Tracking the Toll of Kidney Disease

Anushree C. Shirali* and Daniel R. Goldstein†

Divisions of *Nephrology and †Cardiology, Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut

ABSTRACT

Since the discovery of the Toll-like receptors and their crucial role as modulators of innate immunity, there has been increasing appreciation of their role in human health and disease. Toll-like receptor signaling is critical in defending against invading microorganisms, but sustained receptor activation is also implicated in the pathogenesis of inflammatory diseases. Here we review the role of Toll-like receptors and their endogenous ligands in various renal diseases, particularly their activation in the inflammatory response of ischemic kidney injury, organ transplantation, and immune-mediated glomerulonephritis.


The Toll-like receptors (TLR) were discovered more than a decade ago as sentinel receptors for the mammalian innate immune system.1 TLR are among a growing number of receptors that recognize pathogen-associated molecular patterns as infectious non-self ligands and, in response, ignite an inflammatory cascade that includes activation and maturation of dendritic cells (DC), the most potent antigen-presenting cells of innate immunity.2 TLR-activated DC induce naive T lymphocytes to mature into antigen-specific effector T cells, particularly of the Th1 lineage.2 Thus, TLR link innate and adaptive immune responses, both of which are critical to host defense against pathogens. TLR are also implicated in the pathogenesis of several inflammatory diseases, including kidney diseases. In particular, increasing evidence suggests that endogenous ligands activate TLR, resulting in the antigen-independent inflammation that accompanies ischemic acute kidney injury (AKI), solid organ transplantation, and immune-mediated glomerulonephritis.

TLR: STRUCTURE, EXPRESSION, AND SIGNALING PATHWAYS

TLR are mammalian homologues of the Toll receptor, first described in Drosophila as playing a critical role in embryogenesis and antifungal immunity.1 At present, 11 human and 13 mouse TLR have been identified,3 and all are structurally similar to the Toll receptor, highlighting in terms of evolution that this is an ancient system for immune protection. TLR are type I integral membrane glycoproteins. They contain a cytoplasmic domain named the Toll/IL-1R (TIR) domain because of sequence homology with the IL-1 receptor (IL-1R).4 The extracellular portion of TLR contains unique tandem areas of leucine-rich repeats in contrast to the Ig-like domains found in the IL-1R.4 TLR are found on a variety of cell types, including epithelial cells, endothelia, DC, monocytes/macrophages, and B and T cells.5 One particular cell type may express only a limited number of TLR.6 For example, plasmacytoid DC, specialized DC that secrete type I IFN in response to viral infection, express TLR7 and 9 but not other TLR.6 In the kidney, tubular epithelial cells and mesangial cells express TLR 1 through 4 and 6.7,8 Data on TLR expression in podocytes and other glomerular components are sparse, although a recent study found constitutive TLR2 staining in glomerular capillary endothelial cells.9 Most TLR reside on the cell surface, except TLR 3 and 7 through 9, which are found intracellularly to encounter ligands in endosomes or lysosomes.3 Thus, by achieving wide distribution in different tissues, cells, and subcellular compartments, TLR are exceptionally well-positioned sentinels against invading pathogens.

The cytosolic domains of TLR recruit adaptor proteins and initiate signaling pathways in response to ligand binding (Figure 1). Five adaptor proteins have been discovered: Myeloid differentiation factor 88 (MyD88), MyD88 adaptor–like (MAL; also known as TIRAP), TIR-domain-containing adaptor protein–inducing IFN-β (TRIF; also known as TICAM1), TRIF-related adaptor molecule (TRAM; also known as TICAM2), and sterile α- and armadillo motif–containing protein.10 All TLR use the MyD88 pathway, except TLR3, which signals
through TRIF. TLR2 and TLR4 recruit MyD88 through MAL/TIRAP, whereas TLR4 also engages TRIF through TRAM.\(^\text{10}\) Once stimulated, the MyD88-dependent pathway leads to activation of the NF-κB, mitogen-activated protein kinase, and IFN regulatory factor pathways of inflammation, cell growth, and differentiation.\(^\text{10}\) The TRIF-dependent pathway also activates NF-κB with delayed kinetics and additionally induces IFN regulatory factor 3, a transcription factor necessary for production of type I IFN.\(^\text{11}\) TLR 1, 2, and 6 contain a phosphatidylinositol 3-kinase (PI3K) binding motif\(^\text{11}\) and activate NF-κB through PI3K independent of MyD88. Thus, depending on the specific combinations of TLR ligand, cell type, receptor, and adaptor protein, different signaling cascades result in a diverse range of cellular responses, all of which support a robust innate immune response and subsequently shape adaptive immunity.

### TLR: LIGANDS SIGNAL “STRANGER” AND “DANGER”

TLR respond to a variety of activators (Table 1), including DNA, RNA, lipids, and peptide products from bacteria, viruses, fungi, and synthetically derived compounds.\(^\text{5,12,13}\) Among the best characterized ligands, LPS is the cell wall component of Gram-negative bacteria that plays a prominent role in the pathogenesis of sepsis.\(^\text{14}\) LPS activates TLR4, and this interaction contributes to the inflammation that characterizes LPS-induced AKI, as elevated blood urea nitrogen (BUN) in a murine model.\(^\text{15}\) As demonstrated by Cunningham \textit{et al.},\(^\text{15}\) this is a systemic effect. Kidneys from wild-type mice transplanted into TLR4\(^{−/−}\) recipients resisted LPS-induced AKI, whereas TLR4\(^{−/−}\) kidneys transplanted into wild-type recipients sustained severe AKI triggered by LPS.

The identity of TLR agonists has grown to include endogenous ligands (Table 1) such as heat-shock proteins (HSP), high mobility group box 1 (HMGB1) nuclear protein, and hyaluronan (HA).\(^\text{5,12,13}\) Although there is concern that microbial contamination may be a confounding factor,\(^\text{16}\) the weight of experimental evidence supports that these molecules activate TLR. Although this presents a paradigm shift in the traditional concept of immune surveillance as self/non-self discrimination (the “stranger” hypothesis), it more readily explains a role for the innate immune system in AKI, organ transplantation, and autoimmune disease, as Matzinger\(^\text{17}\) proposed in the “danger” model. Concerning AKI and organ transplantation, these models support the idea that antigen-independent injury, such as ischemia reperfusion or vessel attachment of ischemic allografts, initiates a common pathway of innate immune activation and inflammation.

### ENDOGENOUS LIGANDS IN ISCHEMIA REPERFUSION INJURY

Ischemia reperfusion injury is a complex pathophysiologic process that occurs...
when blood flow is restored to ischemic tissues. It occurs with profound hypovolemia, sepsis, and transplantation of vascularized allografts. Ischemia reperfusion injury leads to poor clinical outcomes, for example, the association of delayed graft function and allograft rejection with prolonged cold ischemia time. Renal ischemia reperfusion injury is associated with an influx of neutrophils, macrophages, and T cells, which cause inflammation and lead to chronic renal dysfunction.18 Various endogenous ligands are implicated as mediators of this process.16,19 Table 1 lists several of these substances, including two extensively studied ligands: HA and HMGB1.

HA is a glycosaminoglycan component of the extracellular matrix and is abundantly present in the renal medulla.20 HA is the major ligand for CD44, a transmembrane glycoprotein receptor, but studies suggest HA also transduces inflammatory signals through TLR2 alone or in synergy with TLR4.21 A recent review21 discussed HA in depth, including its role as an innate immune activator.

Studies of murine models reported increased renal expression of HA as well as the HA receptor, CD44, after kidney ischemia reperfusion injury.22,23 HA-CD44 interactions are critical for inflammation induced by renal ischemia. Rouschop et al.24 demonstrated that 24 h after bilateral kidney ischemia reperfusion injury, CD44+/− mice had better renal function, as measured by BUN and creatinine (BUN/Cr) levels, and less histologic evidence of tubular necrosis and brush border loss compared with wild-type mice. Renal inflammation was also abrogated without CD44, because CD44−/− mice had decreased infiltration of neutrophils compared with wild-type mice. This effect is independent of cytokine and chemokine levels. Interestingly, although mutant mice displayed decreased inflammation, they also displayed a slower tempo of neutrophil clearance compared with wild-type mice.24 Perhaps CD44 plays a role in clearing renal inflammation in later stages of ischemia reperfusion injury, as had been demonstrated in an experiment model of acute lung injury.25

HMGB1 is another endogenous molecule increasingly implicated in ischemia reperfusion injury. HMGB1 is a highly conserved nuclear protein that binds DNA and facilitates interactions between DNA and nuclear proteins, which regulate transcription.26 It is released during cell necrosis and binds to the receptor for advanced glycation end products, TLR2, and TLR4. In addition, HMGB1 is released during late phases of LPS-induced septic shock in mice and is present in patients with sepsis.27 Moreover, administration of anti-HMGB1 antibodies protects against lethality from established endotoxemia.28 Recent studies found that HMGB1 plays a central role in mediating the inflammatory response to ischemia reperfusion injury, particularly in models of liver ischemia. The Billiar laboratory demonstrated the expression of HMGB1 increases in murine livers soon after ischemia reperfusion injury and remains elevated up to 24 h.28 In vitro studies with cultured hepatocytes found hypoxia was sufficient to stimulate HMGB1 upregulation.28 Use of a neutralizing antibody against HMGB1 after ischemia reperfusion injury decreased local TNF-α and IL-6 production and afforded protection of liver function, as shown by decreased serum alanine aminotransferase levels.28 HMGB1 uses TLR signaling in mediating hepatic ischemia reperfusion injury; TLR4 knockout mice were resistant to the effects of the anti-HMGB1 antibody. Whether HMGB1 is an endogenous ligand activating innate immune signaling during kidney ischemia reperfusion injury is unclear, although a recent study reported increased renal HMGB1 expression after kidney ischemia.29

**TLR SIGNALING IN AKI**

Several reports in the past few years proposed a role for TLR signaling in ischemic kidney injury. Using *in situ* hybridization, Wolfs et al.30 found that TLR2 and TLR4 are constitutively expressed in healthy, wild-type murine kidneys, primarily in proximal and distal tubule epithelial cells (TEC) and in the epithelium of Bowman’s capsule. During unilateral ischemia, expression of both TLR rapidly increased in ischemic kidneys, up to four- to five-fold over basal levels at 5 d after ischemia, especially in distal tubular epithelia.30 Leemans et al.31 explored the functional significance of these findings by comparing bilateral kidney ischemia reperfusion injury in TLR2 null mice versus wild-type mice and found that ischemia-induced renal dysfunction, as assessed by BUN/Cr and histologic evidence of acute tubular necrosis, was TLR2 dependent. Experiments with bone marrow chimeras demonstrates that TLR2 expression in renal parenchyma mediates inflammation in this experimental model.31

Within the past year, the role of other TLR as well as TLR adaptor proteins has been investigated during renal ischemia. One study found that bilateral renal ischemia reperfusion injury in TLR2−/− mice resulted in less renal impairment than in wild-type mice but through MyD88-independent mechanisms.9 This suggests involvement of MyD88-independent pathways for TLR2 signaling activated by kidney ischemia reperfusion injury, through either PI3K or TIRAP. Wu et al.29 suggested that TLR4 also participates in ischemia-induced inflammation. In an *in vivo* model of renal ischemia reperfusion injury, the authors demonstrated that TLR4−/− mice had lower creatinine levels, less histologic evidence of tubular injury, and decreased neutrophil influx compared with wild-type controls at several time points after renal ischemia.29 Studies with bone marrow chimeric mice confirmed that TLR4 signaling on renal parenchyma is necessary for the complete pathologic profile of ischemia reperfusion injury.29 In contrast to the previous study,9 MyD88−/− mice in this model of ischemia reperfusion injury have a similar phenotype of renal function and tubular injury as TLR4−/− mice.29 Furthermore, renal expression of several endogenous ligands increases after ischemia reperfusion injury, including biglycan, HMGB1, and HA, but not HSP 70.29 but there were no mechanistic data linking the release of these ligands to the altered phenotype.
seen without TLR4. Clearly, this is an area that merits further investigation.

**INNATE IMMUNE ACTIVATION IN ORGAN TRANSPLANTATION**

Experimental and clinical evidence suggests an association between endogenous ligands and allograft rejection.\(^20,32,33\) We previously hypothesized that antigen-independent ischemia reperfusion injury after organ implantation would release innate immune ligands, and this would initiate TLR signaling on either host or recipient DC, leading to DC maturation and priming of alloimmune responses. In testing this hypothesis in a murine minor mismatch (H-Y) skin allograft model, we found that acute allograft rejection depended on MyD88 signaling.\(^34\) Specifically, MyD88\(^{-/-}\) females were unable to reject skin grafts from MyD88\(^{-/-}\) males, whereas rejection was preserved in wild-type controls.\(^34\) In addition, MyD88\(^{-/-}\) mice displayed reduced numbers of mature DC in draining lymph nodes after transplantation, suggesting the defective alloimmune response in MyD88\(^{-/-}\) mice occurs in the initiation phase of the immune response to transplantation.\(^34\) Lack of MyD88 signaling also leads to defective CD8 alloimmune priming and Th1 alloimmune responses.\(^34\) The identity of the upstream TLR that initiate MyD88 signaling is not clear, although we did find that TLR2\(^{-/-}\) mice have a delayed tempo of graft rejection,\(^34\) whereas, in agreement with other reports of a skin allograft model,\(^35\) TLR4 did not play a significant role. Perhaps multiple TLR act in concert to deliver the immune response in clinical organ transplantation. Alternatively, LPS contamination during organ transplantation in humans may be a confounding factor.\(^36\)

Indeed, clinical studies of renal\(^37,38\) or lung transplant recipients\(^39\) with hyporesponsive TLR4 polymorphisms have decreased acute allograft rejection rates. In subsequent studies, we determined that MyD88 signaling is not critical for rejection of fully allogeneic skin or cardiac allografts, although Th1 alloimmune responses are decreased in these models without MyD88 signaling.\(^40\) TLR-dependent MyD88 signaling impairs the induction of transplantation tolerance.\(^41\) In this work, MyD88 signaling activates inflammatory responses by DC during transplantation, which subsequently primes alloreactive T cells.\(^41\) These primed T cells are resistant to the immunoregulatory properties of regulatory T cells. Similar findings have been found in other studies.\(^42,43\) In summary, multiple innate immune pathways—TLR-dependent/MyD88 independent and/or non-TLR pathways—are necessary for acute allograft rejection; however, MyD88 signaling inhibits the induction of transplantation tolerance.

We also investigated the role of endogenous ligands in organ transplantation. There is no increase in levels of HSP 70 in fully mismatched skin grafts undergoing acute rejection\(^44\); however, in the H-Y–incompatible murine skin graft model, HA levels were increased during acute rejection.\(^45\) We also found increased levels of HA in the bronchial lavage fluid of lung transplant recipients with clinical evidence of rejection compared with patients who remained free of rejection.\(^45\) In vitro studies showed that stimulation of DC with proinflammatory low molecular weight HA fragments leads to DC maturation, including upregulation of co-stimulatory molecules CD40, CD86, and CCR7 and production of TNF-α.\(^45\) Interestingly, although HA-induced TNF-α production is MyD88 dependent, upregulation of co-stimulatory molecules is MyD88 independent and depends on TIRAP signaling.\(^45\) The in vivo importance of TIRAP signaling to transplantation is unclear.

**TLR INVOLVEMENT IN AUTOIMMUNE GLOMERULONEPHRITIS**

Many of the autoantibodies used clinically as markers of autoimmune disease activity, particularly in systemic lupus erythematosus (SLE), target nucleic acids and their associated substructures. This clinical observation supports an involvement for TLR, particularly TLR 3, 7, and 9, which recognize nucleic acids (Table 1), in mediating the inflammatory response in autoimmune disease. Mar-oak-Rothstein\(^36,47\) seminal work in murine models of systemic autoimmune disease established in vitro that self-IgG2a antibodies complex with chromatin or RNA autoantigens to stimulate B cell proliferation and autoantibody formation through sequential engagement of the B cell receptor and TLR9 or TLR7, respectively; however, Patole et al.\(^48\) found that in vitro binding of synthetic dsRNA to TLR3 failed to activate B cells and produce anti-DNA antibodies. To clarify the role of TLR in autoimmunity, subsequent studies have investigated the in vivo importance of TLR for autoimmune disease.

Christensen et al.\(^49,50\) backcrossed Fas-deficient MRL/Mp\(^{lpr/lpr}\) (MRL/lpr) mice, an accepted murine model of genetic susceptibility to SLE characterized by spontaneous development of an SLE-like syndrome and immune complex glomerulonephritis, to TLR3\(^{-/-}\), TLR7\(^{-/-}\), and TLR9\(^{-/-}\) mice. They found that TLR9 but not TLR3 is required for DNA or chromatin autoantibody production.\(^49,50\) In the absence of TLR9, MRL/lpr mice shift their autoantibody repertoire, with nuclear staining patterns suggestive of increased anti-RNA antibodies.\(^50\) In contrast, MRL/lpr mice crossed to a TLR7\(^{-/-}\) background lack antibodies directed against RNA autoantigens but have intact anti-DNA antibodies.\(^50\) Despite the antibody profiles, clinical disease activity diverges with TLR7 versus TLR9 deficiency. Lupus-prone, TLR7-deficient mice display fewer skin lesions and have modestly less renal disease, as defined by a composite score of glomerular lesions and interstitial infiltrates, than TLR7-sufficient control MRL/lpr mice.\(^50\) In contrast, TLR9 deficiency increases disease activity in MRL/lpr mice, with mice displaying more severe skin lesions and greater scoring for glomerular and interstitial renal lesions compared with wild-type littermates. These findings correlate with distinct differences in immune activity,\(^50\) because immune cells from MRL/lpr TLR7\(^{-/-}\) mice, including T cells, B cells, and plasmacytoid DC have
an immature phenotype, whereas the same cells in MRL/lpr TLR9\(^{-/-}\) mice, particularly plasmacytoid DC, have an activated phenotype. Taken together, these results suggest in experimental SLE that TLR7 promotes whereas TLR9 dampens inflammation in target organs, including the kidney. In partial agreement with this finding, Pawar et al.\(^5\) found that inhibitory synthetic oligodeoxynucleotides with immunoregulatory sequences specific for TLR7 decrease interstitial and glomerular injury in MRL/lpr mice. Dual inhibition of TLR7 and TLR9 by a different immunoregulatory sequence do not have an additive renal-protective role but do not abrogate the protection afforded by lone TLR7 blockade, either.

The increased numbers of activated plasmacytoid DC in TLR9\(^{-/-}\)/MLR/lpr mice partly explain the results by Christensen et al.\(^5\) Plasmacytoid DC secrete type I IFN in response to immune complexes containing either RNA or DNA, although IFN-\(\alpha\) responses to RNA are more robust.\(^6,5\) Indeed, TLR9\(^{-/-}\) mice have higher serum levels of IFN-\(\alpha\) compared with wild-type controls.\(^5\) Clinically, higher levels of type I IFN correlate with progression and severity of disease, including renal manifestations.\(^5\) Perhaps the shift in RNA-specific antibodies in TLR9\(^{-/-}\) animals induces stronger type I IFN responses and more severe disease. Clearly, downstream effects of TLR signaling in immune cell subsets and their specific effects on renal pathology in lupus are potential areas of investigation.

The studies by Christensen et al.\(^5\) contrast with earlier in vitro\(^5\) and in vivo\(^4,5\) studies supporting an immunostimulatory role of TLR9 agonists in MRL/lpr mice. The in vivo findings by Christensen et al.\(^5\) confirmed by other reports,\(^8,3\) clearly indicate TLR9 interactions are more complex than previously thought. Emerging data suggest TLR9 activation on plasmacytoid DC by nucleic acids and subsequent IFN-\(\alpha\) production is regulated by endogenous ligands other than nucleic acids. Popovic et al.\(^5\) found that recombinant HMGB1 inhibits in vitro production of IFN-\(\alpha\) and other proinflammatory cytokines in human plasmacytoid DC activated by TLR9 agonists. Tian et al.\(^9\) found opposite results in a similar study in mice with purified HMGB1 complexing with CpG oligonucleotides and stimulating enhanced inflammatory cytokine production through TLR9-MyD88 and receptor for advanced glycogenesis end products-dependent pathways.

Although these studies showed contrasting results, both studies suggested that cross-talk between endogenous ligands modulates TLR9 responses. TLR activation in such an interlinked system to maintain tissue homeostasis after injury by allowing effector mechanisms of inflammation and repair while avoiding loss of self-tolerance to neointeragens created by the same mechanisms. Understanding the pathways that govern these systems is critical in determining safe and effective therapeutics for lupus and lupus-associated kidney disease.

Several laboratories point to the importance of TLR signaling in models of glomerulonephritis besides lupus nephritis. In particular, Brown et al. found that both activation of TLR2\(^20,2\) and TLR4\(^6,4\) in a murine model of crescentic glomerulonephritis induced by an anti-mouse glomerular basement membrane antibody exacerbated the severity of nephritis. These studies are particularly interesting because of the known clinical association between infection and subsequent development or exacerbation of glomerulonephritis in susceptible individuals.\(^6\) Because TLR mediate immune responses against both infection and injury, they may prove useful targets in modulating immune activation in several models of glomerulonephritis.

**CONCLUSIONS**

Experimental and clinical evidence supports the involvement of TLR in development of kidney diseases, including AKI, transplant rejection, and autoimmune glomerulonephritis. Although these pathologic states are diverse, they share a common pathway of TLR activation that begins with tissue injury. In this model (Figure 2), various forms of renal injury, including ischemia reperfusion injury and immune complex deposition, induce the release of endogenous ligands to activate TLR. As a result, an adaptive immune response is primed, culminating in robust effector cell responses that cause renal inflammation. With controlled inflammatory signals, the kidney undergoes repair and recovers function; however, with unresolved inflammation, TLR activation persists, resulting in a cycle of chronic renal injury, inflammation, and dysfunction. Further studies are needed to determine how the immune system modulates TLR activation to maintain the balance between tissue repair and injury. Such lines of investigation may lead to novel therapeutics for kidney diseases.

**Figure 2.** Role of TLR in the cycle of renal injury induced by ischemia, organ transplantation, or autoimmunity. AKI, active kidney disease; CKD, chronic kidney disease.
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DISCLOSURES

None.

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