Leptospirosis: A Toll Road from B Lymphocytes

Catherine Werts, PhD

Leptospirosis is a widespread zoonosis caused by *Leptospira interrogans*, a pathogen transmitted by asymptomatic infected rodents. Leptospiral lipoproteins and lipopolysaccharide (LPS) have been shown to stimulate murine cells via Toll-like receptors (TLRs) 2 and 4. Host defense mechanisms remain obscure, although TLR4 has been associated with clearing *Leptospira*. In a recent study, we showed that in response to *Leptospira* infection, double (TLR2 and TLR4) knock-out (DKO) mice unexpectedly developed TLR-independent pro-inflammatory responses, and rapidly died from severe hepatic and renal failure. Subsequent analysis of chimeric and transgenic mice identified B-cells as the crucial lymphocyte subset responsible for the clearance of *Leptospira*, initially through the production of specific TLR4-dependent immunoglobulin M (IgM), directed against the LPS of *Leptospira*, and subsequently through specific IgG production, which is impaired in DKO mice. We also identified the protective, tissue-compartmentalized, TLR2/TLR4-mediated production of interferon-γ (IFN-γ) by B- and T-lymphocytes. Overall, our recent findings demonstrate that TLR2 and TLR4 both play a key role in the early control of leptospirosis, and constitute the first line of defense against *Leptospira*, confirming previous *in vitro* data showing that both LPS and lipoprotein play a crucial role in cell activation. However, if this first line of defense is by-passed, *Leptospira* can induce a deleterious inflammation in the target organs, and this does not rely on TLR activation. *(Chang Gung Med J 2010;33:591-601)*

Dr. Catherine Werts

Key words: leptospirosis, Toll-like receptors, TLR-deficient mice

Leptospira interrogans is a spirochete responsible for a zoonotic disease known as leptospirosis. Rodents constitute the main reservoir and asymptomatically excrete the bacteria in their urine. Humans get infected via contaminated water. This common, reemerging disease is frequent in highly-populated, poor, urban centers where flooding occurs, and in East Asia among paddy field workers. Human leptospirosis can take many different forms, ranging from a 'flu'-like syndrome to multi-organ failure leading to death. *Leptospira* are motile, and migrate via a breach in the skin into the bloodstream, where they are rapidly disseminated and subsequently colonize the liver, the lungs and the kidneys, which constitute the main target organs of *Leptospira*.[1]

Microbes possess conserved microbial compo-

From the Institut Pasteur, Group: Biology & Genetics of Bacterial Cell Wall; Avenir Group INSERM.
Received: Feb. 2, 2010; Accepted: May 28, 2010
Correspondence to: Dr. Catherine Werts, Institut Pasteur, BGPB. 28 rue du Dr Roux, 75724 Paris CEDEX 15, France.
Tel.: 033-144389508; Fax: 033-140613640; E-mail:cwerts@pasteur.fr

nents (pathogen molecular patterns or PAMPs), such as their hypomethylated DNA or lipopolysaccharide (LPS) that are recognized by the host's pattern recognition receptors (PRRs), such as the Toll-like receptors (TLRs). This innate sensing system alerts the host to the microbial intrusion, and initiates an inflammation that triggers the recruitment of leukocytes to combat the microbes.[2] Besides heat-shock proteins, the major antigens of pathogenic *Leptospira* are LPS and the lipoproteins.[3] Both are PAMPs found in the kidneys of *Leptospira*-infected animals,[4,5] and their presence has been linked to *Leptospira*-induced tubulo-interstitial nephritis.[6] Elucidating the mechanism by which leptospiral components are detected by TLRs and the subsequent initiation of inflammation seems crucial to achieve a better understanding of the events leading to leptospirosis.

This review aims to recapitulate what we have learned about *Leptospira* recognition and signaling by Toll-like receptors in the context of *in vitro* studies or *in vivo* studies using murine models.

Leptospira are peculiar spirochetes that possess LPS

Mammalian infection by Gram-negative bacteria is usually characterized by a systemic pro-inflammatory syndrome, mainly due to LPS recognition by the host. A complex of different proteins including CD14, a glycosyl phosphatidylinositol anchored protein, TLR4, a trans-membrane receptor, present either on the cell surface of macrophages or localized intracellularly in epithelial cells, and the cofactor MD2, constitute the receptor of the lipid A moiety of the LPS of classical Gram-negative bacteria. The subsequent intracellular signaling involves activation of the nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) transcription factor and its nuclear translocation, and mitogen-activated protein (MAP) kinase phosphorylation, and leads to the expression of pro-inflammatory cytokines and chemokines, which in turn initiates the host immune response.[7]

Leptospira species are peculiar in possessing a LPS which is an abundant cell surface glycolipid component present in most Gram-negative bacteria,[8-11] but which is usually missing in spirochetes, such as the strict parasitic species *Borrelia* or *Treponema*. Early diagnosis of leptospirosis is difficult, mainly because of lack of any sign of inflammation (no pain, redness, or heat) at the site where *Leptospira* has penetrated the skin. We hypothesized that the LPS of *Leptospira* could be peculiar, and so not recognized in the same way as conventional LPS.

TLR2 is the main receptor of lipoproteins and LPS from *Leptospira* in human cells

LPS from *Leptospira interrogans* icterohaemorragiae, Verdun strain, has been purified and its reactivity has been compared to that of the LPS from *Escherichia coli* or *Salmonella minesotta*. In collaboration with the group of Ulevitch, we first demonstrated that LPS from *Leptospira* needs CD14, but that it uses TLR2 instead of TLR4 to stimulate TNF production in the human monocyte-like cell line, THP1-CD14.[11] TLR2 is a receptor of lipoproteins, including LipL32,[11] the most abundant *Leptospira* lipoprotein, which is only expressed by pathogenic *Leptospira*.[4] The unexpected reactivity of leptospiral LPS did not seem to be due to lipoprotein contamination, as only polymixin B reduced leptospiral LPS activity, whereas proteinase K treatment, which abolished the activity of LipL32 towards TLR2, did not. We therefore conclude that TLR2 is the main TLR receptor involved in sensing *Leptospira* in human macrophages. On the other hand, work by Yang's group has also shown that outer membrane protein extracts from *Leptospira* stimulate the translocation of NF-κB and activation of MAP kinases in medullary ascending limb cells.[12] The same group subsequently showed that TLR2 plays an important role in kidney epithelial cell signaling by recognizing outer membrane proteins and LipL32 from *Leptospira* in mouse proximal tubules cells.[13,14] Interestingly, other lipoproteins such as LipL31 or LipL41, which are also expressed by the saprophytic *Leptospira* Patoc strain, were tested for their reactivity towards TLR2 but only LipL32, which is expressed by the pathogenic strains, activated TLR2.[14]

The lipid A moiety of leptospiral LPS is atypical

The peculiar reactivity of the leptospiral LPS drove us to study the structure of the lipid A moiety, which constitutes the endotoxin component of the LPS, and the portion usually recognized by TLR4. In collaboration with the group of Raetz, we deciphered

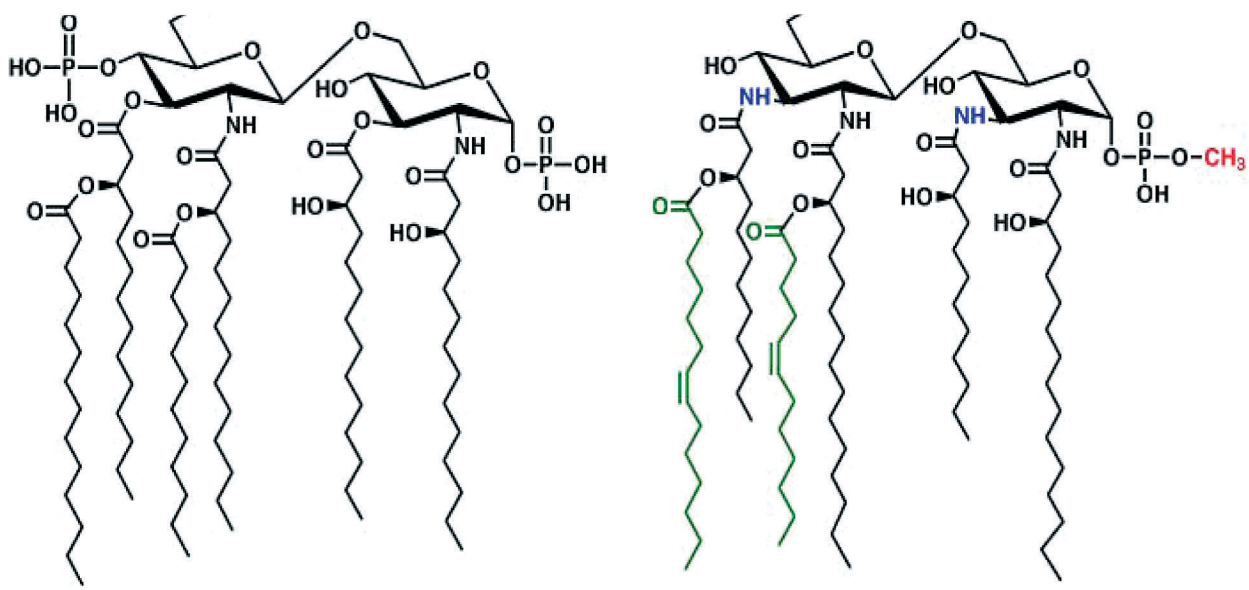
the structure of leptospiral lipid A, and showed that it has an overall hexa-acylated disaccharide backbone similar to that typical of *E. coli* lipid A. However, several features distinguished the forms of lipid A found in *Leptospira* and in *E. coli* (Fig. 1). (1) The four principal and two secondary acyl chains are of different lengths in *Leptospira* and *E. coli*., (2) The secondary acyl chains display some unsaturations in *Leptospira*. (3) Two important positions at the extremities of the disaccharide backbone that are phosphorylated in *E. coli*, are devoid of phosphate, or are replaced by a methyl group in *Leptospira*. (4) Only amide bonds link the sugar backbone to the acyl chains in *Leptospira*.[9] Compared with lipid A from *E. coli, leptospiral* lipid A differs not only in structure but also in function, since leptospiral lipid A does not coagulate limulus amoebocyte lysate,[15] the reference test for endotoxin detection. This is consistent with the low endotoxicity reported for whole leptospiral LPS.[16]

Murine TLR4, but not human TLR4, recognizes lipid A from Leptospira

The cell stimulatory properties of leptospiral lipid A have also been studied in comparison to lipid A from *E. coli* and also to whole leptospiral LPS. We found an unexpected difference in TLR recognition of leptospiral lipid A and of LPS in murine and human cells.[15] We found that leptospiral lipid A was not recognized by human TLR4, although it was detected by murine TLR4. Furthermore, TLR2 of human or murine origin did not recognize leptospiral lipid A, which is in line with numerous studies demonstrating that only TLR4 acts as a receptor of lipid A. Moreover, the whole leptospiral LPS molecule is not detected via TLR4 in human cells, whereas it is recognized by both TLR4 and TLR2 in murine cells.[15] A point of particular interest is whether the relative resistance of mice towards leptospirosis compared to that of humans is linked to this differential sensing of LPS by TLR4. Our data suggest that the O antigen or the 2-keto-3-deoxy-octonoic acid (KDO) part of the LPS, and not the lipid A portion, is responsible for recognition by the TLR2. The actual agonist of leptospiral LPS that is recognized by TLR2 is still unknown.

Murine TLR4 plays a critical role in leptospirosis

Rodents are considered to be a reservoir of path-



E. coli
Mol. Wt.: 1798.37

L. interrogans
Mol. Wt.: 1726.41

Fig. 1 Comparative structure of lipid A from *Escherichia coli* and *Leptospira interrogans*. Colors on the *Leptospira* lipid A structure denote differences from that of *E. coli*. On the *Leptospira* lipid A, note the absence of a free phosphate group at both extremities of the disaccharide backbone.

ogenic *Leptospira*. Mice are usually resistant to acute leptospirosis. Just before the discovery of the TLR family, Pereira et al demonstrated that the mouse C3H/HeJ line, known for decades to be hyporesponsive to LPS, was highly sensitive to *Leptospira interrogans*, Fiocruz strain. Indeed, 100% of C3H/HeJ mice infected intraperitoneally before the age of 3 weeks died within a few weeks from acute *Leptospirosis*, reproducing the features of human disease.[17] Beutler's group then mapped the C3H/HeJ genome, and showed that the mutated allele was a homologue of the Toll gene, already known to be involved in the innate response of Drosophila against fungi.[18] As a result, the long sought-after gene involved in LPS recognition was designated TLR4. Later. Vinetz et al compared the *Leptospira* infection of C3H/HeJ mice to that of C3H/HeN mice, which do not possess the TLR4 mutation, and showed that only C3H/HeJ and not C3H/HeN mice were sensitive to *Leptospira*, thus confirming the crucial role played by TLR4 in *Leptospira* clearance in mice.[19]

Double TLR2/TLR4 deficient (DKO) mice are a useful model for studying acute leptospirosis

Since only very young, and not adult C3H/HeJ mice are sensitive to *Leptospira*, TLR4 cannot be the only receptor involved in resistance to leptospirosis. Moreover, our results and data from other authors showing that leptospiral LPS and lipoproteins are recognized by TLR2[11,14,15] led us to hypothesize that mice deficient for both TLR2 and TLR4 would be more sensitive to *Leptospira* than C3H/HeJ mice. Indeed, we showed recently that adult DKO mice rapidly died from severe hepatic and renal failure following intraperitoneal infection with 2×10^8 *L. interrogans*, Fiocruz strain (Fig. 2).[20] We next sought to determine the respective contributions of the TLR2 and TLR4 receptors in the pathogenesis by studying the consequences of leptospiral infection in *tlr2*$^{-/-}$ and/or *tlr4*$^{-/-}$ mice compared with those in their resistant, C57BL/6J wild-type (WT) counterparts. Interestingly, no *tlr2*$^{-/-}$ mice died, whereas the *tlr4*$^{-/-}$ deficient mice all died, albeit later than the DKO mice. This confirms the major role played by TLR4 in the clearance of *Leptospira*, but also shows that TLR2 contributed to the *Leptospira*-induced lethality. The leptospiral load, plasma biochemical parameters, and cytokine and chemokine mRNA expression were measured in the target organs, i.e. lungs, liver and kidneys, 3 days post infection, just before the infected DKO mice died. Interestingly, the clearance of *Leptospira* from the liver was impaired in both *tlr4*$^{-/-}$ and DKO mice, whereas in the kidneys, clearance was impaired only in DKO mice, revealing a compartmentalized host response, with TLR4 playing a preeminent role in the liver, and TLR2 and TLR4 playing complementary roles in kidney defenses against pathogenic *Leptospira*.[20]

TLR2/4 dependent expression of IFNγ and iNOS

Next the levels of interferon-γ (IFN-γ) and inducible nitric oxide synthase (iNOS) mRNA, two important mediators of the host defense involved in the bacterial killing were measured on day 3 post infection in the target organs. IFN-γ and iNOS mRNA expression was dramatically lower in the liver, kidneys and lungs from DKO mice compared with those in WT mice, in which expression of these mediators was upregulated post infection. In the kidneys, TLR2 or TLR4 deficiency alone did not impair the production of IFN-γ mRNA, whereas in the liver, deficiency of TLR2 or TLR4 resulted in very low IFN-γ mRNA expression. These findings suggest that TLR2 and TLR4 have overlapping roles in the kidney, whereas they act synergistically in the liver to produce IFN-γ. Importantly, both TLR2 and TLR4 contribute to iNOS expression in both the kidneys and lungs.

IFN-γ and iNOS are double-edged swords, as these mediators can also cause severe cell damage in infected organs. Indeed, Yang and Hsu reported the clinical case of a patient who died from pulmonary leptospirosis and displayed iNOS mRNA overexpression, resulting in the overproduction of nitric oxide in the lungs, leading to cell damage and subsequent acute respiratory failure.[21] We showed that the global defective iNOS and IFN-γ response in DKO mice correlates with an increased number of *Leptospira* in the kidneys and lungs, which suggests that the upregulation of iNOS and IFN-γ in *Leptospira*-infected WT mice is protective rather than deleterious. Our results are in line with findings reported by Yang *et al*., who demonstrated that the outer membrane lipoproteins extracts from pathogenic *Leptospira* stimulated iNOS expression in the proximal tubule in a TLR2-dependent manner.[14]

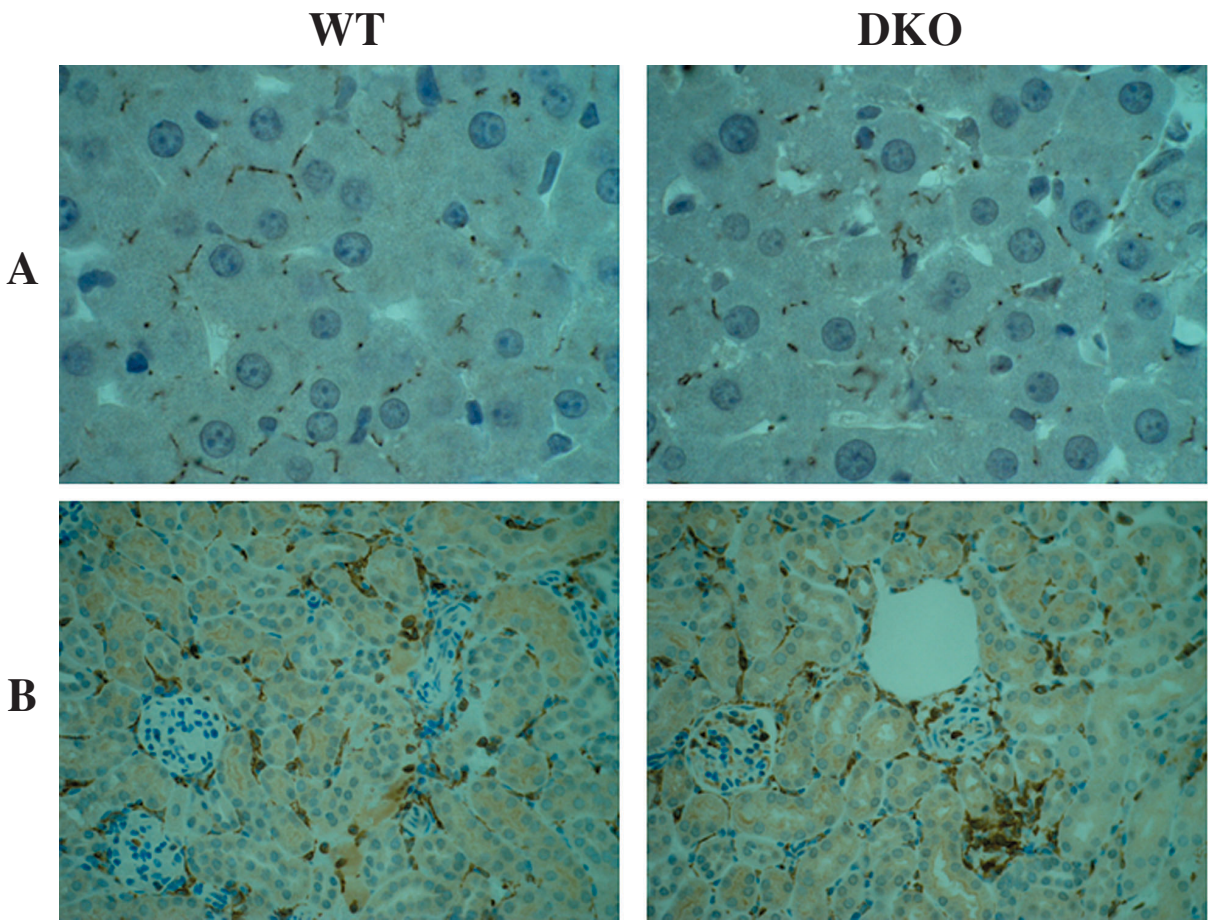


Fig. 2 Immunohistology of organs of WT and DKO (double TLR2/ TLR4 deficient) mice on day 3 post infection with 2 x 10^8 *Leptospira interrogans* Fiocruz strain. (A) *Leptospira* antigen LipL32 immunolabeling in the liver. (B) Anti-CD3 labeling showing T cells infiltrating the cortex of infected kidneys.

MyD88-independent inflammation

In contrast to the data described above, on day 3 post infection the expression of cytokines (IL 1-β, TNF, IL6) and chemokines (RANTES, KC, MIP-2) mRNA were all found to be up-regulated in the organs of DKO mice compared with those of infected WT mice. This demonstrates that the expression of pro-inflammatory cytokines and chemokines is independent of both TLR2 and TLR4. This result also did not depend on any other TLR, since mice deficient for myeloid differentiation primary response gene (88) (MyD88), the main cytosolic adaptor of TLRs, displayed the same type of inflammation in response to *Leptospira* infection.[20] This result was not expected from the previous *in vitro* work performed by our group and others that had demonstrated that macrophages depend completely on TLR2 and TLR4 to produce pro-inflammatory cytokines in response to whole, heat-killed *Leptospira*, LPS and LipL32 protein.[11,15,19] Furthermore, Yang *et al*. also showed that outer membrane proteins (OMPs) extracted from pathogenic *Leptospira santarosai* serovar Shermani stimulate the secretion of pro-inflammatory mediators in renal tubule epithelial cells,[13] as well as the production of CXCL1/KC via a TLR2-mediated pathway. Another study from the same group also showed that the expression of the chemokine CCL2/MCP-1 induced by pathogenic leptospiral OMPs, in particular LipL32, requires TLR2 in the proximal tubule cells.[14]

How can we reconcile these *in vitro* and *in vivo*

data? Part of the explanation could lie in the measurement time used, typically 24 h *in vitro*, whereas our *in vivo* study measurements were performed 3 days post infection, just before the death of the DKO mice. We checked the kinetics of the production of MIP-2, KC and RANTES chemokines, as well as of TNF in the liver and kidneys of *Leptospira*-infected mice 1, 2 or 3 days post infection (Fig. 3). On Day 1 post infection, the levels of cytokines and chemokines were higher in the organs of WT than DKO mice. Then, around 2 days post infection, the levels of cytokines began to rise in the organs of DKO mice, whereas they fell in those of WT mice that had already cleared the bacteria (Fig. 3). These findings suggest that the defect in early TLR2 and TLR4 activation allowed the bacteria to initiate secondary, uncontrolled and deleterious inflammation in the target organs, which was independent of the activation of TLR/MyD88.

CR3 is not the *Leptospira* receptor involved in MyD88 independent inflammation

Complement receptor-3 (CR3) (aka Mac1, CD11b/CD18) is an integrin expressed at the surface

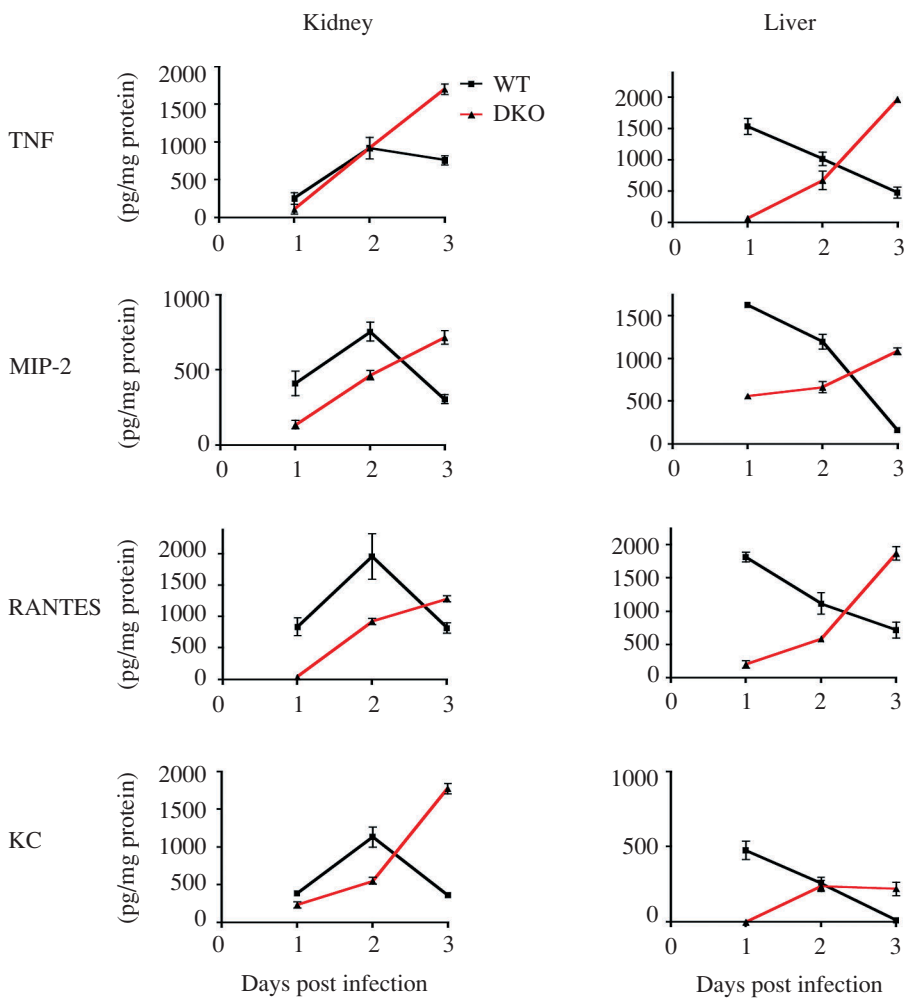


Fig. 3 Kinetics of cytokine and chemokine expression in organs of WT and DKO infected mice. Homogenates of liver and kidneys from WT or DKO mice infected intraperitoneally with 2 x 10^8 *Leptospira interrogans*. Fiocruz strain were analyzed by ELISA on days 1, 2 and 3 post infection for TNF, MIP-2, KC and RANTES expression. Values are expressed as mean ± SD, n = 4 in each group.

of most leukocytes. CR3 is involved in the phagocytosis of bacteria, and also in LPS recognition.[22] Interestingly, CR3 has recently been shown to be involved in binding *Leptospira*.[23] We therefore investigated the contribution of CR3 to the inflammatory response induced by *Leptospira* by blocking the CR3 receptor in DKO mice according to a recently published procedure using a neutralizing CD11b antibody.[24] Bacterial loads and inflammatory cytokine mRNA levels measured 3 days after infection were the same in anti-CR3 treated and non-treated DKO mice (Data not shown). This finding suggests that the MyD88-independent inflammation cannot be attributed to the CR3 integrin binding of *Leptospira*.

Bone marrow-derived cells expressing TLR2 and TLR4 control *Leptospira* clearance

Since the WT and the DKO mice were completely resistant and sensitive to lethal infection by *Leptospira* respectively, reciprocal chimeras of WT and DKO mice were constructed to find out which cell compartment controls susceptibility or resistance towards pathogenic *Leptospira*. The data suggest that cells from the hematopoietic compartment expressing TLR2 and TLR4 play a major role in controlling bacterial clearance.[20]

B-lymphocytes are major players in leptospirosis

We then tried to figure out which cell subset from the hematopoietic compartment could be responsible for controlling the infection. The first candidate was the polymorphonuclear (PMN) cell. This cell subset is known to be involved in bacterial phagocytosis and killing, and to express TLR2 and TLR4.[25] PMN depletion in WT mice induced using a specific antibody did not change any of the parameters measured, with the exception of the *Leptospira* load in the liver, which was increased in the depleted mice, suggesting that the neutrophils make only a minor contribution (personal data). We then excluded the monocyte/macrophage subsets, because in transfer experiments, bone-marrow derived monocytes from WT mice did not protect the DKO mice from *Leptospira*-induced death. Since Pereira *et al*. showed that T-cell depletion can aggravate experimental leptospirosis in C3H/HeJ,[17] and because the humoral response is known to protect against *Leptospira*,[26] we first tested *Rag*$^{-/-}$ mice, in a C56BL/6J background, which are devoid of both B- and T-lymphocytes.[26] In contrast to the WT mice, which survived the infection, all the *Rag*$^{-/-}$ mice died as a result of the infection, but later than the DKO mice.[27] Transgenic *CD3*$^{-/-}$ mice do not express T-lymphocytes. These mice were not susceptible to the lethal leptospirosis, although μMT mice, which are deficient only for B cells, died from leptospirosis with the same kinetics as the TLR4-deficient mice.[27] This finding suggests that B-cells are critical in the defense against leptospirosis, which is in line with the pioneering work of Adler et al. four decades ago, which showed that mice treated with cyclophosphamide, a B cell depleting agent, are sensitive to *Leptospira*.[27]

Compartmentalized response in kidneys and liver

Analyses of the expression profiles of cytokine and chemokine mRNA in the various different transgenic mice have shown that the TLR2- and TLR4-independent inflammation measured in the liver and kidneys was mostly due to lymphocytes. More specifically, we showed that in the liver, the production of IL-6 and TNF was dependent on the presence of B cells, whereas TNF expression in the kidneys was mostly due to T-lymphocytes (Fig. 2).[27] In contrast, RANTES chemokine production does not depend on lymphocytes, but on another subset of the hematopoietic compartment, which has yet to be identified. On the other hand, the lymphocyte subsets responsible for the inflammation are also involved in host defense through the TLR2 and TLR4-dependent production of IFN-γ.

Early TLR4-dependent, anti-*Leptospira* IgM response

B cells, TLR2 and TLR4 all seem to be crucial in the host's defense against *Leptospira*. The first functional link found between these different partners is the production of the protective IFN-γ nmediator by TLR2- and TLR4-expressing B-cells. Another relationship between B cells and TLRs emerged from rescue experiments involving μMT mice devoid of antibodies.[20] Indeed, an intraperitoneal injection of heat-inactivated sera from infected WT mice, which had been bled 20 days post infection, was able to rescue μMT mice and prevent

them from dying, thus confirming the important role of antibodies in leptospirosis.[3,26,27] As the death of infected DKO mice occurs from day 4 post infection, we studied the antibody response 3 days post infection, and found that the production of specific IgMs against *Leptospira* was already measurable in sera from WT mice, whereas it was markedly impaired in *tlr4*$^{-/-}$ and DKO mice, but not in *tlr2*$^{-/-}$ mice. Interestingly, the serum from day-3 post infected WT could not rescue the μMT infected mice, but did delay their death, something that was not observed with the sera from either day-3 infected DKO or naive mice. This outcome indicates that even if not fully protective, the early TLR4-dependent IgM response does play a role in protecting the host against *Leptospira*. We also showed that purified leptospiral LPS could induce an early, TLR4-dependent, specific IgM response, suggesting that the antigen is the lipid A moiety, as the response was not impaired in *tlr2*$^{-/-}$ mice. Moreover, in sub-lethal experiments we also demonstrated that the protective IgG response measured 20 days post infection required the presence of either TLR2 or TLR4, as it was only impaired in DKO, but not in *tlr4*$^{-/-}$ or *tlr2*$^{-/-}$ mice.[20] We have therefore demonstrated for the first time that TLR4 and TLR2 are both involved in the protective antibody response against pathogenic *Leptospira*.

Conclusions

To sum up, our recent *in vivo* study has revealed the crucial role of B-cells in the clearance of *Leptospira*.[20] This response of B cells is compartmentalized, as in the circulation, infection by *Leptospira* results in the TLR4-dependent production of specific IgMs against LPS from *Leptospira*, whereas in the liver, it results in TLR2/4-dependent production of IFN-γ. We have also demonstrated that, in the kidney, *Leptospira* activates T-cells and parenchymal renal cells through TLR2 and TLR4 to produce IFN-γ and iNOS. Aside from these protective responses, B- and T-cells also participate in the TLR-independent inflammation observed in the liver and kidneys of infected susceptible mice (Fig. 4). These findings reveal that whereas the initial onset of inflammation in response to *Leptospira* infection is primarily dependent on TLR activation, and is certainly required to attract leukocytes and kill bacteria, a secondary pro-inflammatory response occurs in a MyD88-independent manner and contributes to the severe and deleterious organ damage responsible for the death of sensitive mice.

Future perspectives

The complementary role of TLR4 and TLR2 in combating *Leptospira* by recognizing their principal PAMPs, LPS and lipoproteins has now been established (Table 1). *Leptospira* can adapt to many different hosts. One of the reasons for this may be its ability to modify its LPS, which contributes to its ability to escape the host's immune responses. Indeed, since the TLR4 sensing of leptospiral LPS is impaired in humans, it could be helpful to study TLR2 polymorphism to find out whether there is any correlation between the severity of the clinical outcome from leptospirosis and the TLR status of the patient.

Another avenue of future research will be to identify the *Leptospira* receptor, which is responsible for the TLR- and MyD88-independent response. Aside from other PRRs, such as the nucleotide-binding oligomerization (Nod) receptors, which are cytosolic proteins involved in innate immunity against microbes,[2,28,29] and which we are studying for their role in *Leptospira* recognition, no other obvious candidate is currently under investigation. Proteomic and microarray studies should therefore be very useful in the search for the receptor(s) involved in the deleterious inflammation. Moreover, *Leptospira* infected C57BL6/J mice provide a very good model for studying renal fibrosis, an adverse event observed in kidneys from patients recovering from leptospirosis which was found one month after infection in WT and in surviving DKO mice. The different steps that lead to the development of this fibrosis will be studied in relationship with the Smad3/TGF pathway that Yang's group has already shown to be involved in the fibrosis process triggered in human cells by pathogenic *Leptospira*.[30]

Acknowledgements

We are grateful to Béatrice Fernandez, Nathalie Quellard and Jean-Michel Goujon (CHU Poitiers, France) for expert immunohistochemistry. We thank Cécilia Chassin for ELISA experiments, and Alain Vandewalle (INSERM U773, Centre Bichat, Paris, France) for his careful and critical reading of the manuscript.

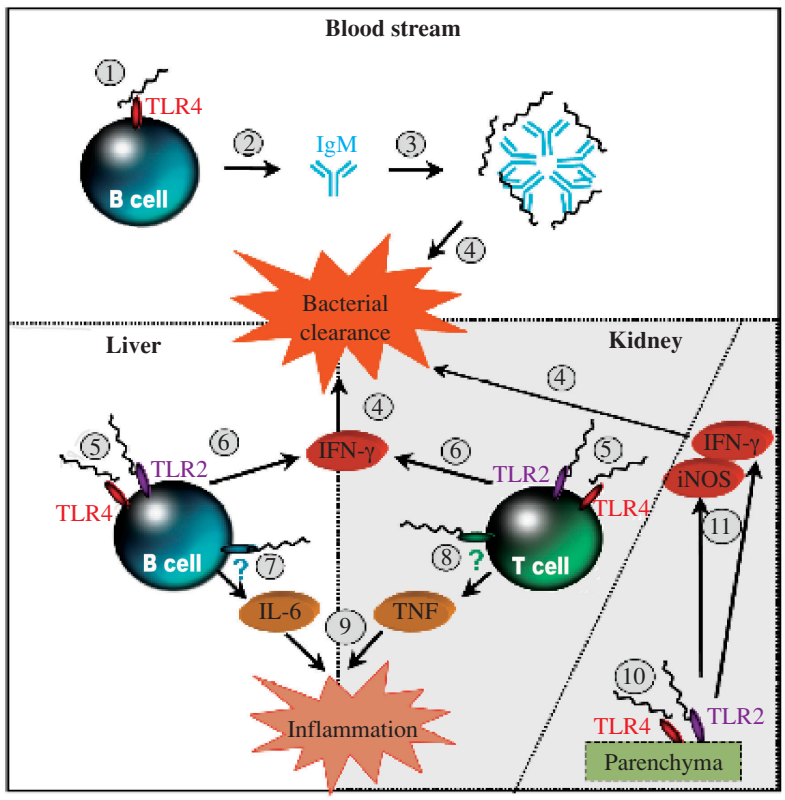


Fig. 4 Proposed model of compartmentalized TLR4 and TLR2 activation in the clearance of *Leptospira*. In the blood stream, infection by *Leptospira* results in the stimulation of B cells (1) and leads to the TLR4-dependent production of specific IgM directed against the LPS of *Leptospira* (2), that can opsonize the bacteria (3) and promote their phagocytosis and killing (4). Bacteria that escape antibody opsonization reach the liver and kidneys, where they stimulate B-cells and T-cells, via TLR2 and TLR4 (5). This activation leads to the production of IFN-γ (6), promoting the phagocytosis and killing of the bacteria (4). Moreover, in the kidney, *Leptospira* activate parenchymal renal cells through TLR2 and TLR4 (10) to produce IFN-γ and iNOS (11), which also contribute to bacterial clearance (4). In addition to these protective responses, B-cells from the liver and T-cells from the kidney are also responsible for a TLR-independent inflammation (9), via unknown receptor(s) (7) and (8) that recognize *Leptospira*.

Table 1. *In vitro* Studies Involving TLR and PAMPs from *Leptospira*

PAMP	TLR Involved	*Leptospira* strain	Cell type	Read-out	Ref
LPS	TLR2	*L. interrogans* Verdun	Human THP1-CD14 macrophages	TNF, IL-8, NF-κB	(11)
LipL32	TLR2	*L. interrogans* RZ11	Human THP1-CD14 macrophages	TNF, IL-8	(11)
LPS	TLR2/TLR4	*L. interrogans* Verdun	peritoual macrophages	TNF, IL-6, MAP Kinase	(15)
LipidA	TLR4	*L. interrogans* Verdun	peritoual macrophages	TNF, IL-6, MAP Kinase	(15)
OMPs	TLR2	*L. santarosai* Shermani *L. interrogans* Bratislava	Mouse renal proximal tubule cells	MCP-1, iNOS	(21)
recLipL32	TLR2	*L. santarosai* Shermani	Mouse renal proximal tubule cells	MCP-1, iNOS	(21)

REFERENCES

1. Yang CW. Leptospirosis in Taiwan--an underestimated infectious disease. Chang Gung Med J 2007;30:109-15.
2. Werts C, Girardin SE, Philpott DJ. TIR, CARD and PYRIN: three domains for an antimicrobial triad. Cell Death Differ 2006;13:798-815.
3. Guerreiro H, Croda J, Flannery B, Mazel M, Matsunaga J, Galvao Reis M, Levett PN, Ko AI, Haake DA. Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans. Infect Immun 2001;69:4958-68.
4. Haake DA, Chao G, Zuerner RL, Barnett JK, Barnett D, Mazel M, Matsunaga J, Levett PN, Bolin CA. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. Infect Immun 2000;68:2276-85.
5. Barnett JK, Barnett D, Bolin CA, Summers TA, Wagar EA, Cheville NF, Hartskeerl RA, Haake DA. Expression and distribution of leptospiral outer membrane components during renal infection of hamsters. Infect Immun 1999;67:853-61.
6. Yang CW, Wu MS, Pan MJ, Hsieh WJ, Vandewalle A, Huang CC. The Leptospira outer membrane protein LipL32 induces tubulointerstitial nephritis-mediated gene expression in mouse proximal tubule cells. J Am Soc Nephrol 2002;13:2037-45.
7. Fitzgerald KA, Rowe DC, Golenbock DT. Endotoxin recognition and signal transduction by the TLR4/MD2-complex. Microbes Infect 2004;6:1361-7.
8. Ren SX, Fu G, Jiang XG, Zeng R, Miao YG, Xu H, Zhang YX, Xiong H, Lu G, Lu LF, Jiang HQ, Jia J, Tu YF, Jiang JX, Gu WY, Zhang YQ, Cai Z, Sheng HH, Yin HF, Zhang Y, Zhu GF, Wan M, Huang HL, Qian Z, Wang SY, Ma W, Yao ZJ, Shen Y, Qiang BQ, Xia QC, Guo XK, Danchin A, Saint Girons I, Somerville RL, Wen YM, Shi MH, Chen Z, Xu JG, Zhao GP. Unique physiological and pathogenic features of Leptospira interrogans revealed by whole-genome sequencing. Nature 2003;422:888-93.
9. Que-Gewirth NL, Ribeiro AA, Kalb SR, Cotter RJ, Bulach DM, Adler B, Girons IS, Werts C, Raetz CR. 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 2004;279:25420-9.
10. Sweet CR, Williams AH, Karbarz MJ, Werts C, Kalb SR, Cotter RJ, Raetz CR. Enzymatic synthesis of lipid A molecules with four amide-linked acyl chains. LpxA acyltransferases selective for an analog of UDP-N-acetylglucosamine in which an amine replaces the 3”-hydroxyl group. J Biol Chem 2004;279:25411-9.
11. Werts C, Tapping RI, Mathison JC, Chuang TH, Kravchenko V, Saint Girons I, Haake DA, Godowski PJ, Hayashi F, Ozinsky A, Underhill DM, Kirschning CJ, Wagner H, Aderem A, Tobias PS, Ulevitch RJ. Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. Nat Immunol 2001;2:346-52.
12. Yang CW, Wu MS, Pan MJ. Leptospirosis renal disease. Nephrol Dial Transplant 2001;16 Suppl 5:73-7.
13. Hung CC, Chang CT, Chen KH, Tian YC, Wu MS, Pan MJ, Vandewalle A, Yang CW. Upregulation of chemokine CXCL1/KC by leptospiral membrane lipoprotein preparation in renal tubule epithelial cells. Kidney Int 2006;69:1814-22.
14. Yang CW, Hung CC, Wu MS, Tian YC, Chang CT, Pan MJ, Vandewalle A. Toll-like receptor 2 mediates early inflammation by leptospiral outer membrane proteins in proximal tubule cells. Kidney Int 2006;69:815-22.
15. Nahori MA, Fournie-Amazouz E, Que-Gewirth NS, Balloy V, Chignard M, Raetz CR, Saint Girons I, Werts C. Differential TLR recognition of leptospiral lipid A and lipopolysaccharide in murine and human cells. J Immunol 2005;175:6022-31.
16. Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, Levett PN, Gilman RH, Willig MR, Gotuzzo E, Vinetz JM. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis 2003;3:757-71.
17. Pereira MM, Andrade J, Marchevsky RS, Ribeiro dos Santos R. 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 1998;50:191-8.
18. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 1998;282:2085-8.
19. Viriyakosol S, Matthias MA, Swancutt MA, Kirkland TN, Vinetz JM. Toll-like receptor 4 protects against lethal Leptospira interrogans serovar icterohaemorrhagiae infection and contributes to in vivo control of leptospiral burden. Infect Immun 2006;74:887-95.
20. Chassin C, Picardeau M, Goujon JM, Bourhy P, Quellard N, Darche S, Badell E, d’Andon MF, Winter N, Lacroix-Lamande S, Buzoni-Gatel D, Vandewalle A, Werts C. TLR4- and TLR2-mediated B cell responses control the clearance of the bacterial pathogen, Leptospira interrogans. J Immunol 2009;183:2669-77.
21. Yang GG, Hsu YH. Nitric oxide production and immunoglobulin deposition in leptospiral hemorrhagic respiratory failure. J Formos Med Assoc 2005;104:759-63.
22. Perera PY, Mayadas TN, Takeuchi O, Akira S, Zaks-Zilberman M, Goyert SM, Vogel SN. CD11b/CD18 acts in concert with CD14 and Toll-like receptor (TLR) 4 to elicit full lipopolysaccharide and taxol-inducible gene

expression. J Immunol 2001;166:574-81.
23. Cinco M, Cini B, Perticarari S, Presani G. Leptospira interrogans binds to the CR3 receptor on mammalian cells. Microb Pathog 2002;33:299-305.
24. Leon F, Contractor N, Fuss I, Marth T, Lahey E, Iwaki S, la Sala A, Hoffmann V, Strober W, Kelsall BL. Antibodies to complement receptor 3 treat established inflammation in murine models of colitis and a novel model of psoriasiform dermatitis. J Immunol 2006;177:6974-82.
25. Sabroe I, Dower SK, Whyte MK. The role of Toll-like receptors in the regulation of neutrophil migration, activation, and apoptosis. Clin Infect Dis 2005;41 Suppl 7:S421-6.
26. Adler B, Faine S. Host immunological mechanisms in the resistance of mice to leptospiral infections. Infect Immun 1977;17:67-72.
27. Adler B, Faine S. Susceptibility of mice treated with cyclophosphamide to lethal infection with Leptospira interrogans Serovar pomona. Infect Immun 1976;14:703-8.
28. Werts C, le Bourhis L, Liu J, Magalhaes JG, Carneiro LA, Fritz JH, Stockinger S, Balloy V, Chignard M, Decker T, Philpott DJ, Ma X, Girardin SE. Nod1 and Nod2 induce CCL5/RANTES through the NF-kappaB pathway. Eur J Immunol 2007;37:2499-508.
29. Le Bourhis L, Werts C. Role of Nods in bacterial infection. Microbes Infect 2007;9:629-36.
30. Tian YC, Chen YC, Hung CC, Chang CT, Wu MS, Phillips AO, Yang CW. Leptospiral outer membrane protein induces extracellular matrix accumulation through a TGF-beta1/Smad-dependent pathway. J Am Soc Nephrol 2006;17:2792-8.