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TLR2 Is Constitutively Expressed within the Kidney and Participates in Ischemic Renal Injury through Both MyD88-Dependent and -Independent Pathways[1]

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TLRs are an evolutionarily conserved family of cell membrane proteins believed to play a significant role in innate immunity and the response to tissue injury, including that induced by ischemia. TLR signaling pathways activate transcription factors that regulate expression of prosurvival proteins, as well as proinflammatory cytokines and chemokines through one of two proximal adapter proteins, MyD88 or Toll/IL-1R domain-containing adaptor-inducing IFN-β (Trif). Our study defines the constitutive protein expression of TLR2 in kidneys of humans and mice, and provides insight into the signaling mechanisms by which a deficiency of TLR2 protects from ischemic organ injury. Our study compared and contrasted the effects of renal ischemia in wild-type mice and mice deficient in TLR2, MyD88, Trif, and MyD88 × Trif. TLR2 protein was evident in many cell types in the kidney, including renal tubules of the outer stripe of the medulla, glomeruli, and in the renal vasculature. The pattern of protein expression was similar in humans and mice. The absence of TLR2, MyD88, and MyD88 × Trif conferred both physiologic and histologic protection against sublethal ischemia at 24 h. Interestingly, TLR2-deficient mice were better protected from ischemic renal injury than those deficient for the adapter protein MyD88, raising the intriguing possibility that TLR-2-dependent/MyD88-independent pathways also contribute to kidney injury. We conclude that TLR2 protein is constitutively expressed in the kidney and plays an important role in the pathogenesis of acute ischemic injury by signaling both MyD88-dependent and MyD88-independent pathways. *The Journal of Immunology,* **2007, 178: 6252–6258.**

Many studies have pointed to the evolutionarily conserved family of TLRs as crucial sensors of tissue damage. As such, there has been intense interest in their function in normal and disease states. Studies over the past few years have shown that TLRs recruit intracytoplasmic adapter proteins to relay extracellular signals to the nucleus. All TLRs are thought to be dependent on signaling through the proximal molecule MyD88, except TLRs 3 and 4 (1). TLR3 is solely dependent upon Toll/IL-1R domain-containing adapter-inducing IFN-β (Trif),[4] and TLR4 signals through MyD88 and Trif (2). Simultaneous blockade of MyD88 and Trif is thought to prevent all TLR-dependent signaling (1, 3). TLRs are expressed on parenchymal cells as well as on cells of the innate and adaptive immune systems (1, 3), positioning them as molecular sentinels of tissue damage.

Damaged tissue is thought to release molecules called damage-associated molecular patterns (DAMPs) that activate TLRs and lead to downstream activation of transcription factors regulating the expression of survival genes or proinflammatory cytokines and chemokines (4–6). Several molecules have been implicated as DAMPs, including uric acid crystals (7), nucleic acids (8, 9), fragments of extracellular matrices (hyaluronic acid, fibronectin, heparin sulfate) (10), components of the clotting cascade (such as fibrin (11) and fibrinogen (10)), the nuclear protein high-mobility group B1 (12), and heat shock proteins (13, 14).

Because ischemic tissue releases DAMPs, several reports have pointed to an important role for TLRs in ischemic organ damage (15–27). In particular, the absence of TLR2 has been shown to decrease kidney injury in response to prolonged ischemia (28) and to nephrotoxic Ab-induced glomerulonephritis (29). The expression of mRNA for TLR2, as well as other TLRs, has been shown by in situ hybridization to increase with experimental ischemia reperfusion (IR) injury (30, 31). However, the constitutive expression within the kidney and the membrane distribution has not been well defined due to limited reagents for detection of TLR2 protein. Furthermore, recent reports have suggested that TLR2 might also engage non-TLR-dependent adapter molecules (32, 33), suggesting that blockade of TLR2 might involve both MyD88-dependent and -independent signaling pathways.

To define TLR2 protein expression within the kidney and establish a linkage to human renal disease, we examined human and mouse kidneys using high power imaging that allowed delineation of membrane and intracellular TLR2 expression. We also evaluated the dependence of kidney injury and chemokine/chemokine secretion

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[4] Abbreviations used in this paper: Trif, Toll/IL-1R domain-containing adaptor-inducing IFN-β; DAMP, damage-associated molecular pattern; DAPI, 4′,6-diamidino-2-phenylindole; IR, ischemia reperfusion; KC, keratinocyte-derived cytokine; WT, wild type.

on MyD88 and Trif, the two major adapter proteins necessary for transmission of TLR signaling.

Materials and Methods

Induction of IR injury

All mice used in the IR experiments were housed in the vivarium at The Scripps Research Institute and approved for use by the Laboratory Animal Care and Use Committee of the Animal Research Center at The Scripps Research Institute. All animals were handled according to the recommendations of the Humanities and Sciences and the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care. C57BL/6 mice were obtained from The Jackson Laboratory. MyD88-deficient, Trif-deficient, and (MyD88 × Trif)-deficient mice were obtained from B. Beutler (The Scripps Research Institute, La Jolla, CA). TLR2-deficient mice were obtained from P. Tobias (The Scripps Research Institute, La Jolla, CA). Wild-type (WT; C57BL6), TLR adapter protein-deficient (MyD88-deficient, Trif-deficient, and (MyD88 × Trif)-deficient), and TLR2-deficient mice were used in these experiments, and all mice had been bred onto a C57BL/6 background by >10 generations. All mice were matched for age (8–12 wk), and only male mice were used. As previously published (34), the following methods were used to induce nonlethal IR injury. The mice were anesthetized with methoxyflurane and injected i.p. with ketamine (100 mg/kg)/xylazine (8 mg/kg) in saline. Core body temperatures were maintained between 36°C and 37.5°C during surgery by continuous monitoring with a rectal thermometer and automatic heating blanket. Both kidneys were exposed with bilateral flank incisions and ischemia induced by clamping both renal arteries with nontraumatic microvessel clamps (S&T) for 25 min. Renal veins remained unoccluded. Cessation of blood flow was documented by visual inspection. After 25 min of ischemia, the clamps were released and reflow was verified by visual inspection of the kidneys. All mice received 200 μl of saline dripped over the open flanks during surgery to keep the tissue moist and 30 μl of saline per gram body weight injected s.c. after surgery to replenish for fluid loss.

Measurement of renal function

Measurement of renal function was conducted 24 h following reperfusion. The mice were anesthetized before sacrifice, and blood was collected from the inferior vena cava into a syringe preloaded with 3.8% sodium citrate. Plasma was isolated by centrifugation at 4000 × *g* for 10 min at 4°C. Renal function was assessed using the Sigma Diagnostics creatinine kit (Sigma-Aldrich), running all samples in duplicates, and repeating measurements three times for each sample. Baseline (before laparotomy) and terminal (at the time of sacrifice) serum creatinines were measured in all animals.

Confocal imaging and analysis

To prepare tissue for confocal imaging, experimental and control groups of WT and TLR2-deficient mice were sacrificed and perfused in situ with 2% paraformaldehyde. Immediately following the perfusion, the kidneys were dissected, removed, and fixed in a solution of 4% paraformaldehyde overnight. The next morning, the kidneys were placed through a sucrose gradient and then frozen in OCT medium (Sakura). Sections (6 μm thick) were stained with anti-TLR2 (T2.5; a gift from P. Tobias, The Scripps Research Institute, La Jolla, CA), rhodamine phalloidin (to visualize F-actin and thereby delineate cellular architecture), anti-CD31 (to distinguish endothelial cells), and 4′,6-diamidino-2-phenylindole (DAPI) (to distinguish nuclei). To assay for tissue injury, groups of mice were sacrificed 24 h after reperfusion, and their kidneys were dissected and halved sagitally. One-half was placed in a solution of 10% buffered zinc formalin (Sigma-Aldrich) for routine morphological examination by H&E staining and periodic acid-Schiff staining. The kidneys from five mice per group were blindly evaluated by the pathologist, and at least 10 fields (×400) were scored for epithelial necrosis, loss of brush border, tubular dilation, cast formation, and leukocyte infiltration (35). Human tissue was prepared by placing fresh sections in 10% formalin for overnight fixation and then placing through a sucrose gradient, sectioning, and staining as above for the mouse tissue. Human tissue was obtained through Institutional Review Board approval (Scripps Clinic and Green Hospital) for use of discarded tissue and was in adherence to the Declaration of Helsinki.

Fixed and stained samples were prepared as described above and then viewed using Rainbow Radiance 2100 laser scanning confocal system attached to a Nikon TE2000-U inverted microscope (Bio-Rad-Zeiss). Images were acquired using Laser Sharp 2000 software and then imported and further analyzed for quantitative colocalization and average fluorescence intensity, using the three following independent software packages: Laser Sharp (Bio-Rad-Zeiss), LSM examiner (Zeiss), and Image J packages (NIH Imaging: http://rsb.info.nih.gov/ij). Colocalization between two fluorescently labeled reagents was quantified by obtaining the threshold range of real over background (autofluorescence) signal and then using the average real threshold range to calculate the correlation coefficients (M values) for at least 30 cells in four separate experiments. To quantitate intracellular TLR2, >250 cells were compared between groups. Comparisons between mean fluorescence intensities were obtained by measuring the total area of each cell with a fluorescence signal in Image J software. The average fluorescent intensity of the cytoplasmic, lateral, and basolateral staining of TLR2 was obtained by outlining these regions in detail at the pixel level, using Image J.

Detection of cytokines and chemokines

For cytokine/chemokine measurements, animals were sacrificed at 24 h after ischemic injury and a sample of blood was taken from the inferior vena cava, and then kidneys were harvested and halved kidneys were snap frozen in liquid nitrogen. The kidneys were homogenized in extraction buffer (10 mM Na-phosphate (pH 7.5), 0.1 Mm amino caproic acid, 10 U/ml heparin Na-salt, complete protease inhibitor tablet with EDTA) for 30 s and incubated overnight at 4°C on a rotator. Supernatants were collected after centrifugation at 10,000 × *g* for 10 min at 4°C. Cytokine and chemokine levels were measured in tissue homogenates using commercial ELISA kits (R&D Systems), according to manufacturer instructions. The detection limits were 16 pg/ml for IL-6, keratinocyte-derived cytokine (KC), and MIP-2; 31 pg/ml for TNF-α and IFN-γ; and 4 pg/ml for MCP-1. Plasma concentrations of mouse IL-6 were measured using ELISA kits (limited to 16 pg/ml).

Results

TLR2 protein expression within normal and ischemic mouse kidney

To detect TLR2 protein within the normal unmanipulated mouse kidney, we examined WT (C57BL/6) murine kidneys, sampling tissue from the cortex to the papilla (Fig. 1). Fig. 1, *A* and *B*, shows low power black and white panels, demonstrating the expression of both CD31 and TLR2 in the kidneys of a TLR2-deficient mouse (*A*) vs a WT mouse (*B*). CD31 is widely expressed throughout the kidney and its expression follows the vascular network of the normal kidney, and it does not differ between TLR2-deficient and WT mice. TLR2 is highly expressed in the WT kidney, with the highest amount of TLR2 expressed within the renal cortex and the outer stripe of the medulla (Fig. 1*B*; TLR2). A WT kidney stained with H&E is shown between *B* and *C* as a reference for the location of TLR2 staining seen in the low power images (*B*) and the location of sampling in the high power images (*C*). The negative staining for TLR2 in the TLR2-deficient kidney confirms the anti-TLR2 Ab specificity.

To delineate TLR2 expression on individual cells within regions of the kidney, sections were obtained from the indicated regions of the kidney and were costained with TLR2, F-actin (to delineate cell structure), CD31 (to detect endothelial cells), and DAPI to detect nuclei. High power resolution imaging was then performed using laser scanning confocal microscopy (Fig. 1, *C* and *D*). Fig. 1*C* shows that TLR2 was expressed within glomeruli, where it colocalized with CD31 (endothelial cells), and was also seen along Bowman's capsule and possibly on mesangial cells (*C*, *top panel*). The *middle* and *lower panels* of Fig. 1*C* show TLR2 is expressed on the basolateral membranes of renal tubule cells within the ischemia-sensitive regions of the kidney (outer stripe of the medulla (*middle panel*)), but not on renal tubules from deeper in the medulla (*lower panel*). TLR2 was also expressed on the endothelial lining of blood vessels, where it colocalized with the endothelial marker CD31 (Fig. 1*D*).

Fig. 2*A* shows high power confocal imaging of TLR2 staining 24 h after reperfusion, comparing uninjured to injured kidneys. Images of renal tubules from WT, MyD88-deficient, and TLR2-deficient mice are shown (Fig. 2*A*, WT vs MyD88 vs TLR2 deficient). Following IR injury, TLR2 is no longer seen on the lateral wall of the cells (intercellular membranes). In contrast to

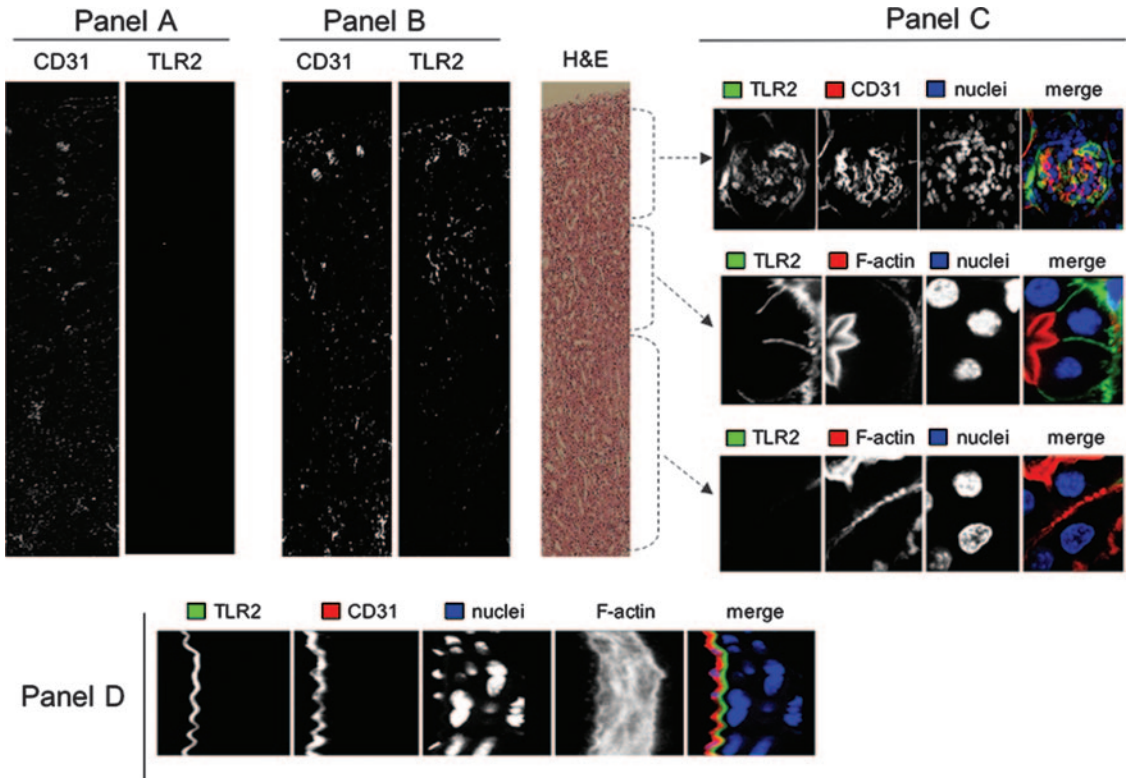

FIGURE 1. TLR2 expression within normal mouse kidney. Fig. 1 shows four panels (labeled *A–D*) and a section of WT kidney stained for H&E for architectural reference (labeled H&E). *A* and *B*, Low power imaging of kidney sections stained for CD31 (endothelial marker) and TLR2. *A* sections are from a TLR2-deficient mouse, and *B* sections are from a WT mouse. *C* and *D*, High power images of WT kidney sections. *C*, Shows a glomerulus in the *top panel* stained with anti-TLR2 (labeled TLR2 in the black and white panel, and green in the merge panel), anti-CD31 (labeled CD31 in the black and white panel, and red in the merge panel), and DAPI nuclear stain (labeled nuclei in the black and white panel, and blue in the merge panel). The merged panel shows the confluence of all three stains. The *middle group* of images in *C* show renal tubule cells obtained from the inner stripe of the medulla, stained for TLR2 (TLR2 in the black and white panel, and green in the merge panel), F-actin to show the cell borders (F-actin in the black and white panel, and red in the merge panel), and DAPI nuclear stain (labeled nuclei in the black and white panel, and blue in the merge panel). The *bottom group* of images in *C* has the same labeling key as the *middle panels*. *D*, An arteriole stained for TLR2 (labeled TLR2 in the black and white panel, and green in the merge panel), anti-CD31 (labeled CD31 in the black and white panel, and red in the merge panel), and DAPI nuclear stain (labeled nuclei in the black and white panel, and blue in the merge panel), and F-actin (labeled F-actin in the black and white panel). The merged panel shows the confluence of the TLR2, CD31, and nuclear stains. Tissue was examined using laser scanning confocal microscope, and images were processed using Laser Sharp, LSM examiner, and Image J software. The images represent one of six independent experiments.

the staining pattern observed in the uninjured tubules, we now see a different pattern of TLR2 staining, with intracellular distribution, suggesting that ligand binding results in internalization of the protein receptor. Fig. 2*B* compares the mean fluorescence intensity of TLR2 within the cytoplasm of IR injured WT vs MyD88-deficient tubules (>250 cells per group), showing that there were no detectable differences in intracellular TLR2 between ischemic WT and MyD88-deficient tubules.

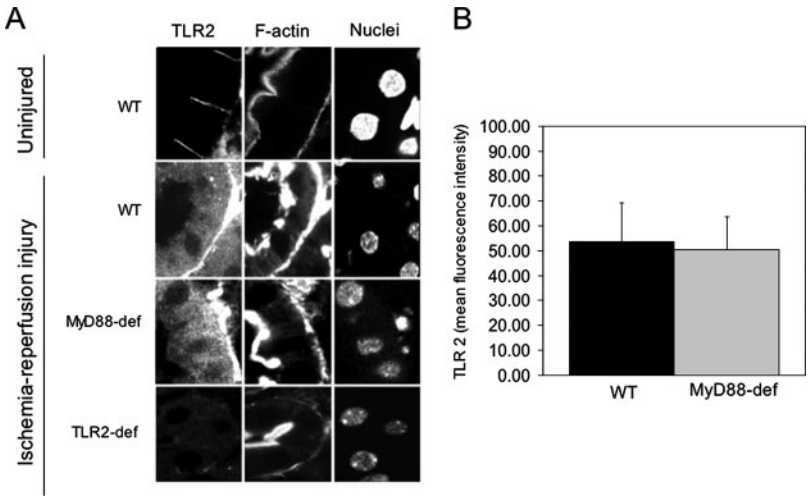

FIGURE 2. TLR2-deficient mice are protected from renal IR injury. *A*, From *left* to *right*, a series of black and white panels demonstrating individual stains for TLR2, F-actin, nuclei stained with DAPI (Nuclei). WT, uninjured kidneys (*top panels*, labeled uninjured, WT) are compared with WT, MyD88-deficient, and TLR2-deficient kidneys subjected to IR injury (second through fourth panels, labeled WT, MyD88-def, TLR2-def, IR injury). Tissue was examined using laser scanning confocal microscope, and images were processed using Bio-Rad LaserSharp 2000 software and ISEE software (Innovision). The confocal images were the same in three different TLR adapter protein-deficient animals imaged following IR injury. *B*, Means fluorescence intensity of TLR2 staining in >250 tubular cells compared between IR injured WT vs MyD88-deficient kidneys. Error bars represent SDs, and there was no significant difference between the results ($p = 0.18$).

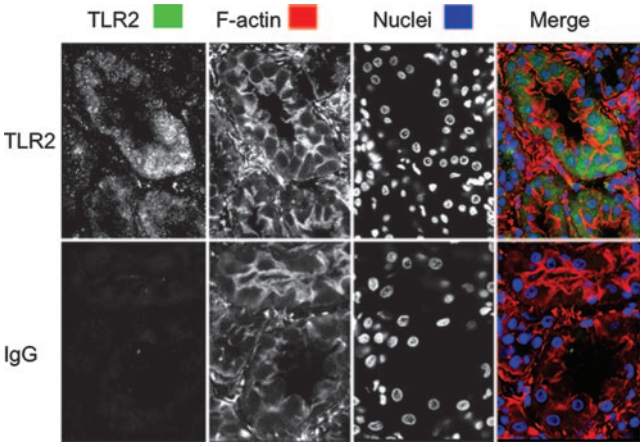

FIGURE 3. TLR2 protein expression in the human kidney. TLR2 protein expression was examined in tissue from a human kidney that had been removed 30 min earlier, thus representing ~30 min of ischemia. *Top panels*, From *left* to *right*, black and white panels depicting TLR2 expression (TLR2), F-actin expression (F-actin), and nuclei stained with DAPI (Nuclei). A merge figure is shown on the *right* that demonstrates the confluence of all three stains, with TLR2 in green, F-actin in red, and nuclei in blue (Merge). *Lower panels*, Stained with the IgG1 control for TLR2, confirming the specificity of the TLR2 stain. Tissue was examined using laser scanning confocal microscope, and images were processed using Laser Sharp, LSM examiner, and Image J software.

TLR2 protein expression within human kidney

Because TLR2 was expressed on murine tubules, we asked whether TLR2 was also expressed on renal tubules of human kidneys. To detect TLR2 protein, we examined tissue from nephrectomy specimens removed from patients with renal cell carcinoma (Fig. 3), a standard way to obtain normal human kidney tissue. Tissue was taken from a site distal to the encapsulated tumor and represented kidney tissue uninvolved by tumor. The specimen shown in this figure was from a core biopsy of a kidney removed 30 min earlier and represents the same results seen in four individual patient samples. Tissue could not be obtained at an earlier time point due to risk of bleeding during the nephrectomy procedure. The *top panels* show sequential images from *left* to *right* showing (in black and white) TLR2, F-actin, and DAPI (nuclei), respectively. The panel on the *far right* shows the color merge of all the stains (TLR2 in green, F-actin in red, and nuclei in blue). The same images were also stained for an isotype control IgG Ab (IgG1, clone MOPC-31C; BD Biosciences) to control for the specificity of TLR2 staining (Fig. 2; IgG). As can be seen in Fig. 3, TLR2 protein appeared to be expressed within renal tubule cells. Whether the intracellular distribution represents cytoplasmic internalization of TLR2 protein in response to ischemia during the course of nephrectomy is not known, and is the subject of ongoing investigations in our laboratory.

TLR2 and IR injury

TLRs have been shown to play a role in ischemic organ injury, and proposed to provide the trigger for inflammatory responses to danger signals released from necrotic/apoptotic tissues. Because TLR2 was expressed on the basolateral membranes of renal tubular cells in the mouse, we next asked whether TLR2-dependent signals influenced murine experimental ischemic renal injury. All mice were on the same genetic background (C57BL/6, for >10 generations). To investigate the role of TLR2-dependent signals, we induced nonlethal IR injury in WT, Trif-deficient, MyD88-deficient, (MyD88 × Trif)-deficient, and TLR2-deficient mice. MyD88 and Trif are intracellular adapter molecules necessary for transmission of TLR signaling. Although it is known that TLR2 does not use the intracellular adapter protein Trif, the Trif-deficient mice provided an additional control, demonstrating the specificity of kidney protection to MyD88 and TLR2.

Fig. 4 shows serum creatinine levels 2 wk before and 24 h after injury for each group of mice (pretreatment creatinines in the ▨ and postischemia creatinines in the ■ of each group). In this nonlethal IR model, we found that TLR2-deficient mice were significantly protected from injury at 24 h ($p < 0.0003$), as were the MyD88-deficient ($p = 0.001$) and MyD88 × Trif)-deficient ($p = 0.003$) mice. As expected, the absence of Trif ($p = 0.4$) had no influence on IR injury. Sham-operated mice showed no changes in creatinine, as expected (data not shown). Histologic scoring for necrosis and leukocyte infiltration (35) did not significantly differ between TLR-deficient vs WT mice (data not shown). Furthermore, there was no correlation between inflammatory cell infiltrates and serum creatinine levels (correlation coefficient = 0.09), suggesting recruitment of inflammatory cells occurred independently of TLR-mediated signals. Because it is known that inflammatory cells (e.g., T cells (1)) express TLR2, studies are ongoing in our laboratory to determine whether activation of inflammatory cells is inhibited in the absence of TLR2-dependent signals. Interestingly, the functional data suggest that a deficiency in TLR2-mediated signals provided more protection than a deficiency in the

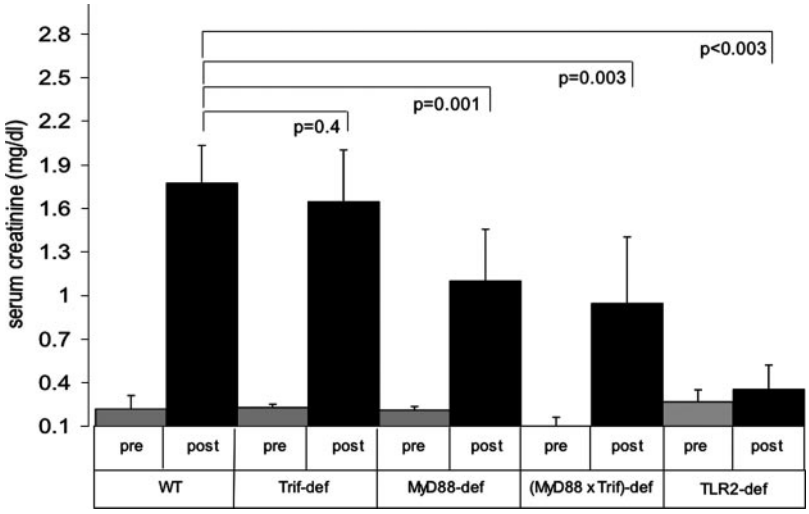

FIGURE 4. Renal IR injury in WT vs TLR adapter protein-deficient mice. This figure shows serum creatinines from WT ($n = 7$), Trif-deficient ($n = 7$), MyD88-deficient ($n = 6$), (MyD88 × Trif)-deficient ($n = 6$), and TLR2-deficient ($n = 6$) mice that were subjected to 25-min ischemia/24 h of reperfusion (■). Preinjury creatinines were obtained 2 wk earlier (▨). Error bars represent SD of creatinines, and statistical significance was determined with two-tailed Student's *t* test.

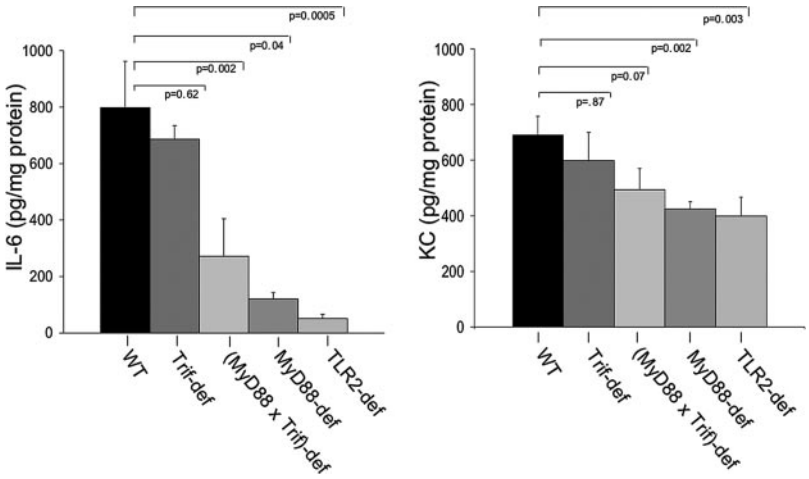

FIGURE 5. Proinflammatory cytokine/chemokine production following IR injury in WT vs Trif-deficient, (MyD88 × Trif)-deficient, MyD88-deficient, and TLR2-deficient mice. IR injury was induced in the WT vs TLR-deficient strains listed in the figure below each bar. *Left panel*, Serum IL-6 production. *Right panel*, KC in tissue homogenates. Protein was detected in either serum or kidney homogenates using a commercial ELSIA kit for the respective murine cytokines/chemokines (R&D Systems). The error bars represent SDs of six mice per group, and statistical significance was determined with two-tailed Student's *t* test.

proximal adapters thought to be necessary for transmission of TLR2-dependent signals, suggesting there are additional TLR2-dependent/MyD88-independent signaling pathways that contribute to renal IR injury in this model.

Chemokine and cytokine levels in mice after renal IR injury

To investigate proinflammatory cytokines and chemokines known to participate in the inflammatory response to kidney IR injury (28, 36), we compared and contrasted cytokine and chemokine secretion in serum and tissue homogenates 24 h after IR injury in WT, Trif-deficient, (MyD88 × Trif)-deficient, MyD88-deficient, and TLR2-deficient mice (Fig. 5). As shown in Fig. 5, there were significant reductions in both serum IL-6 and tissue KC levels 24 h following IR injury between WT mice and mice with deficiencies in MyD88 (MyD88-def and (MyD88 × Trif)-def)- and TLR2-deficient mice. KC has been validated as an early biomarker for renal IR injury (36, 37), and serum IL-6 levels are highly correlated with the severity of the physiologic response to tissue injury (38–40), possibly because of the high levels of IL-6 released from tissue-resident macrophages activated by molecules released from ischemic tubules (41). We also compared TNF-α, IFN-γ, MIP-2, and MCP-1 levels at 24 h following renal injury and did not find any significant differences between the groups (data not shown). Previous studies also showed no changes in MIP-2 and TNF-α using TLR2-deficient mice (28).

Discussion

TLRs are expressed throughout the animal and plant kingdom (1, 3), and therefore there has been an intense interest in defining their role in health and disease. TLR2-deficient mice have recently been shown to experience only mild renal injury following prolonged ischemic injury (28). We have expanded on these results by defining the pattern of TLR2 protein expression in mice and humans, by confirming a dominant role of TLR2 in sublethal ischemic renal injury, and by demonstrating that TLR2 mediates renal injury through both MyD88-dependent and MyD88-independent pathways.

We found that TLR2 protein was constitutively expressed in the kidney. However, the distribution of TLR2 was not uniform throughout the nephron. TLR2 was highly expressed within the glomeruli, where it was associated with endothelial cells, epithelial cells of Bowman's capsule, and mesangial cells. TLR2 protein was also found on endothelial cell membranes throughout the rest of the kidney, both in peritubular capillaries and arterioles. TLR2 was not, however, uniformly expressed on renal tubule cells. Renal tubule cells within the cortex and outer stripe of the medulla expressed abundant TLR2 on their basolateral membranes, but renal tubule cells within the deeper medulla showed no TLR2 expression. The TLR2 expression pattern (on endothelium, glomeruli, and tubules in the outer medulla) raises the possibility that protection from ischemic injury may well involve the renal vasculature and the glomerulus. This possibility is supported by the finding of preserved glomerular filtration rate despite similar degree of necrosis between the WT and TLR-deficient animals.

TLRs have been suspected participants in renal IR injury for some time based on studies showing that ligation of TLR2 and 4 induced NF-κB-dependent production of proinflammatory cytokines and chemokines from cultured tubular epithelium (42). Tsuboi et al. (43) showed that murine tubular epithelial cells constitutively expressed mRNA for TLR1, 2, 3, 4, and 6, but not for TLRs 5 or 9. Murine tubular epithelial cells have also more recently been shown to express the TLR coreceptors MD-2 and CD14, as well as the adapter protein MyD88 (44).

Our studies are the first to define the distribution of TLR2 protein throughout the human and mouse kidney. We show that TLR2 is highly expressed on several cell types within the kidney, including within the glomeruli, on endothelial cells, and on renal tubular cells located within the outer stripe of the medulla. Wolfs et al. (30) localized TLR2 and TLR4 by examining mRNA levels in various parts of the mouse kidney using in situ hybridization. Interestingly, they were able to detect both TLR2 and TLR4 mRNA in epithelial cells of proximal and distal tubules as well as in Bowman's capsular epithelium, but did not find TLR2 or TLR4 mRNA in either glomeruli (endothelial cells or mesangial cells) or endothelial cells within the kidney. One explanation for the discrepancy between our findings of abundant TLR2 protein in the glomeruli, and that of Wolfs, is that protein turnover might occur more frequently in renal tubule cells than in the endothelial or mesangial cells. Functional studies have recently provided somewhat contradictory findings to that of Wolfs, showing that glomerulonephritis associated with nephrotoxic Abs was primarily dependent upon glomerular TLR2, and that TLR2 mRNA was detectable in mesangial cells (29).

We also found that the constitutive TLR2 membrane expression (on basolateral membranes) changed following IR injury. TLR2 protein was no longer expressed on the lateral membrane; rather, it was internalized into the cytoplasm, a finding common to both WT and MyD88-deficient mice. TLR2 protein was also highly expressed within the cytoplasm of ischemic tubules of human kidneys: these kidneys had experienced ~30 min of ischemia during

the course of nephrectomy. Several investigators have reported that TLR2 mRNA expression increased by 3–5 days following renal IR injury (30, 31), which might reflect increased protein synthesis on regenerating tubular epithelium. We did not study TLR2 expression on regenerating tubules, although this will be pursued in future studies. The marked constitutive expression of TLR2 protein throughout the kidney suggests an important role for this receptor in not only regulating the response to renal injury, but also strongly suggests it may play an important, as yet unknown, role under normal physiologic conditions.

Because TLR2 protein was prominently expressed in normal kidneys and TLR2-deficient mice were protected from IR injury, we predicted that a deficiency in MyD88 signaling would strongly protect from IR injury. Upon ligation, all TLRs recruit intracellular adapter proteins that divide TLR-mediated signaling into two pathways, as follows: a MyD88-dependent and a MyD88-independent/Trif-dependent pathway. All TLRs, except TLR3, are dependent upon intact MyD88 signaling, and therefore, we expected profound protection in the presence of a MyD88 deficiency. Surprisingly, we found that the absence of TLR2 protected kidneys from ischemic injury to a greater degree than the absence of MyD88. Because TLR2-deficient mice were protected to a greater degree than the adapter protein-deficient mice, it is likely that ligation of TLR2 by DAMPs not only activates traditional TLR adapters, but it also activates TLR2-dependent/MyD88-independent signaling pathways. A TLR2-dependent/MyD88-independent pathway involving TLR2/Rac-1/PI3K/Akt activation of NF-κB gene transcription has been described in TLR2-transfected cell lines (45–47), and TLR2 has been found to contain a consensus-binding motif (YXXM) (48) for the p85 subunit of PI3K, which is distinct from the binding site for MyD88 (46). Furthermore, TLR2 activation of this pathway is thought to affect NF-κB activity by reversible actylation of the p65 Rel family member at Ser^{536} (46). Studies are underway in our laboratory to determine whether a Rac-1/PI3K, or other alternative pathways, induces TLR2-dependent/MyD88-independent proinflammatory signals.

It is quite likely that renal IR injury triggers multiple pathophysiologic mechanisms that are interrelated and culminate in the expression of survival genes, proinflammatory cytokines, and chemokines (and ultimately activation of inflammatory cells). The prominent constitutive expression of TLR2 protein throughout the kidney, and the profound protection afforded by its blockade, positions this receptor as not only a major regulator of renal injury, but suggests an important role in normal renal physiology.

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Disclosures

The authors have no financial conflict of interest.

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