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Toll-like receptor 2 mediates early inflammation by leptospiral outer membrane proteins in proximal tubule cells

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Tubulointerstitial nephritis is a cardinal renal manifestation in leptospirosis and LipL32, the major lipoprotein component of leptospiral outer membrane proteins (OMPs), induces a robust inflammatory response in cultured renal proximal tubule cells through a nuclear factor-κB-related pathway. Here, we investigated whether Toll-like receptor (TLR), known to play a pivotal role in innate immunity, could mediate the inflammatory response induced by leptospiral OMPs in renal proximal tubule cells. TLR expression was analyzed by flow cytometry and indirect immunofluorescence in cultured mouse proximal tubule (pyruvate kinase simian virus 40-proximal straight (PKSV-PR)) cells. Reverse transcription-competitive polymerase chain reaction and enzyme-linked immunosorbent assay were undertaken to analyze the inducible effects of inducible nitric oxide synthase (iNOS) and monocyte chemoattractant protein-1 (MCP-1 also termed CCL2) by pathogenic and non-pathogenic leptospiral OMPs and recombinant lipoproteins in either PKSV-PR cells or TLR-transfected human embryonic kidney (HEK) 293 cells. Anti-TLR antibodies were used for blocking experiments. *Leptospira santarosai* serovar *Shermani* OMPs and LipL32 induced a significant increase in TLR2 but not TLR4 expression in PKSV-PR cells. The increase in iNOS and CCL2/MCP-1 mRNA expressions could be prevented by an anti-TLR2 antibody, but not by an anti-TLR4 antibody. Furthermore, leptospiral OMPs stimulated both CCL2/MCP-1 mRNA and secreted protein in transfected HEK 293 cells with a TLR2-expressing plasmid, but had no effect in cells with a TLR4-expressing plasmid. In conclusion, these findings indicate that the stimulation of iNOS and CCL2/MCP-1 caused by pathogenic leptospiral OMPs, in particular LipL32, in proximal tubule cells requires TLR2 for the early inflammatory response.

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Leptospirosis is a re-emerging and widespread zoonosis in many tropical regions.[1,2] In the infected host, pathogenic leptospires can disseminate hematogeneously and invade kidneys, which represents one of the most frequently affected organs, and through the effects of leptospiral endotoxins and immunologic responses favor the development of tubulo-interstitial nephritis.[2–4]

Leptospiral outer membrane proteins (OMPs) and lipopolysaccharide (LPS) are the major antigens that confer immunity to leptospires and are thought to be involved in host–pathogen interactions.[5] We have previously shown that pathogenic leptospiral OMPs activate the expression of proinflammatory genes related to tubulointerstitial nephritis in mouse renal medullary thick ascending limb cells.[6] We have also shown that LipL32, the major leptospiral outer membrane lipoprotein, can induce a robust inflammatory response in cultured mouse renal proximal tubule cells.[7] The identification of virulent OMP components have represented an important step in the understanding of the pathogenesis of leptospirosis tubulointerstitial nephritis.[4,8]

Microbial invasion of the host triggers a series of events designed to control and eventually eliminate the infection. A number of pattern recognition receptors, which recognize defined conserved microbial structures or products of microbial metabolism, have been shown to play a key role in innate immunity. Among them, the family of Toll-like receptors (TLRs) participate in the innate defense against a variety of bacterial infections by initiating acute inflammatory responses.[9,10] Recent studies have also shown that renal tubule cells express TLRs,[11] mainly TLR2 and TLR4, that are upregulated following the reperfusion of ischemic kidneys.[12] TLR4 recognizes LPS of Gram-negative bacteria,[13] whereas TLR2 provides responsiveness to bacterial lipoproteins and peptidoglycan of Gram-positive bacteria.[14–16] Recognition of bacterial components by their respective TLRs will allow the activation of the nuclear factor-κB pathway and the stimulation of chemokines and cytokines production.[17]

Although leptospirosis is an important cause of acute renal failure, the mechanism of renal dysfunction caused by this microorganism is not fully understood. We have shown that leptospiral OMPs and the main antigenic component, LipL32 lipoprotein, certainly play a role in the pathogenesis of leptospirosis renal disease by mediating the activation of the nuclear factor-κB-associated pathways and the release of cytokines.[6,7] Werts *et al.*[18] have provided the first demonstration that TLR2 is required for the activation of macrophages by leptospiral outer membrane constituents. Because this TLR is highly expressed in renal tubule cells, the question arises as to whether TLR2 participates in the inflammatory response triggered by pathogenic leptospires in renal proximal epithelial tubule cells. To answer this question, experiments were carried out to analyze the expression of TLR2 stimulated by leptospiral OMPs and its participation in the induction of the inflammatory response caused by pathogenic leptospiral OMPs in cultured mouse proximal tubule PKSV-PR cells[19] and in human embryonic kidney (HEK) 293 cells transiently expressing TLR2. The results of this study indicate that OMPs from pathogenic leptospires and purified LipL32 increased the expression of TLR2 and stimulated the release of the monocyte chemoattractant protein-1 (CCL2/MCP-1) in renal proximal tubule cells. These results strongly suggest thus that TLR2 initiate the inflammatory response caused by pathogenic leptospires in renal tubule epithelial cells.

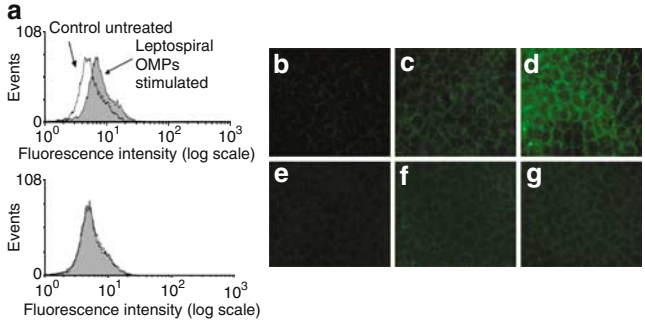

Figure 1 | Effects of leptospiral outer membrane proteins on TLR2 and TLR4 expression in renal proximal tubule cells. (**a**) Flow cytometry analysis of mouse proximal tubule cells stimulated with leptospiral OMP extracts. TLR2 and TLR4 fluorescence-activated cell sorting analyses using monoclonal rat anti-mouse TLR2 antibody, monoclonal rat anti-mouse TLR4 antibody or rat immunoglobulin G2a isotype as control were performed on confluent PKSV-PR cells incubated with or without 0.2 μg/ml *L. santarosai* serovar *Shermani* OMPs for 48 h. Note the increase in TLR2 fluorescence signal following the addition of leptospiral OMPs (**a**) while the profile of TLR4 fluorescence did not change in leptospiral OMPs-treated cells. (**b**–**g**) Immunodetection of TLR2 in PKSV-PR cells. Immunofluorescence studies using anti-TLR2 and TLR4 antibodies were performed on confluent PKSV-PR cells incubated (**c**, **f**) without or (**d**, **g**) with 0.2 μg/ml *L. santarosai* serovar *Shermani* OMPs for 48 h. Note the marked TLR2 staining delineating cell peripheries in (**d**) leptospira-treated cells as compared to that of (**c**) untreated cells. (**f**, **g**) In contrast, leptospiral OMPs did not affect the TLR 4 staining. As controls, only a faint labeling was observed by using (**b**) preimmune rabbit serum or (**e**) rat isotype immunoglobulin G control. Original magnification: × 1000.

RESULTS

Pathogenic leptospiral OMP extract stimulates the expression of TLR2 in cultured late proximal tubule cells

We first analyzed the effects of leptospiral OMPs on TLR2 and TLR4 expression in cultured mouse PKSV-PR cells by using flow cytometry. Adding 0.2 μg/ml OMPs extracted from *Leptospira santarosai* serovar *Shermani* for 48 h increased TLR2 expression by 28% as compared to untreated cells ($P<0.05$). In contrast, the stimulation by leptospiral OMPs did not induce any increase in the expression of TLR4 (Figure 1a). Consistent with these results, indirect immunofluorescence studies revealed that leptospiral OMPs induced a marked redistribution of the cytoplasmic TLR2 at the cell peripheries of proximal tubule cells, without affecting the weak cytoplasmic TLR4 staining (Figure 1b).

The levels of mRNA expression for TLR2, TLR4, and β-actin were then analyzed by using reverse transcriptase (RT)-competitive polymerase chain reaction (PCR). Incubation of PKSV-PR cells with 0.2, 0.3, 0.5, or 1 μg/ml leptospiral OMPs for 48 h induced significant 1.8-, 1.8-, 1.9-, and 1.8-fold increase, respectively, in the level of TLR2/β-actin mRNA expression as compared to that of untreated cells. Conversely, no changes in TLR4/β-actin mRNA levels were observed when incubating cells with increasing concentrations of leptospiral OMPs (Figure 2a). The levels of TLR2 and β-actin mRNA expression were further analyzed with the two recombinant LipL32 and LipL41 leptospiral lipoproteins. Incubating cells with increasing concentrations (0.6–12 ng/ml) of LipL32 for 48 h induced significant 1.5- to 1.9-fold increases in the level of TLR2/β-actin mRNA expression as compared to that of untreated cells. Conversely, no changes in TLR2/β-actin mRNA levels were observed when incubating cells with increasing concentrations of recombinant LipL41 (Figure 2b).

To ensure that the observed increase in TLR2 expression was directly related to pathogenic leptospiral OMPs, additional experiments were carried out using OMPs extract from another pathogenic serovar *Leptospira interrogans* serovar *Bratislava*, and compared to that from the non-pathogenic serovar *Leptospira biflexa* serovar *Patoc*. PKSV-PR cells were incubated with these different OMPs extracts (0.2 μg/ml) for 48 h. The results from RT competitive-PCR revealed that both OMPs extracted from *L. santarosai* serovar *Shermani* and *L. interrogans* serovar *Bratislava* induced significant ($P<0.05$) 1.8- and 1.6-fold increases, respectively, in the level of TLR2/β-actin mRNA, whereas OMPs from *L. biflexa* serovar *Patoc* did not affect the levels (Figure 3a). Overall these results suggested that leptospiral OMPs activate the innate immune receptor TLR2 in renal proximal tubule cells.

Effects of recombinant LipL32 and antibody raised against LipL32 on TLR2 expression in late proximal tubule cells

To answer whether LipL32, a major component of pathogenic leptospires, could *per se* stimulate the expression of TLR2 in renal proximal tubule cells, cultured PKSV-PR cells were

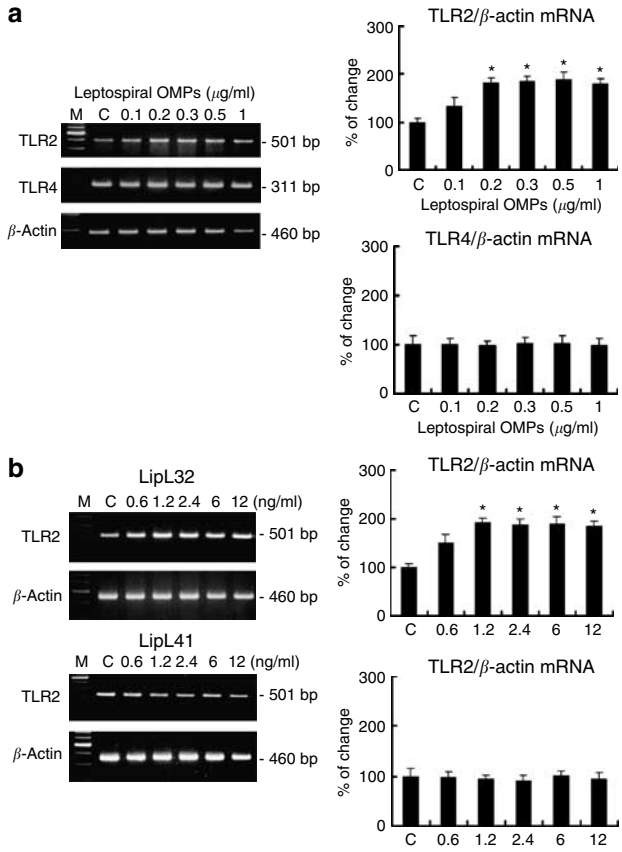

Figure 2 | Effects of increasing concentrations of leptospiral OMPs, LipL32, and LipL41 on the expression of TLR2 and TLR4 mRNAs. PKSV-PR cells were incubated with increasing concentrations of purified OMPs extracted from (**a**) *L. santarosai* serovar *Shermani* or (**b**) increasing concentrations of LipL32 or LipL41 for 48 h. The results from RT-competitive RT-PCR were expressed as percent changes of (**c**) untreated control cell values and corrected by the values of β-actin mRNA, used as internal standard. M: marker; C: control. The bars are the means ± s.e.m. from three independent experiments. *$P<0.05$ versus control untreated (C).

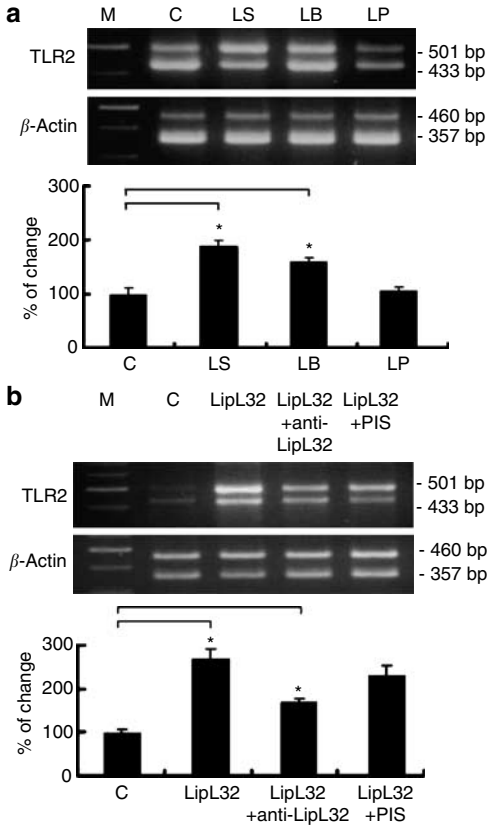

Figure 3 | Effects of pathogenic and non-pathogenic leptospiral OMP and purified LipL32 on TLR2 expression in renal proximal tubule cells. (**a**) Effects of outer membrane protein extracts from pathogenic *L. santarosai* serovar *Shermani*, *L. interrogans* serovar *Bratislava* and non-pathogenic serovar *L. biflexa* serovar *Patoc* on TLR2 mRNA expression in renal proximal tubule cells. β-Actin was used as internal standard. PKSV-PR cells were incubated with 0.2 μg/ml of different OMPs extracts for 48 h. The results from RT-competitive PCR and were expressed as TLR2/β-actin mRNA ratio and the percent changes of untreated cell values (control, C). (**b**) Effects of LipL32 on TLR2 expression in proximal tubule cells. PKSV-PR cells were incubated with recombinant LipL32 alone or with LipL32 plus an antiserum raised against LipL32 (anti-LipL32) or with a preimmune serum (PIS). Note that the recombinant LipL32 increased the expression of TLR2 and that the anti-LipL32 antibody blunted the increase in TLR2 expression caused by LipL32. M: marker; C: control; LS: *Leptospira santarosai* serovar *Shermani*; LB: *Leptospira interrogans* serovar *Bratislava*; LP: *Leptospira biflexa* serovar *Patoc*. The bars are means ± s.e.m. from three independent experiments. *$P<0.05$ versus control untreated (C).

therefore incubated with the recombinant LipL32 alone or with antisera raised against LipL32 (anti-LipL32). The recombinant LipL32 (2.4 ng/ml for 48 h) stimulated by 2.7-fold the level of TLR2 mRNA expression in confluent cultures of PKSV-PR cells. Co-incubation of the cells with recombinant LipL32 and the anti-LipL32 antibody partially prevented the increase in TLR2 expression: the anti-LipL32 antibody significantly reduced ($P<0.05$) by 50% the expression of TLR2 mRNA (Figure 3b). As control, replacement of the anti-LipL32 antibody by preimmune serum had almost no effect on the induction of TLR2 mRNA expression induced by LipL32. Altogether, these results indicate that LipL32 can directly affect TLR2 gene expression in renal proximal tubule cells.

Role of TLR2 in iNOS and CCL2/MCP-1 stimulation caused by leptospiral OMPs or LipL32

Adding *L. santarosai* serovar *Shermani* OMPs (0.2 μg/ml for 48 h) to cultured PKSV-PR cells significantly increased the expression of inducible nitric oxide synthase (iNOS) (× 6.3-fold, $P<0.01$) and CCL2/MCP-1 (× 2.8-fold, $P<0.05$) mRNAs as compared to those measured in untreated cells. PKSV-PR cells were further incubated with *L. santarosai* serovar *Shermani* OMPs alone or with antisera raised against TLR2 and/or TLR4 (gifts from Tularik Inc., San Francisco, CA, USA) to evaluate the respective roles of TLR2 and TLR4. Preincubation of the cells with the anti-TLR2 antibody significantly reduced ($P<0.05$) by 46 and 69% the increase in iNOS and CCL2/MCP-1 mRNAs, respectively, caused by leptospiral OMPs. On the other hand, the anti-TLR4 antibody did not significantly reduce the levels

of iNOS and CCL2/MCP-1 stimulated by leptospiral OMPs. Furthermore, preincubating cells with the anti-TLR4 antibody plus the anti-TLR2 antibody did not further reduce the increased levels of iNOS and CCL2/MCP-1 mRNAs caused by leptospiral OMPs. These findings indicate thus that TLR2, but not TLR4, is specifically involved in the induction of iNOS and CCL2/MCP-1 caused by leptospiral OMPs (Figure 4a).

Cultured renal proximal tubule cells were then further incubated with the recombinant LipL32 (2.4 ng/ml) and the anti-TLR2 antiserum. Recombinant LipL32 significantly increased the mRNA levels of iNOS by 2.0-fold ($P<0.05$) and CCL2/MCP-1 by 1.7-fold ($P<0.05$), respectively. In contrast, preincubation of the cells with the anti-TLR2 antiserum prevented ($P<0.05$) the increase in iNOS and CCL2/MCP-1 caused by LipL32 by 52 and 35%, respectively. Again, the replacement of the anti-TLR2 antibody by the preimmune serum had almost no inhibitory action on LipL32-stimulated iNOS or CCL2/MCP-1 expressions (Figure 4b). These results strongly suggest that TLR2 mediates the induction of tubulointerstitial nephritis-related mRNAs caused by leptospiral OMPs or LipL32.

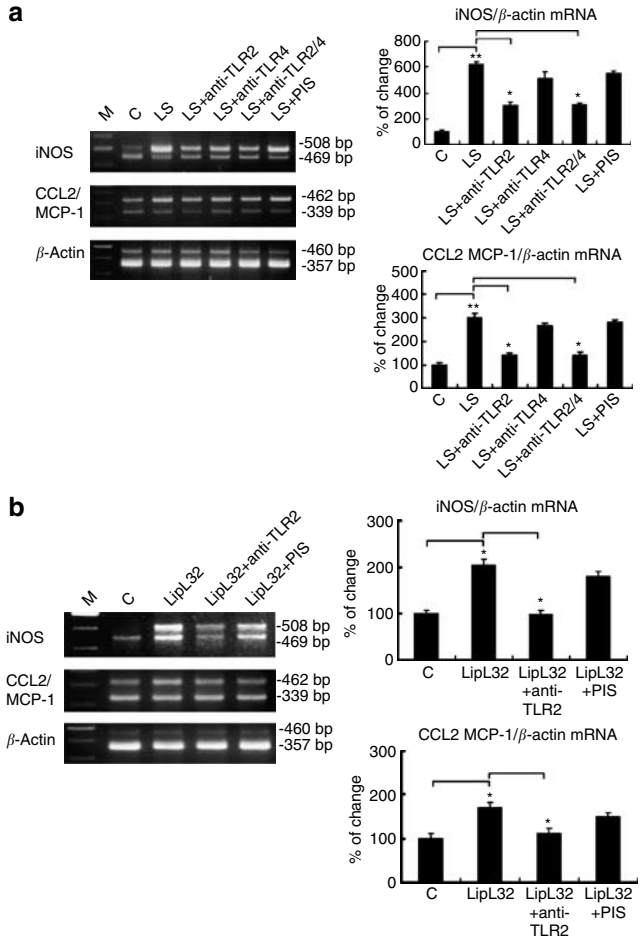

Figure 4 | Effects of *L. santarosai* serovar *Shermani* OMPs or LipL32 and antibody raised against TLR2 or TLR4 on iNOS and CCL2/MCP-1 mRNA expression in proximal tubule cells. β-Actin was used as internal standard. (**a**) Dissociated effects of TLR2 and TLR4 on the induction of iNOS and CCL2/MCP-1 mRNA expression caused by leptospiral OMPs in proximal tubule cells. β-Actin was used as internal standard. PKSV-PR cells were incubated with *L. santarosai* serovar *Shermani* OMPs (LS) alone (0.2 μg/ml for 48 h), LS plus an antibody directed against TLR2 or TLR4, LS plus the two TLR2 and TLR4 antibodies or LS plus a preimmune serum (PIS). (**b**) PKSV-PR cells were incubated or not with LipL32 alone, LipL32 plus the anti-TLR2 antibody or LipL32 plus a preimmune serum (PIS). Note that in both cases the blocking anti-TLR2 antibody prevented the rise in iNOS and CCL2/MCP-1 mRNA expression induced by LS or LipL32 and that PIS had no effect. The bars are means $\pm$ s.e.m. from three independent experiments. M: marker; C: control. *$P<0.05$; **$P<0.01$ versus control untreated (C).

Time course of leptospiral OMPs induced TLR2 and CCL2/MCP-1 expression in proximal tubule cells

Confluent PKSV-PR cells grown in serum-free medium for 24 h were then incubated with leptospiral OMPs (0.2 μg/ml) for various periods. Expression of TLR2, CCL2/MCP-1, and β-actin mRNAs was analyzed by competitive RT-PCR as a function of time. The TLR2 mRNA was activated within 2 h of stimulation followed by the increase of CCL2/MCP-1 mRNA and a progressive increase of CCL2/MCP-1 secreted protein measured by enzyme-linked immunosorbent assay (ELISA) in the culture supernatant (Figure 5).

Effects of leptospiral OMPs on CCL2/MCP-1 in TLR2- and TLR4-transfected HEK 293 cells

To further assess the role of TLR2 in the induction of CCL2/MCP-1 caused by leptospiral OMPs, HEK 293 cells normally

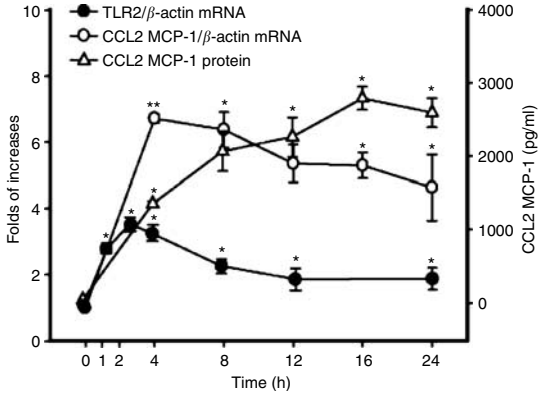

Figure 5 | Time course of leptospiral OMPs induced TLR2 and CCL2/MCP-1 expression in proximal tubule cells. Confluent PKSV-PR cells grown in serum-free medium for 24 h were then incubated with leptospiral OMPs (0.2 μg/ml) for various periods. Expression of TLR2, CCL2/MCP-1, and β-actin mRNAs was analyzed by competitive RT-PCR as a function of time. As a control, β-actin was utilized as the internal standard. The secretion of CCL2/MCP-1 recovered in culture supernatants from PKSV-PR cells stimulated for various periods was measured by ELISA. The TLR2 mRNA was activated within 2 h of stimulation followed by the increase of CCL2/MCP-1 mRNA and a progressive increase of CCL2/MCP-1 secreted protein in the cultures supernatant. Values are means $\pm$ s.e.m. of duplicate measurements from three independent experiments. In all cases, values were significantly higher (**$P<0.01$; *$P<0.05$) than those of untreated cells (time, 0).

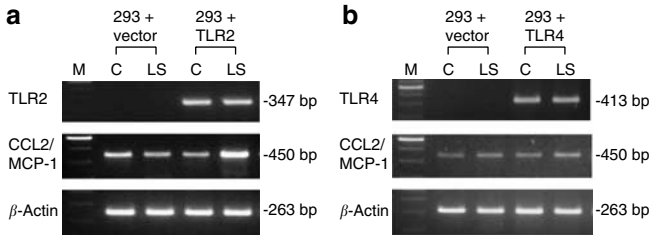

Figure 6 | Effects of transient expression of TLR2 or TLR4 in HEK 293 cells. (**a**) TLR2 and CCL2/MCP-1 mRNA expression was measured in HEK 293 cells transiently transfected with a TLR2-expressing plasmid. β-Actin was used as internal standard (**a**, upper panel). As control, no TLR2 mRNA was detected in HEK 293 cells transfected with an empty vector Transfected HEK 293 cells were then incubated or not with leptospiral OMPs (0.2 μg/ml) for 48 h. Thereafter, CCL2/MCP-1 mRNA expression was found increased in the TLR2-transfected cells. (**b**) In HEK 293 cells transfected with TLR4-expressing plasmid, LS stimulated did not affect CCL2/MCP-1 mRNA expression in TLR4-expressing HEK 293 cells. The bars are means ± s.e.m. from four independent experiments. C: control; LS: *Leptospira santarosai* serovar *Shermani*. *$P<0.05$; **$P<0.01$ versus control untreated (C).

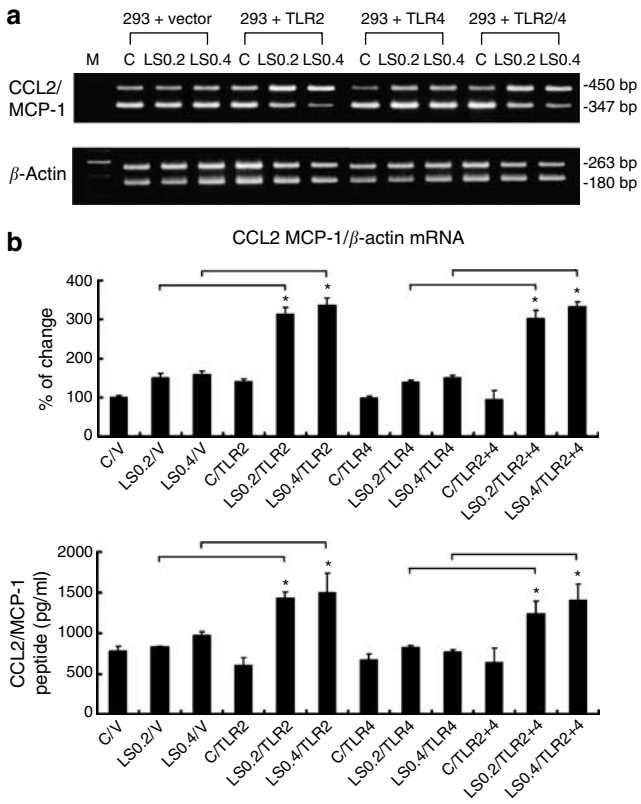

Figure 7 | Effects of various concentrations of *L. santarosai* serovar *Shermani* OMPs in TLR2- and TLR4-expressing HEK 293 cells. HEK293 cells were transfected with TLR2 or TLR4 alone or in combination. (**a**) Results from RT-competitive PCR for CCL2/MCP-1 and β-actin, used as internal standard. Note that only 0.2 and 0.4 μg/ml leptospiral OMPs stimulated the expression of CCL2/MCP-1 mRNA in TLR2-expressing HEK 293 cells. (**b**) The bars represent the quantitative results for the levels of CCL2/MCP-1 mRNA and the amount of CCL2/MCP-1 protein recovered in supernatants. Note that TLR2-expressing HEK 293 cells responded to 0.2 and 0.4 μg/ml leptospiral OMP with increased expression of CCL2/MCP-1 mRNA and protein, and that TLR4-expressing HEK 293 cells remain unresponsive. Double transfection of TLR2 and TLR4 did not cause further increase in CCL2/MCP-1 expressions as compared to that of single TLR2 transfection. The bars are means ± s.e.m. from four independent experiments. C: control; LS: *Leptospira santarosai* serovar *Shermani*. *$P<0.05$ versus control untreated.

lacking TLR2 and TLR4 were transiently transfected with TLR-expressing plasmids. We first checked that HEK 293 cells transfected with an empty vector do not express TLR2 or TLR4. In contrast, strong TLR2 or TLR4 mRNA expressions were detected in TLR2- or TLR4-transfected cells, respectively. Interestingly, leptospiral OMPs only increased the expression of CCL2/MCP-1 mRNA levels in TLR2-transfected cells but not in TLR4-transfected cells or in empty vector-transfected cells (Figure 6a and b).

Additional experiments were then carried out on HEK 293 cells transiently transfected with TLR2- or TLR4-expressing plasmids or with both plasmids and incubated with two concentrations (0.2 and 0.4 μg/ml) of leptospiral OMPs. In both cases, OMPs significantly increased ($P<0.05$) the CCL2/MCP-1 mRNA expression by 3.1- to 3.4-fold, respectively (Figure 7a). Consistent with these results, 0.2 and 0.4 μg/ml OMPs also significantly increased by 1.9- and 2.1-fold, respectively, the secretion of the CCL2/MCP-1 protein recovered in medium supernatants from HEK 293 cells transfected with the TLR2-expressing plasmid (Figure 7b). Conversely, HEK 293 cells transfected with the TLR4-expressing plasmid remained unresponsive to leptospiral OMPs. Additionally, double transfection of TLR2 and TLR4-expressing plasmids did not result in further increase in CCL2/MCP-1 expression (mRNA and protein) as compared to that achieved with the TLR2-expressing plasmid alone (Figure 7a and b). Thus, these experiments provide additional evidence that the innate immunity TLR2 pathway mediates the induction of CCL2/MCP-1 caused by pathogenic leptospiral OMPs in proximal tubule epithelial cells.

DISCUSSION

The results of this study provide lines of evidence that TLR2 mediates the early inflammatory response caused by pathogenic leptospires in renal proximal tubule cells. At least 11 TLRs have been identified so far in immune cells and in a variety of epithelial cells.[20–23] Renal tubule epithelial cells are among the non-immune cells that express TLR1, TLR-2, TLR-3, TLR-4, and TLR-6;[12,24] TLR2 and TLR-4, the main receptors of Gram-positive and -negative bacteria,[25,26] are constitutively expressed in both proximal and distal tubular renal epithelial cells *in vivo*.[27] Renal inflammation caused by warm ischemia–reperfusion was shown to cause a marked increase in TLR2 and -4 mRNA synthesis dependent on the action of tumor necrosis factor-α and interferon-γ.[12] In the kidney, TLR4 is required for the resistance to Gram-negative bacteria, as assessed by the persistence of Gram-negative bacteria in experimental models of pyelonephritis induced in C3H/HeJ mice harboring a non-functional TLR4, when compared to the rapid renal clearance of bacteria in wild-type counterparts.[28] TLR2 has a broader specificity, as it

recognizes microbial components from bacteria, mycobacteria, mycoplasma, and also spirochetes. Werts *et al.*[18] have demonstrated that the activation of macrophages by leptospiral LPS requires CD14 and TLR2. The components of the leptospiral outer membrane certainly determine the virulence of pathogenic leptospires. Many of the most abundant proteins in the spirochetal outer membrane are lipoproteins that participate in the pathogenesis of tubulointerstitial nephritis.[8,29] Previous studies have shown that the leptospiral outer membrane has a relatively complex protein profile.[30–32] Outer membrane antigenic proteins along with leptospiral LPS are expressed in infected kidneys,[8] suggesting their roles in the induction and persistence of leptospirosis tubulointerstitial nephritis. The OMP extraction method used in this study enhanced the harvest of lipoprotein components. Although one cannot exclude a role for contaminant leptospiral LPS in OMPs preparations, it remains that glycolipoprotein are responsible for major leptospiral toxicity.[33] In this line, we had previously shown that heat and proteinase K digestion of leptospiral OMP extracts significantly reduced their inducible action on iNOS and CCL2/MCP-1 expressions in renal tubule cells.[6] Moreover, polymyxin B, which inhibits the LPS activity, did no significantly impair the inducible action of leptospiral OMPs (data not shown). To further rule out any participation of contaminant LPS from *Escherichia coli* preparations in experiments using LipL32 and LipL41, the recombinant lipoproteins were treated with endotoxin-removing gel. LipL32, but not LipL41, stimulated in a dose-dependent manner the expression of TLR2 mRNA in proximal tubule cells. These results are in agreement with a previous study, which showed that LipL41 did not stimulate iNOS or CCL2/MCP-1 expression in proximal tubule cells.[7] Overall, these results strongly suggest that LipL32 represents the main leptospiral outer membrane lipoprotein responsible for the induction of the proinflammatory response in renal tubule cells. Here, we also show that the neutralizing anti-TLR2 antibody used, but not the anti-TLR4 antibody, inhibits the inducible action of leptospiral OMPs on the iNOS and CCL2/MCP-1 downstream products. The results from TLR transfection studies also provide additional evidence that TLR2 is the innate receptor for leptospiral OMPs in renal proximal tubule cells. In HEK 293 cells that do not express TLRs, the transient expression of TLR2 allows leptospiral OMPs to increase the expression levels of CCL2/MCP-1. In contrast, no stimulation of CCL2/MCP-1 could be detected in TLR4-transfected HEK 293 cells. Thus, these results strongly suggest that TLR2 mediates the induction of the inflammatory response of renal epithelial cells caused by pathogenic leptospires. This is the first demonstration that pathogenic leptospires, which specifically invade and reside in the kidney, may trigger an innate immune response through TLR2-dependent pathway in proximal tubule cells.

Chemotactic chemokines responsible for the recruitment of leukocytes to the site of inflammation are upregulated in a variety of glomerular and interstitial renal injuries.[34,35] We have previously shown that leptospiral OMPs and LipL32 stimulate the expression of CCL2/MCP-1 and RANTES (regulated upon activation normal T-cell expressed and secreted) in association with increased generation of iNOS and tumor necrosis factor-α.[6,7] These findings have revealed that leptospiral OMPs are able to provoke an inflammatory response in injured renal tubule cells and thereby permit the recruitment of infiltrating cells into the renal interstitium.[6,7] Recent studies have also shown that the expression of TLR2 in human HEK 293 cells mediates the activation of nuclear factor-κB and enables stress-activated mitogen-activated protein kinase p38 phosphorylation in response to LPS, membrane lipoproteins or Gram-positive bacterial products.[36] In accordance with these findings, we found that leptospiral OMPs stimulate the phosphorylated mitogen-activated protein kinase p38 in proximal tubule cells (manuscript in preparation). Together with the present findings, these results therefore support the hypothesis that pathogenic leptospiral OMPs conceivably initiate tubulointerstitial nephritis through the components contained in the outer membrane that binds to TLR2 in proximal tubule cells. The present results also show that TLR4 may not be required in the recognition of leptospiral OMPs. These results are in accordance with previous studies which showed that the LPS isolated from *L. interrogans* as well as LipL32 stimulate the production of IL-8 and tumor necrosis factor-α in human macrophages via a TLR2-mediated pathway, and that TLR2-deficient mice are insensitive to leptospiral LPS.[18] Koizumi *et al.*[37] have also shown that TLR4-deficient C3H/HeJ mice remain sensitive to leptospiral infection, suggesting that TLR4 is not a major mediator in leptospirosis infection. Consistent with these studies, the present work provides additional evidence that TLR2 represents the main pattern recognition receptor recognizing pathogenic leptospiral OMPs. In conclusion, the present findings provide a basis for understanding the molecular mechanisms controlling the innate immune response caused by leptospirosis in renal tubule cells, which will serve for future studies devoted to the understanding of the pathogenesis of microorganism-induced tubulointerstitial nephritis.

MATERIALS AND METHODS

Preparation of OMPs extract of leptospires

Two frequently encountered pathogenic leptospires serovar *L. santarosai* serovar *Shermani* (ATCC number 43286™) and *L. interrogans* serovar *Bratislava* (ATCC number 23578™), and a non-pathogenic *L. biflexa* serovar *Patoc* (ATCC number 23582™), were obtained from ATCC (Rockville, MD, USA) and grown in 10% Ellinghausen McCullough Johnson Harris leptospiral enrichment medium (Difco, Detroit, MI, USA). The OMPs were extracted with 1% Triton X-114 and more than 90% of OMPs were extracted in the Triton X-114 detergent phase.[6]

Cloning LipL32 and LipL41

Standard recombinant DNA procedures were undertaken to clone LipL32 and LipL41 as described.[7] LipL32, but not LipL41, can induce a robust inflammatory response in cultured mouse proximal

tubule cells.[7] Thus, LipL41 was used to compare the effect of LipL32 in TLR activation. The recombinant proteins were passed through an endotoxin-removing gel, Detoxigel (Pierce Biotechnology Inc., Rockford, IL, USA), to remove possible contamination by LPS, and controlled by the Limulus Amebocyte Lysate assay.

Cultured proximal tubule cells

The PKSV-PR cells employed herein were derived from isolated late proximal tubules (PR) dissected from the kidney of an L-PK/Tag1 transgenic mouse as specified earlier.[19] Experiments were undertaken on exponentially growing or confluent cells between the 40 and 55th passages. Cells were shifted to a serum-free medium 24 h before adding OMP extract or recombinant lipoproteins to the cell culture medium for 48 h. Total RNA was extracted to undergo RT-PCR, and supernatant was collected to measure the amounts of protein. All measurements were made at least in triplicate.

Flow cytometry

PKSV-PR cells (1×10^6/ml) were incubated or not with leptospiral OMPs for 48 h. After washing, 2×10^5 cells were resuspended in 200 μl phosphate-buffered saline containing 1% bovine serum albumin and 0.01% sodium azide. The cells were preincubated with 1 μg/ml of Fc block (BD PharMingen, San Diego, CA, USA) before incubating them with a monoclonal rat anti-mouse TLR2 antibody or a monoclonal rat anti-mouse TLR4 antibody (eBioscience, San Diego, CA, USA). Similar experiments were performed using a rat immunoglobulin G2a isotype control antibody. Cells were washed and incubated with a fluorescein isothiocyanate-labeled anti-rat immunoglobulin G antibody and analyzed using a FACS Calibur System (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) flow cytometer. The data were analyzed using Win MDI 2.8 software.

Immunofluorescence studies

Confluent cultures of PKSV-PR cells incubated or not with leptospiral OMP were first incubated with rabbit anti-mouse TLR2 or rat monoclonal anti-mouse TLR4 antibodies (eBioscience, San Diego, CA, USA) followed by fluorescein isothiocyanate species-specific secondary antibodies and were examined using an inverted microscope equipped with epifluorescence optics.

Competitive RT-PCR assay

RNA was extracted from confluent proximal tubule cells using RNA-zol (Cinna/Biotecx Laboratories International Inc., Friendwood, TX, USA) and was reverse transcribed as described.[6] The sets of iNOS, CCL2/MCP-1, and β-actin primers used for RT-PCR were the same as described previously.[38,39] The mouse TLR2 primer pair was 5′-GTG CCA CCA TTT CCA CGG GC-3′(sense) and 5′-CAA AAC ACT TCC TGC TGG CC-3′ (antisense), yielding a 501 bp PCR product. The mouse TLR4 primer pair was 5′-AGT GGG TCA AGG AAC AGA AGC A-3′(sense) and 5′-CTT TAC CAG CTC ATT TCT CAC C-3′ (antisense), yielding a 311 bp PCR product. PCR primers were also used to detect human cDNA in HEK 293 cells. The human TLR2 primer pair was 5′-GCC AAA GCT TTG ATT GAT TGG-3′ (sense) and 5′-TTG AAG TTC TCC AGC TCC TG-3′ (antisense), yielding a 347 bp PCR product. The human TLR4 primer pair was 5′-TGC GGG TTC TAC ATC AAA-3′(sense) and 5′-CCA TCC GAA ATT ATA AGA AAA GTC-3′ (antisense), yielding a 413 bp PCR product. The human CCL2/MCP-1 primer pair was 5′-CCT GCT GTT ATA ACT TCA CC-3′(sense) and 5′-ACA TCC CAG GGG TAG AAC TG-3′ (antisense), yielding a 450 bp PCR product. The human β-actin primer pair was 5′-CCC CAG GCA CCA GGG CGT GAT-3′ (sense) and 5′-GGT CAT CTT CTC GCG GTT GGC CTT GGG GTT-3′ (antisense), yielding a 263 bp PCR product. The PCR products were analyzed initially by amplifying at the exponential phase. Competitive PCR assays were performed to evaluate the mRNA levels and the mean values of each measured mRNA/β-actin ratio were further expressed as a percentage change of the control values in each experiment.[40,41] Competitive PCR was performed to measure mouse TLR2, CCL2/MCP-1, iNOS, β-actin, and for human CCL2/MCP-1, β-actin, using deletion cDNA mutant.

Enzyme-linked immunosorbent assay

HEK 293 cells were incubated without or with *L. santarosai* serovar *Shermani* OMPs and the CCL2/MCP-1 protein recovered in culture medium was measured by ELISA using the commercially available kit Quantikine (R&D systems, Minneapolis, MN, USA).

LipL32 antisera

Antiserum to LipL32 was prepared by immunizing New Zealand White rabbits with purified His6-LipL32 fusion proteins. These proteins were expressed by *E. coli* BL21 (DE3) pLysS transformed with the pRSET plasmid that contained the LipL32 gene as described previously.[7]

Transient transfection of TLR in HEK 293 cells

The pUNO-hTLR2, pDUO-MD2/TLR4, and pUNO empty plasmids were obtained from InvivoGen Inc. (San Diego, CA, USA). The HEK 293 cells (ATCC, Manassas, VA, USA) were plated into six-well tissue culture plates and maintained in Dulbecco's minimum essential medium supplemented with 10% FCS. Cells were transfected using the Lipofectamine™ 2000 transfection protocol (Life Technologies Inc., Carlsbad, CA, USA) with 4.0 μg pUNO, pUNO-hTLR2 or/and pDUO-MD2/TLR4, and 6.0 μl Lipofectamine. Transfected cells were selected in medium containing 10 μg/ml blasticidin S (InvivoGen, San Diego, CA, USA) and maintained in 1–3 μg/ml blasticidin S-supplemented medium. Transfected cells were cultured in normal medium for 36–48 h, and then in serum-free medium for additional 18 h before adding leptospiral OMPs for 24 h. Total mRNA was then extracted for RT-PCR and supernatants were tested for CCL2/MCP-1 by ELISA. All of the transfection experiments were repeated at least twice.

Statistical analysis

All measurements were made at least in triplicate and the results were expressed as means $\pm$ s.e.m. Differences among groups were analyzed by the unpaired Student's *t*-test or analysis of variance when appropriate. A $P < 0.05$ was considered significant.

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