Toll-Like Receptor 4 Protects against Lethal *Leptospira interrogans* Serovar Icterohaemorrhagiae Infection and Contributes to In Vivo Control of Leptospiral Burden

Suganya Viriyakosol,1,2 Michael A. Matthias,1 Mark A. Swancutt,1 Theo N. Kirkland,1,2 and Joseph M. Vinetz1*

Division of Infectious Diseases, Department of Medicine, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0640,1 and Veterans Administration San Diego Healthcare System and Department of Pathology, University of California, San Diego, 3350 La Jolla Village Drive, La Jolla, California 921252

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The roles of innate immune responses in protection from or pathogenesis of severe leptospirosis remain unclear. We examined the role of Toll-like receptors (TLRs) in mouse infection and macrophage responses to *Leptospira*. C3H/HeJ mice (TLR4 deficient) and C3H/HeJ-SCID mice, but not C3H/OU1 mice (TLR4 intact), died after intraperitoneal infection with *Leptospira interrogans* serovar Icterohaemorrhagiae. Death in both C3H/HeJ mouse strains was associated with jaundice and pulmonary hemorrhage, similar to the patient from whom the isolate was obtained. In chronic sublethal infection, TLR4-deficient mice harbored more leptospires in liver, lung, and kidney than control mice. Heat-killed *Leptospira* stimulated macrophages to secrete proinflammatory cytokines, tumor necrosis factor alpha, interleukin-6, and macrophage inflammatory protein 2 not inhibited by polymyxin B, suggesting that leptospiral lipopolysaccharide (LPS) did not drive these responses. Anti-TLR4 and anti-MD-2 but not anti-CD14 monoclonal antibodies inhibited cytokine production. Peritoneal macrophages from CD14−/− and TLR2−/− mice exhibited no defect in cytokine responses to *Leptospira* compared to controls. Macrophages from C3H/HeJ, TLR4−/−, and MyD88−/− mice secreted far-lower levels of cytokines than wild-type macrophages in response to *Leptospira*. TLR4 plays a crucial role in protection from acute lethal infection and control of leptospiral burden during sublethal chronic infection. Cytokine responses in macrophages correlated with leptospiral clearance. These TLR4-dependent but CD14/TLR2-independent responses are likely mediated by a leptospiral ligand(s) other than LPS.

Leptospirosis is a zoonotic disease of global importance caused by more than 11 species and 200 serovars of spirochetes of the genus *Leptospira* (6). In recent years, a grave form of leptospirosis, pulmonary hemorrhage, has emerged worldwide as an important clinical syndrome (25, 29, 30). Mechanisms of pathogenesis, host defense, and protective immunity in leptospirosis remain poorly understood (6). Given that case fatality rates in the disease can be as high as 25% (31), an improved understanding of host defense mechanisms in leptospirosis may help to more effectively treat and prevent this disease.

The most informative small-animal models of leptospirosis have been hamsters and guinea pigs (5, 9, 17, 22), in which manifestations of disease recapitulate those of severe human disease, including jaundice, hemorrhage, and interstitial nephritis. Infection of mice and rats to reproduce human disease has been inconsistent, often requiring immunosuppressive treatment with drugs such as cyclophosphamide or corticosteroids (1, 2, 19, 24). The number of organisms required to initiate infection in small-animal models varies widely for unknown reasons (7). Some isolates freshly isolated from diseased animals or humans may not be infectious or cause disease. Other isolates are highly virulent, causing death with as few as 10 to 100 organisms. Even low-passage isolates of idiopathic serovars of *Leptospira interrogans* serovar Copenhageni isolated from patients dying from pulmonary hemorrhage have different 50% lethal doses in guinea pigs (J. Nally and M. Lovett, personal communication). Two groups have recently demonstrated that *L. interrogans* can produce lethal infection in C3H/HeJ mice (14, 27), but host response mechanisms predisposing these mice to death were not explored in detail. Furthermore, infection outcomes in C3H/HeJ mice, in which Toll-like receptor 4 (TLR4) function is impaired, were not compared to those in wild-type (WT) mice. Small-animal models have not yet been used to elucidate in vivo mechanisms of innate or acquired immunity, except that serovar-specific anti-lipopolysaccharide (LPS) antibodies can confer complete protection against homologous but not heterologous challenge (33). A recent report of experimental leptospirosis in guinea pigs suggested that the severity of pulmonary hemorrhage parallels the deposition of immunoglobulin and complement in the alveolar septum (22). The role of cellular immunity, either innate or acquired, has just recently begun to be explored, with some evidence that T-cell receptor γδ T cells are associated with vaccine-induced protection in cattle and that *L. interrogans* powerfully stimulates gamma interferon-secreting T-cell receptor γδ T cells in peripheral blood mononuclear cells from *Leptospira*-naïve humans (13) that directly kill *Leptospira* in vitro (G. Klimpel and J. Vinetz, unpublished observations).

The relationship of single genes to susceptibility to bacterial and parasitic diseases has been established in mouse models of infections due to organisms such as *Leishmania major*, *Myco-
bacterium tuberculosis, Salmonella enterica serovar Typhimurium, and Escherichia coli (15). While the NRAMP-1 iron transporter gene has been shown to be important for host resistance to intracellular infections, TLR4 has been shown to be critical in susceptibility to gram-negative LPS. Although the innate immune response to *Leptospira* has been explored in vitro (37), whether protection from or susceptibility to severe leptospirosis is related to a single Mendelian trait has not been explored in vivo. In a report of in vitro *L. interrogans* stimulation of the innate immune system, leptospiiral LPS was shown paradoxically to stimulate the TLR2-dependent pathway but not the TLR4 pathway (37). The potential for TLR2 to be utilized in vivo role in the host response to *Leptospira* has not been shown in challenge models.

In this study, we report results of experiments from an in vivo murine model of severe leptospirosis in which a single gene, TLR4, determined the clinical outcome of infection as well as tissue burden of leptospires in sublethal infection. A lack of antibody and B and T lymphocytes did not prevent jaundice or pulmonary hemorrhage, suggesting that these arms of the immune system are not involved in *Leptospira*-induced immunopathology, at least in mice. In in vitro assays, *Leptospira*-stimulated proinflammatory cytokines and chemokines are dependent on TLR4 and MD-2 present in the TLR4 signaling complex but independent of CD14. These observations indicate that a CD14-independent, TLR4-dependent pathway of the innate immune response contributes an unexpectedly important component of protection against death in severe murine leptospirosis and in controlling leptospirolic proliferation during chronic infection.

**MATERIALS AND METHODS**

**Reagents.** *Salmonella minnesota* Re595 LPS was purchased from List Biological Laboratories. *Leptospira interrogans* LPS was kindly provided by Catherine Wertz (Institut Pasteur, Paris, France). Heat-killed *Syphilis salmoenuerum* was obtained from Calbiochem. An anti-CD14 monoclonal antibody (MAb), 28C5, was a generous gift from P. Tobias (Research Institute of the Scripps Clinic, La Jolla, CA). MAb anti-mouse TLR4-MD-2 clone MTS10 was obtained from Ebisocience. MAb anti-TLR4 clone 14HBE12 was produced in our laboratory. Control mouse immunoglobulin G1 (IgG1) was obtained from Caltag. Polymyxin B (PMB) was purchased from Sigma.

**Mice.** Female C57BL/6, C3H/HeJ, C3H/HeJ-SCID, C3H/OuJ, CD14<sup−/−</sup> (B6), and TLR2<sup−/−</sup> (B6) mice were purchased from the Jackson Laboratories, Bar Harbor, Maine. TLR4<sup−/−</sup> and MyD88<sup−/−</sup> mice on a C57BL/6 background were obtained by Shizuo Akira (Osaka University) and provided by Eyal Raz (University of California, San Diego). All mice were 3 to 8 weeks of age, housed in a pathogen-free facility, and handled according to the recommended guidelines. All animal studies were approved by the Institutional Animal Care and Use Committees of the University of California, San Diego, and the Veterans Affairs Medical Center, San Diego, CA.

**Bacteria.** *Leptospira interrogans* serovar Icterohaemorrhagiae strain HA1188 was isolated from a 15-year-old boy from Iquitos, Peru, who died of leptospirosis characterized by jaundice, renal failure, pulmonary hemorrhage, and refractory shock (29). The isolate was identified as *Leptospira interrogans* serovar Icterohaemorrhagiae by a combination of serological typing, 16S ribosomal gene sequencing, and modified pulsed-field gel electrophoresis (M. A. Matthias and J. M. Vinetz, unpublished data) (8). Low-passage bacteria, expanded to stationary phase in PPM-5 (Serologicals Corp.), were used for all experiments. For in vitro stimulation assays, bacteria were washed five times with pyrogen-free water (BD Biosciences), counted using a Petroff-Hauser counting chamber under dark-field microscopy, and heat killed at 56°C for 30 min. All preparations were tested for endotoxin contamination using a QCL-1000 chromogenic LAL assay (Bio-Whittaker).

**Challenge infections.** For lethal infection, 3-week-old mice were injected intraperitoneally (i.p.) with 6 × 10<sup8</sup> *Leptospira* cells in 1.0 ml of PLM-5. For sublethal infection, 6-week-old mice were infected i.p. with 10<sup8</sup> *Leptospira* cells in 1.0 ml of PLM-5. Control, uninfected animals received the same volume of sterile culture medium. Infected animals were clinically observed twice daily.

**Activation of elicited mouse peritoneal macrophages.** Mice (6 to 8 weeks old) were injected i.p. with 1 ml of 5 mM sodium periodate in phosphate-buffered saline per mouse 4 days prior to harvesting of macrophages. The peritoneal macrophages were harvested in phosphate-buffered saline; washed with a solution containing Dulbecco’s modified Eagle’s medium high-glucose medium, 10% fetal calf serum, 10 mM HEPES, and 2 mM glutamine; and seeded at 2 × 10<sup6</sup> cells per well of 96-well plates. The plate was incubated for 1 to 2 h at 37°C to allow cells to adhere. After washing once with medium, cells were stimulated with various reagents. After 16 h of incubation, the supernatant was harvested and assayed for mouse cytokines using enzyme-linked immunosorbent assay (ELISA) kits for tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) (OptEIA set; Pharmingen), and macrophage inflammatory protein 2 (MIP-2) (Duo set; R&D Systems) according to the manufacturers’ protocols.

**Real-time PCR for quantification of* Leptospira* cells in tissues of mice.** Mice were euthanized with thiopental. Organs were harvested and stored in 70% ethanol at −20°C until used for extraction. Three sections of approximately 25 mg were accurately weighed (for normalization of bacterial concentrations) and used for genomic DNA extraction. DNA extraction was done using the QIAamp tissue DNA extraction kit according to the manufacturer’s directions (QIAGEN). Five microliters of DNA was added to a PCR reaction mixture containing 25 µl Platinum Quantitative PCR Supermix-UDG (Invitrogen), 0.6 µM each forward and reverse 16S rRNA gene primer, and a FamTam probe (5′-CTC ACC AAG GCG ACG ATC GGT AGC-3′) as described previously by Smythe et al. (32). The concentration of the final PCR product of 87 bp was determined using a standard curve generated with serial dilutions (10<sup6</sup> to 10<sup10</sup>) of genomic DNA extracted from in vitro-cultivated bacteria. Tissue sections from an uninfected control animal were spiked with appropriate numbers of bacteria and then extracted using a standard protocol using the QIAamp tissue DNA extraction kit for use as a template in the standard curve.

**Statistical analysis.** Data were analyzed in a one-way analysis of variance with post hoc Tukey tests for pairwise comparisons to avoid type 1 error inflation using Prism software (GraphPad). Probabilities (P values) less than 0.05 were considered significant.

**RESULTS**

High-dose challenge infection of TLR4 mutant but not TLR4 WT mice with *Leptospira interrogans* serovar Icterohaemorrhagiae strain HA1188 recapitulates severe human leptospirosis. To investigate the role of innate immune responses in leptospirosis infection, we infected 3-week-old C3H/HeJ mice (TLR4 deficient), C3H/HeJ-SCID mice (TLR4 deficient and lacking T and B cells), and C3H/OuJ mice (WT) with a high dose (6 × 10<sup8</sup> cells) of *Leptospira interrogans* serovar Icterohaemorrhagiae strain HA1188. The challenge inoculum used a low-passage isolate (passage 2) obtained originally from a fatal case of human leptospirosis in the Peruvian Amazon which clinically manifested as jaundice, severe hemoptysis, and refractory shock (4). Infected sick mice developed piloerection, progressing to hunching and listlessness before becoming severely moribund, which necessitated euthanasia at 4 to 5 days for C3H/HeJ mice and 3 to 4 days for C3H/HeJ-SCID mice and at day 8 for one C3H/OuJ mouse. The C3H/HeJ and C3H/HeJ-SCID mice all had obvious jaundice (Fig. 1A), pulmonary hemorrhage (Fig. 1B), blood in nares (not shown), mediastinal hemorrhage (not shown), and massive splenic enlargement. C3H/HeJ SCID mice appeared to die at the same rate as or only slightly more rapidly than the C3H/HeJ mice (Table 1). In contrast, eight out of nine C3H/OuJ (wild-type TLR4) mice survived infection without external evidence of
The one C3H/OuJ mouse that died was not jaundiced, nor did it have pulmonary hemorrhage; the cause of death was not able to be determined. The surviving eight C3H/OuJ mice were sacrificed at day 14 after infection. At necropsy, all organs appeared grossly normal. Cultures of kidneys for Leptospira were positive in all groups, including those of kidneys from the wild-type mice at 14 days, confirming infection.

Sublethal infection resulted in higher leptospiral density in kidneys of TLR4 mutant mice than in those of WT mice. When 6-week-old C3H/HeJ and C3H/OuJ mice were infected with a lower dose (1 x 10^8 cells) of Leptospira interrogans serovar Icterohaemorrhagiae strain HAI188, all mice survived. Organs were removed 21 days after infection, and the presence of Leptospira was determined quantitatively by real-time PCR. Leptospira was presented in heart, lung, liver, spleen, and kidneys in both C3H/HeJ and C3H/OuJ mice. However, a significantly higher organism burden was observed in the kidneys and lungs of C3H/HeJ mice than in C3H/OuJ mice (Fig. 2).
These data suggest that intact TLR4 is involved in the control of leptospirosis burden in tissues of sublethally infected mice, which more closely mimics the natural state of infection.

**Leptospira stimulates periodate-elicited mouse peritoneal macrophages to produce proinflammatory cytokines.** To determine whether *Leptospira* stimulated production of proinflammatory cytokines by mouse macrophages, heat-killed *L. interrogans* serovar Icterohaemorrhagiae strain HAI188 at doses varying from $10^4$ to $10^6$ cells/ml was incubated with peritoneal macrophages overnight. *Leptospira* stimulated production of MIP-2, IL-6, and TNF-α in a dose-dependent manner (Fig. 3A). In the presence of PMB, which is known to bind to and inhibit LPS activation, no significant difference was found in the cytokine production stimulated by *Leptospira* (Fig. 3A). As a positive control, PMB inhibited cytokine production induced by *Salmonella minnesota* Re595 LPS (Fig. 3A). It was reported that PMB also bound to leptospiral LPS and inhibited TLR activation (37). To investigate whether the macrophages produced cytokines in response to the LPS component of heat-killed *Leptospira*, we activated mouse peritoneal macrophages with leptospiral LPS in the presence and absence of PMB (Fig. 3B). As a further control, MIP-2, IL-6, and TNF-α responses to *Leptospira* LPS as well as *Salmonella minnesota* Re595 LPS were inhibited by PMB. In contrast, the cytokine responses to heat-killed whole *Leptospira* cells were not inhibited by PMB. This result is consistent with a non-LPS leptospiral component being involved in most of the cytokine induction of mouse macrophages by heat-killed *Leptospira*.

**Leptospira stimulation of MIP-2 production is TLR4 dependent but CD14 independent.** Using MIP-2 production as a sensitive measure of proinflammatory cytokine secretion, we determined whether *Leptospira* stimulation of macrophages from wild-type C57BL/6 mice was dependent on normal TLR4 and CD14 function. Anti-TLR4/MD-2 complex MAb (MTS510) and anti-TLR4 MAb (14H4E12) both significantly decreased MIP-2 production from macrophages stimulated by *Leptospira* (Fig. 4A). As negative controls, neither tissue culture medium alone nor isotype control antibody had a significant effect on MIP-2 production. An anti-CD14 monoclonal antibody, 28C5, did not reduce MIP-2 production in response to heat-killed *Leptospira*. As a positive control, 28C5 did inhibit *Salmonella minnesota* Re595 LPS stimulation of MIP-2 production. This result suggests that *Leptospira* stimulates MIP-2 production in a CD14-independent manner (Fig. 4). As expected, anti-TLR4/MD2, anti-TLR4, and anti-CD14 antibodies significantly inhibited MIP-2 production induced by *Salmonella minnesota* Re595 LPS (Fig. 4). 

As independent confirmation that CD14 does not play a role in leptosomal stimulation of proinflammatory cytokines, peritoneal macrophages from C57BL/6 (WT) and CD14−/− mice were coincubated with heat-killed *Leptospira*. *Salmonella minnesota* Re595 LPS was used as a control. While Re595 LPS stimulated much higher levels of MIP-2 and IL-6 from macrophages of the WT mice than from macrophages of CD14−/− mice, the responses of both MIP-2 and IL-6 production to *Leptospira* stimulation were similar in both the WT and CD14−/− macrophages (Fig. 5).

**The role of TLR2 in macrophage responses to Leptospira.** Previously, Werts et al. showed that *Leptospira* LPS, unlike *Salmonella* or *E. coli* LPS, activates cells through human TLR2 (37). We determined whether intact *Leptospira* isolates behave similarly by using peritoneal macrophages from TLR2−/− and C56BL/6 (WT) mice. Macrophages from both mice secreted similar levels of IL-6 and TNF-α upon activation with heat-killed *Leptospira* and Re595 LPS (Fig. 6). Leptosomal LPS, however, induced much higher levels of cytokine responses from WT murine macrophages than from macrophages from TLR2−/− mice. The cytokine levels were similar to cytokine responses induced by heat-killed *Staphylococcus aureus*, a ligand known to stimulate TLR2-dependent responses. We also observed that 1 µg/ml of leptosomal LPS induced levels of TNF-α similar to those induced by 1 ng/ml Re595 LPS in the WT mice. This observation suggests that leptosomal LPS is at least 1,000-fold less active than Re595 LPS in stimulating cytokine responses.

**Macrophage cytokine responses to Leptospira are dependent on TLR4.** To assess directly whether leptosomal stimulation of peritoneal macrophages was dependent on intact TLR4, we compared cytokine production of peritoneal macrophages

![FIG. 2. Quantitative measurement of *Leptospira* (Leptos) in infected mouse tissues. C3H/OuJ (OuJ) and C3H/HeJ (HeJ) mice were infected with 10⁴ bacteria i.p. The mice were sacrificed 21 days after infection, and the heart, liver, lung, spleen, and kidney were removed for quantitative analysis of bacterial load by real-time PCR of 16S rRNA genes. Three pieces of each organ were analyzed in a triplicate real-time PCR. Bars represent means ± standard errors (SE) of assays from a group of three mice. An * indicates a P value of <0.05.](http://iai.asm.org/)
Leptospira-induced MIP-2 and IL-6 responses in macrophages were abrogated in C3H/HeJ mice, while high levels of these cytokines were observed in C3H/OuJ mice. Heat-killed Staphylococcus and Leptospira LPS induced high levels of the cytokines independent of TLR4 (Fig. 7A). When macrophages from transgenic TLR4−/− mice were compared with those from WT mice, ex vivo-obtained TLR4−/− macrophages failed to produce MIP-2, IL-6, and TNF-α after stimulation with all doses of Leptospira (Fig. 7B). The responses to other control stimuli, Re595 LPS, heat-killed Staphylococcus LPS, and leptospiral LPS, were similar to the results shown in Fig. 7A (data not shown).

Leptospira activates macrophages predominantly through the MyD88 pathway. TLRs signal through MyD88-dependent and -independent pathways. To determine whether the cellular signaling pathway induced by Leptospira is mediated through MyD88, peritoneal macrophages from MyD88−/− mice were stimulated with Leptospira. As with heat-killed Staphylococcus and Re595 LPS, heat-killed Leptospira stimulated MIP-2, IL-6, and TNF-α in WT mice but induced very low levels of cytokines in macrophages obtained from MyD88−/− mice (Fig. 8). This result suggests that Leptospira-stimulated signal transduction pathways leading to cytokine secretion are predominantly mediated through the signaling molecule MyD88.

FIG. 3. Heat-killed Leptospira interrogans serovar Icterohaemorrhagiae strain HAI188-induced cytokine response in periodate-elicited peritoneal macrophages of C57BL/6 mice. (A) The macrophages were unstimulated (Uns) or stimulated with increasing doses of Leptospira (Lepto) per milliliter of culture medium or 1 ng/ml Re595 LPS in the absence (light bar graph) or presence (dark bar graph) of 50 μg/ml PMB for 16 h. (B) Macrophages were unstimulated (Uns) or stimulated with 1 ng/ml Re595 LPS, 107 Leptospira cells/ml (Lepto), or 1 μg/ml purified Leptospira LPS in the absence (light bar graph) or presence (dark bar graph) of 50 μg/ml PMB for 16 h. The culture supernatant was assayed for MIP-2, IL-6, and TNF-α by ELISA. Data are means ± SE of duplicate experiments. *, P < 0.05.

FIG. 4. Antibody inhibition of MIP-2 production in mouse C57BL/6 periodate-elicited peritoneal macrophages. The macrophages were unstimulated (Uns) or stimulated with Leptospira interrogans serovar Icterohaemorrhagiae at the concentration of 104 bacteria/ml (Lepto) or 100 pg/ml Re595 LPS in the absence (None) or presence of 10 μg/ml of monoclonal antibodies against CD14 (28C5), TLR4/MD-2 (MTS510), or TLR4 (14H4E12) or control IgG1 isotype antibody (Control Ig). Bars represent means ± SE of duplicate experiments.

from C3H/HeJ and control C3H/OuJ mice. Leptospira-induced MIP-2 and IL-6 responses in macrophages were abrogated in C3H/HeJ mice, while high levels of these cytokines were observed in C3H/OuJ mice. Heat-killed Staphylococcus and Leptospira LPS induced high levels of the cytokines independent of TLR4 (Fig. 7A). When macrophages from transgenic TLR4−/− mice were compared with those from WT mice, ex vivo-obtained TLR4−/− macrophages failed to produce MIP-2, IL-6, and TNF-α after stimulation with all doses of Leptospira (Fig. 7B). The responses to other control stimuli, Re595 LPS, heat-killed Staphylococcus LPS, and leptospiral LPS, were similar to the results shown in Fig. 7A (data not shown).

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The key finding in this study was that intact TLR4 function in a murine model of severe leptospirosis was critical for mouse survival and for preventing jaundice, pulmonary hemorrhage, and death. This effect was not related to leptospiral LPS since polymyxin B had no effect on Leptospira-induced cytokine production. Leptospiral stimulation of murine peritoneal macrophages was not dependent on CD14, consistent with the lack of LPS stimulation of the TLR4 pathway. In contrast to findings in the guinea pig model of leptospiral pulmonary hemorrhage (22), immunopathology related to either T or B cells in this model system did not occur.

Jaundice and pulmonary hemorrhage are unique clinical syndromes associated with leptospirosis and differ from other bacterial diseases caused by intra- and extracellular pathogens such as E. coli, Salmonella spp., and Listeria. Leptospirosis is therefore a unique system of bacterial pathogenesis. This murine model, which directly recapitulates severe human leptospirosis, suggests a critical role of the innate immune system, particularly the TLR4 gene or its associated components, in early infection by Leptospira.

Our results also demonstrate that intact TLR4 signaling contributes to the control of the tissue burden of Leptospira in nonlethal infection. Natural mammalian reservoir hosts of leptospires generally do not develop severe pathology in leptospiral infection. It has been well documented that leptospires can persist for prolonged periods of time in renal tubules of a wide variety of mammals (cattle, dogs, pigs, rats, mice, and humans) as well as in other tissues, such as brain, even in the absence of antileptospiral antibodies (6, 17, 35). Therefore, our finding that TLR4-deficient mice have significantly higher numbers of leptospires, particularly in the target organs mediating both leptospiral disease (liver, lung, and kidney) and transmission (kidney), is novel and important.

Our studies are consistent with the observations of Werts et al. (37), who previously observed that leptospiral LPS activates mouse macrophages through TLR2 and that there are no differences between mouse and human TLR2 (37) in the ability to recognize leptospiral LPS. Although a high quantity of purified leptospiral LPS can cause death in mice (37), our studies here clearly demonstrate that leptospiral ligands other than LPS play crucial roles in the pathogenesis of intact Leptospira. In contrast to our results, Werts et al. found that intact, heat-killed leptospires activate cells in a CD14-dependent manner using a human THP.1 cell line transfected with a human CD14 construct. We were unable to confirm this finding with murine macrophages when we used intact, heat-killed leptospires either to stimulate ex vivo-obtained CD14-deficient murine macrophages in the presence of an anti-CD14 monoclonal antibody or to stimulate macrophages from wild-type and CD14−/− mice. A similar discrepancy in the CD14 dependence of cytokine production between ex vivo-obtained murine macrophages and CD14-transfected THP.1 cell lines has previously been observed with Aspergillus fumigatus (18). The most likely

**FIG. 5.** Production of cytokines by peritoneal macrophages of CD14-deficient mice. Mouse peritoneal macrophages of C57BL/6 wild-type or C57BL/6 CD14−/− mice either were left unstimulated (Uns) or were stimulated with 10⁶ Leptospira (Lepto) cells/ml or 1 ng/ml of Re595 LPS. MIP-2 (left panel) and IL-6 (right panel) concentrations were measured by ELISA after 18 h of stimulation. Bars represent means ± SE of duplicate experiments. *, P < 0.05.

**FIG. 6.** Production of cytokines by peritoneal macrophages of TLR2-deficient mice. Mouse peritoneal macrophages of C57BL/6 wild-type or C57BL/6 TLR2−/− mice either were left unstimulated (Uns) or were stimulated with Leptospira (Lepto) at 10⁶ bacteria/ml, 1 ng/ml Re595 LPS (LPS), 1 μg/ml Leptospira LPS (Lepto LPS), or 10⁶ cells/ml of heat-killed Staphylococcus aureus (Staph). IL-6 and TNF-α concentrations were measured by ELISA after 18 h of stimulation. Bars represent means ± SE of assay performed in duplicate. *, P < 0.05.
explanation for this discrepancy is that CD14-transfected cell lines may produce biased cytokine responses compared to those of wild-type cells. It is also possible that human and mouse CD14 differ in their ability to interact with intact leptospires. It is also known that there are differences between human and mouse cells in their abilities to respond to gram-negative LPS and taxol in a TLR4-dependent manner and that the species specificity of the recognition lies in the TLR4 adaptor molecule MD-2, which directly binds to the ligand (36). The requirement of MD-2 in leptosiral recognition needs to be investigated in more detail. Our preliminary studies using mouse and human TLR4-transfected cell lines indicate that there are no differences between mouse and human recognition of intact Leptospira (S. Viriyakosol, unpublished observation). Our finding that the TLR4 pathway protects mice from severe leptospirosis has direct implications for understanding leptospirosis in humans. Most cases of leptospiral infection are asymptomatic; the vast majority of symptomatic infections result in self-resolving undifferentiated fever (1). Relatively few,

FIG. 7. Role of TLR4 in peritoneal macrophage response to Leptospira. (A) Mouse peritoneal macrophages of C3H/OuJ (OuJ) and C3H/HeJ (HeJ) mice were stimulated with 1 ng/ml Re595 LPS (LPS), 10^6 cells/ml heat-killed Staphylococcus aureus (Staph), 10^6 cells/ml Leptospira (Lepto), or 1 μg/ml Leptospira LPS (Lepto LPS). MIP-2 and IL-6 concentrations were measured by ELISA after 18 h of stimulation. Bars represent means ± standard errors of a duplicate assay. (B) Mouse peritoneal macrophages of C57BL/6 wild-type and TLR4−/− mice were stimulated with various doses of Leptospira at the indicated number of bacteria per milliliter. MIP-2, IL-6, and TNF-α concentrations were measured by ELISA after 18 h of stimulation. Bars represent means ± SE of assays performed in duplicate. An * indicates a P value of <0.05.

FIG. 8. Production of cytokines by peritoneal macrophages of MyD88-deficient mice. Mouse peritoneal macrophages of C57BL/6 wild-type or C57BL/6 MyD88−/− mice either were left unstimulated (Uns) or were stimulated with 1 ng/ml Re595 LPS (LPS), 10^6 cells/ml heat-killed Staphylococcus aureus (Staph), or Leptospira (Lepto) at 10^6 and 10^7 bacteria/ml. MIP-2, IL-6, and TNF-α concentrations were measured by ELISA after 18 h of stimulation. Bars represent means ± SE of assays performed in duplicate.
perhaps only 5 to 10% of symptomatic cases, manifest as severe disease (Weil’s syndrome [fever, jaundice, and renal failure]) (4), and of these cases, only a minority result in severe pulmonary hemorrhage (12, 29). An understanding of the mechanisms underlying the predisposition of humans to develop severe leptospirosis would be a major advance in the development of new approaches to treat and prevent this disease, a major problem in the developing world. The present study provides a strong rationale for carrying out a population-based analysis of TLR4 gene polymorphisms and their relationship to the clinical expression of leptospirosis.

Of substantial significance is that the data presented here suggest that the magnitude of TLR4 stimulation by Leptospira in vivo likely far outweighs the leptospirosis LPS stimulation of TLR2 after challenge infection, given the need for pharmacological levels of LPS to induce cytokine production but relatively low levels of heat-killed intact leptospires (similar to the blood or tissue burden that would be present in natural infection) that stimulate TLR4.

Unlike experimental infections such as E. coli pneumonia (16) or Pneumocystis carinii (34), our experiments also demonstrated the correlation of macrophage cytokine responses in vitro and in vivo disease outcomes. Macrophages from TLR4-defective mice showed less inflammatory cytokine responses than those from the wild-type mice. The reduced inflammatory response is associated with impaired bacterial elimination in the kidneys in the sublethal infection experiments as well as death in the lethal infection in the TLR4-deficient mice. The role of inflammatory cytokines in the disease process in vivo is being investigated. Although macrophages of TLR2+/− mice showed no defect in responses to intact Leptospira, the role of TLR2 in experimental infection still remained to be determined.

Mechanisms by which Leptospira activates the TLR4 pathway remain to be determined. In other microbiological systems, several ligands of TLR4 have been identified. The best characterized of these ligands is gram-negative bacterial LPS (28), which, depending upon whether it is the smooth or the rough type, stimulates TLR4 in either a CD14-dependent or -independent manner (11). CD14-independent TLR4 stimulation of cytokines is mediated solely by MyD88-dependent responses (11). We have shown that Leptospira activates the murine TLR4 pathway in a predominantly MyD88-dependent, CD14-independent manner. It is possible that the TLR4 responses to Leptospira that we observed were due to a hitherto-uncharacterized leptospirosis LPS that stimulates TLR4 but not TLR2. Bacteria such as Porphyromonas gingivalis have been shown to produce different, structurally distinct lipid A components of LPS (23) that may stimulate different TLRs. However, the TLR4-dependent responses to intact Leptospira were not inhibited by polymyxin B, which was known to inhibit LPS responses (37). Therefore, our data suggest that it is unlikely that the leptospirosis TLR4 ligand(s) in our studies includes LPS. Recently, several non-LPS TLR4 agonists have been identified. These include pathogen- and host-derived ligands such as coat protein F from respiratory syncytial virus, host-derived Hsp60, fibroactin, hyaluronic acid (3), and, most recently, minimally oxidized low-density lipoprotein (21) and anthracyclin O from Bacillus anthracis (26). A non-LPS TLR4 agonist from intact Mycobacterium tuberculosis activates cells in a CD14-independent manner (20). Identifying the Leptospira component(s) that stimulates TLR4 will be essential for understanding the role of innate immunity in human leptospirosis as well as TLR-dependent cellular responses in general.

The experiments presented here establish a clear basis for pursuing several distinct lines of investigation that will provide additional mechanistic insight into the pathogenesis of leptospirosis and in vivo dynamics during asymptomatic leptospirosis infection. Important avenues of further research include elucidating the effector cell type of the TLR4-containing cell, the relationship of human TLR4 polymorphisms to severity of human leptospirosis, the biochemical components of Leptospira that interact with TLR4 and TLR4-containing protein complexes, and whether differences in these leptosporal components between strains explains virulence potential and human predisposition to severe rather than mild clinical outcomes of leptospirosis infection.

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