions (M. Caroff, unpublished observations).

Another possible explanation could be that leptospiral lipid A requires the polysaccharide portion of LPS to signal in human cells. Such a situation is reminiscent of recent work with a hepta-acylated subspecies of *Salmonella* lipid A (26). This lipid A signals normally through TLR4 in murine cells but does not signal in human cells, despite the fact that the whole LPS is able to stimulate both murine and human cells. In further support of this idea, LPS from a rough mutant of *S. minnesota* requires the 2-keto-3-deoxyoctonic acid part of the molecule to enable signaling of the lipid A through TLR4 in human cells (26). We can imagine a similar phenomenon in which leptospiral lipid A would need the polysaccharide portion of the LPS to signal in human cells. However, in this case, the sugar moiety would confer TLR2 rather than TLR4 recognition for this unusual LPS.

Finally, it is also possible that multiple lipid A species that differ in acylation pattern are present in the LPS from *Leptospira*. Several apparently conflicting studies have reported that the LPS from *P. gingivalis* signals through TLR2 or through TLR4, or it has even been shown to be a TLR4 antagonist (25). A recent report provides a possible explanation of these results, demonstrating that LPS preparations of *P. gingivalis* contain different species of lipid A that functionally interact with both TLR4 and TLR2 (27). The heterogeneity in the acylation pattern of *P. gingivalis* lipid A is likely to be critical for TLR specificity, with penta-acylated forms acting as the MD2-TLR4 agonist, whereas tetra-acylated monophosphorylated forms are recognized through TLR2 or TLR4.

In this study, we showed that leptospiral LPS was recognized through both hTLR2 and hTLR1. Strikingly, the same results have been shown for LPS from *P. gingivalis* (27) and lipoarabinomannan from mycobacteria (28). Interestingly, despite very different structures, all of these lipidated structures have been shown to require CD14 and TLR2, but not TLR4 to signal in human cells (8, 29). The fact that they would use the same human TLR2/1 receptor could be attributed to the presence of an unknown common structural motif or to the presence of a common contaminant such as a lipoprotein (6). In support of this idea, a triacylated lipopeptide that is responsible for TLR2 activity has recently been extracted and purified from *P. gingivalis* LPS (30). This lipopeptide is resistant to high temperature and proteases and to the re-extraction procedure with deoxycholate. The presence of such a copurifying component could explain the unusual TLR2 activity of our repurified leptospiral LPS.

Mice are not susceptible to leptospirosis whereas humans are sensitive (2). Results obtained in this study showing a differential species specificity of TLR use by leptospiral LPS (TLR2 and TLR4 in murine cells vs TLR2 and TLR1 in human cells) led us to hypothesize that a very efficient recognition of LPS by murine cells may be part of the effective innate immune protection against this pathogen. On the contrary, human cells would be less efficient to detect leptospiral LPS because of a lack of lipid A recognition by human MD2/TLR4. Innate immune defense would not be triggered properly, which may lead to leptospirosis as a consequence. This hypothesis could be plausible since C3H/HeJ newborn mice, which have a missense mutation in the *tlr*4 gene (31), are susceptible to leptospirosis (32). In contrast, C3H/HeN newborn mice harboring the WT *tlr*4 allele were not susceptible to leptospirosis (C. Werts, unpublished results). In vivo preliminary data have shown that i.p. infection of TLR2$^{-/-}$ or DKO newborn mice by *L. interrogans* could lead to severe leptospirosis and eventually to death in DKO mice (C. Werts, unpublished results). Taken together, these findings suggest that the TLR2/TLR4 double-deficient mice could represent a valuable model for studying leptospirosis.

## Acknowledgments

We thank Dana Philpott for helpful review of this manuscript, Jörg Fritz for providing PBMC and help with FACS experiments, Martine Caroff for interesting discussions and the gift of LPS, and Martine Parmier for plasmid preparations.

## Disclosures

The authors have no financial conflict of interest.

## References

1. Pereira, M. M., M. G. Matsuo, A. R. Bauab, S. A. Vasconcelos, Z. M. Moraes, G. Baranton, and I. Saint Girons. 2000. A clonal subpopulation of *Leptospira interrogans* sensu stricto is the major cause of leptospirosis outbreaks in Brazil. *J. Clin. Microbiol.* 38: 450–452.
2. Bharti, A. R., J. E. Nally, J. N. Ricaldi, M. A. Matthias, M. M. Diaz, M. A. Lovett, P. N. Levett, R. H. Gilman, M. R. Willig, E. Gotuzzo, and J. M. Vinetz. 2003. Leptospirosis: a zoonotic disease of global importance. *Lancet Infect. Dis.* 3: 757–771.
3. Philpott, D. J., and S. E. Girardin. 2004. The role of Toll-like receptors and Nod proteins in bacterial infection. *Mol. Immunol.* 41: 1099–1108.
4. Akashi, S., S. Saitoh, Y. Wakabayashi, T. Kikuchi, N. Takamura, Y. Nagai, Y. Kusumoto, K. Fukase, S. Kusumoto, Y. Adachi, et al. 2003. Lipopolysaccharide interaction with cell surface Toll-like receptor 4-MD-2: higher affinity than that with MD-2 or CD14. *J. Exp. Med.* 198: 1035–1042.
5. Poltorak, A., I. Smirnova, X. He, M. Y. Liu, C. Van Huffel, O. McNally, D. Birdwell, E. Alejos, M. Silva, X. Du, et al. 1998. Genetic and physical mapping of the *Lps* locus: identification of the Toll-4 receptor as a candidate gene in the critical region. *Blood Cells Mol. Dis.* 24: 340–355.
6. Lee, H. K., J. Lee, and P. S. Tobias. 2002. Two lipoproteins extracted from *Escherichia coli* K-12 LCD25 lipopolysaccharide are the major components responsible for Toll-like receptor 2-mediated signaling. *J. Immunol.* 168: 4012–4017.
7. Hirschfeld, M., Y. Ma, J. H. Weis, S. N. Vogel, and J. J. Weis. 2000. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine Toll-like receptor 2. *J. Immunol.* 165: 618–622.
8. Werts, C., R. I. Tapping, J. C. Mathison, T. H. Chuang, V. Kravchenko, I. Saint Girons, D. A. Haake, P. J. Godowski, F. Hayashi, A. Ozinsky, et al. 2001. Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. *Nat. Immunol.* 2: 346–352.
9. Girard, R., T. Pedron, S. Uematsu, V. Balloy, M. Chignard, S. Akira, and R. Chaby. 2003. Lipopolysaccharides from *Legionella* and *Rhizobium* stimulate mouse bone marrow granulocytes via Toll-like receptor 2. *J. Cell Sci.* 116: 293–302.
10. Hirschfeld, M., J. J. Weis, V. Toshchakov, C. A. Salkowski, M. J. Cody, D. C. Ward, N. Qureshi, S. M. Michalek, and S. N. Vogel. 2001. Signaling by Toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. *Infect. Immun.* 69: 1477–1482.
11. Darveau, R. P. 1998. Lipid A diversity and the innate host response to bacterial infection. *Curr. Opin. Microbiol.* 1: 36–42.
12. Que-Gewirth, N. L., A. A. Ribeiro, S. R. Kalb, R. J. Cotter, D. M. Bulach, B. Adler, I. S. Girons, C. Werts, and C. R. H. Raetz. 2004. A methylated phosphate group and four amide-linked acyl chains in leptospira interrogans lipid A: the membrane anchor of an unusual lipopolysaccharide that activates TLR2. *J. Biol. Chem.* 279: 25420–25429.
13. Fritz, J., H., S. E. Girardin, C. Fitting, C. Werts, D. Mengin-Lecreulx, M. Caroff, J. M. Cavaillon, D. J. Philpott, and M. Adib-Conquy. 2005. Synergistic stimulation of human monocytes and dendritic cells by Toll-like receptor 4 and NOD1 and NOD2-activating agonists. *Eur. J. Immunol.* 35: 2459–2470.
14. Muzio, M., G. Natoli, S. Saccani, M. Levrero, and A. Mantovani. 1998. The human Toll signaling pathway: divergence of nuclear factor κB and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6). *J. Exp. Med.* 187: 2097–2101.
15. Blisnick, T., F. Lema, J. C. Mazie, and L. P. Pereira da Silva. 1988. *Plasmodium falciparum*: analysis of B epitopes of a polypeptide antigen expressed in *Escherichia coli*, using monoclonal antibodies. *Exp. Parasitol.* 67: 247–256.
16. Delude, R. L., R. Savedra, Jr., H. Zhao, R. Thieringer, S. Yamamoto, M. J. Fenton, and D. T. Golenbock. 1995. CD14 enhances cellular responses to endotoxin without imparting ligand-specific recognition. *Proc. Natl. Acad. Sci. USA* 92: 9288–9292.
17. Ozinsky, A., D. M. Underhill, J. D. Fontenot, A. M. Hajjar, K. D. Smith, C. B. Wilson, L. Schroeder, and A. Aderem. 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. *Proc. Natl. Acad. Sci. USA* 97: 13766–13771.
18. Hajjar, A. M., D. S. O'Mahony, A. Ozinsky, D. M. Underhill, A. Aderem, S. J. Klebanoff, and C. B. Wilson. 2001. Cutting edge: functional interactions between Toll-like receptor (TLR) 2 and TLR1 or TLR6 in response to phenol-soluble modulin. *J. Immunol.* 166: 15–19.
19. Morr, M., O. Takeuchi, S. Akira, M. M. Simon, and P. F. Muhlradt. 2002. Differential recognition of structural details of bacterial lipopeptides by Toll-like receptors. *Eur. J. Immunol.* 32: 3337–3347.
20. Spitzer, J. H., A. Visintin, A. Mazzoni, M. N. Kennedy, and D. M. Segal. 2002. Toll-like receptor 1 inhibits Toll-like receptor 4 signaling in endothelial cells. *Eur. J. Immunol.* 32: 1182–1187.
21. Mizel, S. B., A. N. Honko, M. A. Moors, P. S. Smith, and A. P. West. 2003. Induction of macrophage nitric oxide production by Gram-negative flagellin involves signaling via heteromeric Toll-like receptor 5/Toll-like receptor 4 complexes. *J. Immunol.* 170: 6217–6223.
22. Tran, A. X., M. J. Karbarz, X. Wang, C. R. H. Raetz, S. C. McGrath, R. J. Cotter, and M. S. Trent. 2004. Periplasmic cleavage and modification of the 1-phosphate group of *Helicobacter pylori* lipid A. *J. Biol. Chem.* 279: 55780–55791.
23. Hajjar, A. M., R. K. Ernst, J. H. Tsai, C. B. Wilson, and S. I. Miller. 2002. Human Toll-like receptor 4 recognizes host-specific LPS modifications. *Nat. Immunol.* 3: 354–359.
24. Ogawa, T., Y. Asai, M. Hashimoto, O. Takeuchi, T. Kurita, Y. Yoshikai, K. Miyake, and S. Akira. 2002. Cell activation by *Porphyromonas gingivalis* lipid A molecule through Toll-like receptor 4- and myeloid differentiation factor 88-dependent signaling pathway. *Int. Immunol.* 14: 1325–1332.

25. Coats, S. R., R. A. Reife, B. W. Bainbridge, T. T. Pham, and R. P. Darveau. 2003. *Porphyromonas gingivalis* lipopolysaccharide antagonizes *Escherichia coli* lipopolysaccharide at Toll-like receptor 4 in human endothelial cells. *Infect. Immun.* 71: 6799–6807.
26. Muroi, M., and K. Tanamoto. 2002. The polysaccharide portion plays an indispensable role in *Salmonella* lipopolysaccharide-induced activation of NF-κB through human Toll-like receptor 4. *Infect. Immun.* 70: 6043–6047.
27. Darveau, R. P., T. T. Pham, K. Lemley, R. A. Reife, B. W. Bainbridge, S. R. Coats, W. N. Howald, S. S. Way, and A. M. Hajjar. 2004. *Porphyromonas gingivalis* lipopolysaccharide contains multiple lipid A species that functionally interact with both Toll-like receptors 2 and 4. *Infect. Immun.* 72: 5041–5051.
28. Tapping, R. I., and P. S. Tobias. 2003. Mycobacterial lipoarabinomannan mediates physical interactions between TLR1 and TLR2 to induce signaling. *J. Endotoxin Res.* 9: 264–268.
29. 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: 6748–6755.
30. Hashimoto, M., Y. Asai, and T. Ogawa. 2004. Separation and structural analysis of lipoprotein in a lipopolysaccharide preparation from *Porphyromonas gingivalis*. *Int. Immunol.* 16: 1431–1437.
31. Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van 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: 2085–2088.
32. Pereira, M. M., J. Andrade, R. S. Marchevsky, and R. Ribeiro dos Santos. 1998. Morphological characterization of lung and kidney lesions in C3H/HeJ mice infected with *Leptospira interrogans* serovar icterohaemorrhagiae: defect of $CD4^+$ and $CD8^+$ T-cells are prognosticators of the disease progression. *Exp. Toxicol. Pathol.* 50: 191–198.