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Toll-like receptor activation: from renal inflammation to fibrosis

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Toll-like receptors (TLRs) are a conserved family of pattern recognition receptors that play a fundamental role in the innate immune system by triggering proinflammatory signaling pathways in response to microbial pathogens through exogenous pathogen-associated molecular patterns or tissue injury through endogenous danger-associated molecular patterns. In the kidney, TLRs are widely expressed in a variety of cell types. Emerging evidence demonstrates the participation of TLRs in the activation of these cells during renal fibrosis. This review highlights the role of TLRs and their endogenous ligands in the pathogenesis of renal fibrosis using ureteral obstruction and diabetic nephropathy as models of chronic kidney disease.

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Renal fibrosis is a wound healing/scarring response following kidney injury that occurs in many forms of chronic kidney disease (CKD). It is also a crucial determinant underlying the progression from CKD to end-stage renal disease. After over a decade of intense study, it is now known that the interstitial accumulation of extracellular matrix (ECM) proteins is not only the aftermath of fibroblast activation, but also a process contributed to by other intrinsic renal cells, including tubular epithelial cells (TECs), mesangial cells, endothelial cells and infiltrating macrophages.[1,2] Following kidney injury, resident fibroblasts are activated by various pro-inflammatory and pro-fibrotic stimuli. Activated fibroblasts, also called myofibroblasts, produce excessive ECM proteins that accumulate in the interstitium, and therefore they are considered as the key mediator of renal fibrosis.[3]

Despite the fact that most myofibroblasts are derived from local resident fibroblasts, recent studies demonstrated that these ECM-producing cells might originate from bone marrow through differentiation, and from TECs and endothelial cells via a process of epithelial-to-mesenchymal transition and endothelial-to-mesenchymal transition, respectively.[4,5] Although there is an ongoing debate about the existence of epithelial-to-mesenchymal transition *in vivo*,[6] these studies illustrated the contribution of TECs in fibrogenesis through a paracrine mechanism. TECs in co-culture with cortical fibroblasts secreted transforming growth factor-β (TGF-β) and the AB-heterodimer of platelet-derived growth factor (PDGF-AB), which in turn stimulated fibroblast proliferation and total collagen synthesis.[7] The production of insulin-like growth factor binding protein from fibroblasts was also enhanced in the presence of TEC-conditioned medium; thus, these cells could modulate the proliferative response during repair.[8]

Regardless of the primary insult leading to renal fibrosis, chronic inflammation appears to be a critical process heralding fibrogenesis. Elevated levels of inflammatory markers were associated with an increased risk of developing CKD.[9] Induction of various pro-inflammatory cytokines (interleukin (IL)-6, IL-8, IL-10, chemokine (C-C motif) ligand 2, and tumor necrosis factor-α) and adhesion molecules (intercellular adhesion molecule-1 and vascular cell adhesion molecule-1) attracted the transmigration of macrophages and T cells from the circulation to the interstitium, thereby further enhancing the inflammatory state.[10]

Toll-like receptors (TLRs) are a conserved family of pattern recognition receptors that play a fundamental role in innate immunity. TLRs mediate host cell inflammatory response by recognizing pathogen-associated molecular patterns with an extracellular domain comprising leucine-rich repeats and triggering the intracellular signal transduction through a cytoplasmic Toll/IL-1 receptor-like domain. In addition, TLRs are involved in non-infectious inflammatory disease, in which they are activated by endogenous danger-associated molecular patterns that are released from injured tissue.[11] Activation of TLR pathways has been implicated in various renal diseases, including acute kidney injury, ischemia–reperfusion injury, allograft rejection and immune complex nephritis,[12,13] in which they participate in the induction of acute inflammation and early tubular injury. Emerging evidence suggests that TLRs may participate in the pathogenesis of renal fibrosis.

THE EXPRESSION OF TLRs DURING CHRONIC KIDNEY INJURY

TLRs are important in innate immunity and are widely distributed on myeloid cells. To date, 10 human TLRs (TLR1–10) have been identified and demonstrated to initiate proinflammatory signaling pathways via the adaptor protein MyD88,[11] except TLR3, which triggers via TRIF/TICAM-1. Other TLR adaptors include TRIF (TIR-domain-containing adaptor-inducing interferon-β), TIRAP (TIR-domain-containing adaptor protein), and TRAM (TRIF-related adaptor molecule).[14] TLRs utilize these adaptor proteins to transmit signals downstream, leading to the activation of nuclear factor-κB, mitogen-activated protein kinases, JNKs (c-Jun NH2-terminal kinases), p38, ERKs and IRF (Figure 1).[15]

So far, most of the TLR studies associated with chronic renal injury have focused on TLR2 and TLR4 (Table 1). In the kidney, interstitial and glomerular macrophages express TLR1, 2, 4, and 6, and dendritic cells express TLR4, 7, 8, and 9. TLR2 and TLR4 are upregulated in monocytes, and TLR4 is upregulated in neutrophils of end-stage renal disease patients. The alteration of TLR expression in immune cells might contribute to the increased susceptibility to microbial infection and prevailing inflammation in these patients.[16] This notion is supported by the observation that TLR2 expression on monocytes was associated with the inflammatory response of patients with stage 3–4 CKD.[17]

In addition to myeloid cells, TLRs are also expressed in intrinsic renal cells. TECs and mesangial cells express TLR1, 2, 3, 4, and 6, and podocytes express TLR1, 2, 3, 4, 5, 6, and 10. Immunohistochemical studies on human renal biopsies demonstrated the upregulation of TLR2[18] in the glomerular endothelial and mesangial area and TLR4[19] expression in the tubules of patients with diabetic nephropathy (DN) compared with normal renal sections. Increased expression of TLR2 was also observed in interstitial myofibroblasts, tubules, and macrophages of the kidney sections from patients with obstructive hydronephrosis and IgA nephropathy.[20] Although TLRs are present in both immune and renal cells, it is likely that TLR signaling predominates in intrinsic renal cells rather than leukocytes. For example TLR4-deficient mice engrafted with competent leukocytes showed less tubular damage than wild-type mice reconstituted with TLR4-deficient bone marrow cells.[21]

ENDOGENOUS TLR LIGANDS RELEASED DURING CHRONIC KIDNEY INJURY

Over the last decade, numerous endogenous ligands have been identified for the activation of TLRs during kidney injury, and some of them have been shown to be closely associated with renal fibrosis.

High-mobility-group box 1 (HMGB1)

HMGB1 protein belongs to a family of non-histone chromosomal proteins that were first identified to bind to DNA and regulate gene transcription. It is also a secreted protein from activated macrophages and dendritic cells during infection and from damaged cells during tissue injury. Increasing evidence supports that extracellular HMGB1 is a key endogenous ligand of TLR signaling that is involved in the initiation of renal inflammation and the subsequent development of progressive renal fibrosis.

Both experimental and clinical models of renal fibrosis have demonstrated that a high HMGB1 level is associated with the development of progressive CKD, including unilateral ureteral obstruction (UUO) injury,[20] 5/6 nephrectomy,[22] and DN.[18,19,23] Extracellular HMGB1 binds to TLR2 and TLR4[24] to elicit inflammatory responses via a nuclear factor-κB-dependent pathway. Anti-HMGB1 treatment has been shown to attenuate the severity of sepsis in CKD.[22] HMGB1 has also been demonstrated to induce epithelial-to-mesenchymal transition in human proximal TECs.[25]

Heat shock proteins (HSPs)

HSPs were originally characterized as intracellular chaperone proteins that are involved in protein folding and stabilization. Interestingly, HSPs also interact with TLRs during the maturation of immune cells[26] and during induction and termination of cytokine secretion. HSP60 and HSP70 are the two best-known HSPs that bind to TLR2 and TLR4 in inflamed tissue. An early study on DN revealed that HSP60 and HSP70 proteins were significantly induced in lymphocytes of type 2 diabetic patients.[27] Together, the serum level of HSP60 and HSP70 was shown to correlate with the increase of TLR2 and TLR4 expression in monocytes of type 2 diabetic patients.[23] This supports the role of HSPs as endogenous ligands for TLR signaling.

ECM degradation product

Progressive renal fibrosis results in increasing matrix turnover and excessive production of ECM degradation products such as fibrinogen, biglycan, heparin sulfate, hyaluroran, and fibronectin, which have been shown to interact with TLR4.[28,29]

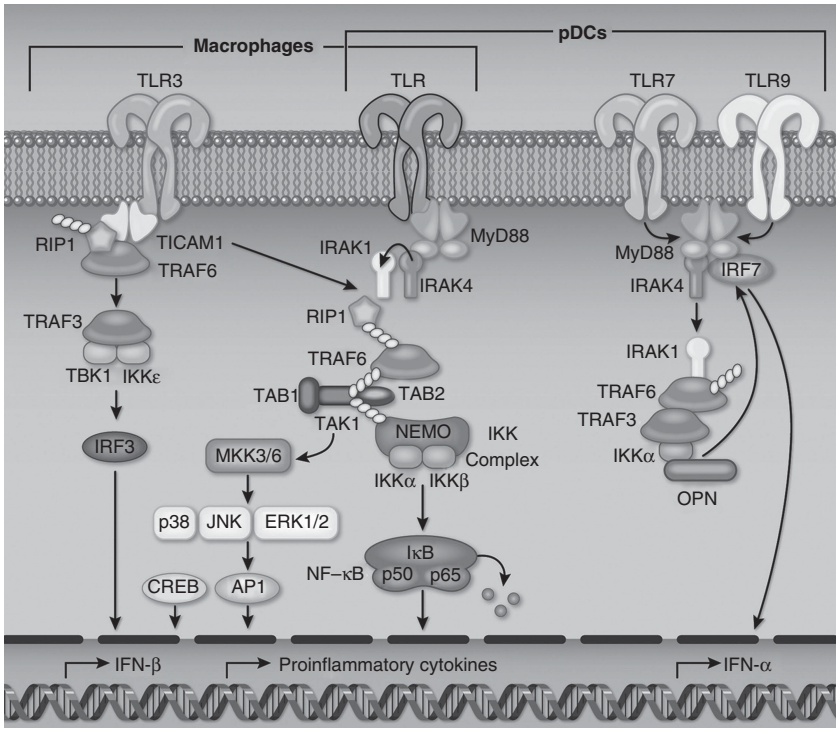

Figure 1 | MyD88-dependent and -independent Toll-like receptor (TLR) signaling pathways. All TLRs (except for TLR3) signal through the adaptor protein MyD88 to activate nuclear factor (NF)-κB and mitogen-activated protein kinases for the induction of proinflammatory cytokines. TLR3 signals through TRIF (TIR-domain-containing adaptor-inducing interferon-β)/TICAM1 (TIR-containing adaptor molecule 1). Other TLR adaptors include TRIF, TIRAP (TIR-domain-containing adaptor protein), and TRAM (TRIF-related adaptor molecule). (Reprinted with permission from Moresco *et al.*,[15] with permission from Elsevier.) IKK, inhibitor of κB-kinase; IRAK, interleukin-1 receptor-associated kinase; NEMO, NF-κB essential modulator; pDCs, plasmacytoid dentritic cells; RIP1, receptor-interacting protein-1; TAB-1, TAK1-binding protein; TAK, TGF-β activated kinase; TRAF, tumor necrosis factor receptor-associated factor.

Table 1 | TLR2 and TLR4 expression in association with chronic kidney diseases

TLR	Disease	Cell type
TLR2	ESRD	Monocyte[16]
	Stage 3-4 CKD	Monocyte[17]
	Obstructive hydronephrosis	Macrophage and myofibroblast[20]
	IgA nephropathy	Macrophage and myofibroblast[20]
	Type 1 DN	Endothelial cell and mesangial cell[18]; monocyte[41]; tubular cell[44]
	UUO	Tubular cell[20]
TLR4	ESRD	Neutrophil and monocyte[16]
	Type 2 DN	Proximal tubular cell[19]
	Type 1 DN	Tubular cell[20]; monocyte[41]

Abbreviations: CKD, chronic kidney disease; DN, diabetic nephropathy; ESRD, end-stage renal disease; IgA, immunoglobulin A; TLR, Toll-like receptor; UUO, unilateral ureteral obstruction.

THE ROLES OF TLRs IN PROGRESSIVE RENAL FIBROSIS

Unilateral ureteral obstruction

UUO is a well-characterized rodent model of progressive renal fibrosis. The diseased kidney exhibits significant inflammation, tubular injury, and even cell death at the first week of obstruction. Release of proinflammatory cytokines such as IL-1, tumor necrosis factor-α, and chemokine (C-C motif) ligand 2 from activated renal cells recruits leukocytes to the damaged tubule that in turn sustains and amplifies inflammation. In fact, secretion of TGF-β1 from both infiltrating leukocytes and intrinsic renal cells plays a crucial role at the onset of UUO as TGF-β1 induces the production of ECM proteins through the activation of fibroblasts and the process of epithelial-to-mesenchymal transition.[30]

There is strong evidence for a role of TLRs in obstructive nephropathy. TLR2 was markedly upregulated in the obstructed kidney after UUO injury.[20,31] Expression of TLR2 was rapidly increased as early as 2 days after surgery, while other TLRs (TLR3, 4, 7, and 9) were only induced 6 days after surgery. This implied the involvement of TLR2 signaling in the early phase of UUO injury. Indeed, the distribution pattern of TLR2 protein was found to be in line with that of the endogenous ligands (e.g. HMGB1 and biglycan) at the apical side of the tubule, suggesting the activation of TLR2 signaling at this site. Results from $TLR2^{-/-}$ mice studies support the idea that TLR2 initiates renal inflammation during the early stage of UUO injury because there was reduced chemokine expression (CXCL1 and chemokine (C-C motif) ligand 2) and leukocyte infiltration compared to wild type.[20] Although TLR2 has also been demonstrated to mediate fibrinogen-induced proliferation of fibroblasts,[32] there is no conclusive evidence to support the role of TLR2 in the progression of fibrosis. There was no difference in ECM accumulation

between TLR2$^{-/-}$ and TLR2$^{+/+}$ mice at the later stage of UUO although less myofibroblasts and reduced MMPs and TIMPs were shown in TLR2$^{-/-}$ mice.[20] Similar findings from a subsequent study also demonstrated that the development of postobstructive renal interstitial fibrosis and tubular atrophy was independent of TLR2-induced MyD88 signaling transduction.[31]

Expression of TLR4 was progressively upregulated after UUO injury. In contrast to TLR2, no significant difference was shown in the proinflammatory response and macrophage infiltration between TLR4$^{-/-}$ and TLR4$^{+/+}$ mice. Instead, TLR4-deficient mice were protected from renal fibrosis with reduced α-SMA protein expression and less fibroblast accumulation.[33] Both *in vivo* and *in vitro* data demonstrated that IL-18 induced pro-fibrotic changes via TLR4 signaling during UUO and in TECs,[34] suggesting the role of TLR4 as a molecular link between inflammation and fibrosis. It has been proposed that TLR4 promotes renal fibrosis by altering the susceptibility of renal cells towards TGF-β signaling. Upon stimulation by TGF-β, TECs and myofibroblasts from TLR4$^{-/-}$ mice produced less collagen type I mRNA than wild-type cells, which was later found to be associated with upregulation of Bambi, the negative regulator of TGF-β signaling.[35] On the other hand, TLR2 and TLR4 were not found to be involved in renal injury following UUO in another study, in which there was no significant difference in collagen IV deposition and macrophage infiltration between TRL2$^{-/-}$, TLR4$^{-/-}$, and wild-type mice.[36] This contradictory finding may be due to the C57BL/6 strain-dependent resistance to UUO.

The role of TLR9 is complicated in the pathogenesis of renal diseases,. On one hand, TLR9 stimulation by its ligand, CpG DNA, aggravated renal injury in mice with IgA nephropathy.[37] Conversely, TLR9-deficient MRL/1pr mice exhibited more severe glomerular and interstitial lesions compared to wild-type mice. Study on renal fibrosis in the UUO model found that TLR9 protected renal tissue from obstructive damage as mice treated with CpG-ODN showed less renal inflammation and fibrosis, which was accompanied by significant reduction in ERK, Smad3, and Stat3 activity.[38]

DIABETIC NEPHROPATHY

DN, the most common cause of end-stage renal disease in the developed world, is characterized by the thickening of glomerular and tubular basement membrane and excessive mesangial matrix expansion. Poor glycemic control is a major risk factor for the development of DN. However, it does not account for all the pathophysiological changes observed in the diseased kidney. Research in the past few years have suggested that inflammatory processes may be important in the development and progression of DN.[39]

Activation of TLR signaling contributes to increased production of proinflammatory mediators, and the sustained chronic inflammatory state associated with diabetes.[40] Clinical studies showed a significant increase in TLR2 and TLR4 protein, which correlated with the upregulation of their ligand and MyD88-dependent nuclear factor-κB expression in monocytes from patients with type 1[41,42] and type 2 diabetes.[23] In addition, stimulation of human monocytes with TLR2 and TLR4 ligands enhanced the production of different cytokines, accounting for the inflammatory state in diabetes. In human renal biopsy studies, sections from diagnosed DN patients also showed an increased expression of TLR2[18] and TLR4[19] in the tubules compared to sections from normal kidneys. The positive correlation between tubular TLR4 expression and serum HbA1c level implicated that high glucose might be a critical determinant for TLR expression. Indeed, results from an *in vitro* study confirmed that high glucose induced TLR2 and TLR4 expression in human monocytes[43] and TLR4 expression in proximal TECs[19] via a PKC-dependent pathway.

Similar to the model of UUO, TLR2 plays a pro-inflammatory role in DN. In one study, short-term exposure to high glucose for 3 days induced both TLR4 and TLR2, but only TLR2 overexpression was sustained upon prolonged exposure for up to 7 days, suggesting a predominant role of TLR2 in mediating chronic inflammation in DN.[44] An animal model of type 1 diabetes supported the progressive induction of TLR2, together with MyD88-nuclear factor-κB expression in the diabetic kidney of rats,[18] while knockout of TLR2 attenuated macrophage-mediated inflammation, albuminuria, and podocyte loss in STZ-induced DN mice.[45]

Recent evidence has suggested that activation of the TLR4 signaling pathway is associated not only with inflammation but also with insulin resistance.[46] Insulin signaling is important for normal renal function in glomeruli and tubules.[47] Mice with altered insulin signaling in podocytes developed significant albuminuria and renal injury resembling the histological features in DN.[48] Blockade of TLR4 signaling in macrophages also improved insulin resistance and reduced albuminuria in *db/db* mice. Consistent with these observations, there was reduced intrarenal expression of pro-fibrotic molecules including TGF-β, PAI-1, collagen IV, and phosphorylated smad2/3 in the diabetic kidney, while TLR4 signaling was inhibited.[49] More recently, our group has demonstrated the pro-fibrotic role of TLR4 in a model of STZ-induced DN in eNOS$^{-/-}$ mice, in which the TLR4 antagonist CRX-526 reduced renal cortical TGF-β, osteopontin production, and collagen deposition, and improved renal function.[50]

CONCLUSION

Renal fibrosis features prominently as an irreversible process of tissue damage in most forms of progressive CKD. Activation of TLR signaling, especially in intrinsic renal cells, is involved in the initiation of the innate immune response to various exogenous and endogenous danger signals. However, aberrant activation of this signaling pathway may lead to chronic inflammation and progression to renal fibrosis. There is strong evidence that TLR2 and TLR4 play distinct roles in the pathogenesis of renal fibrosis; TLR2 initiates proinflammatory responses, whereas TLR4 mediates both

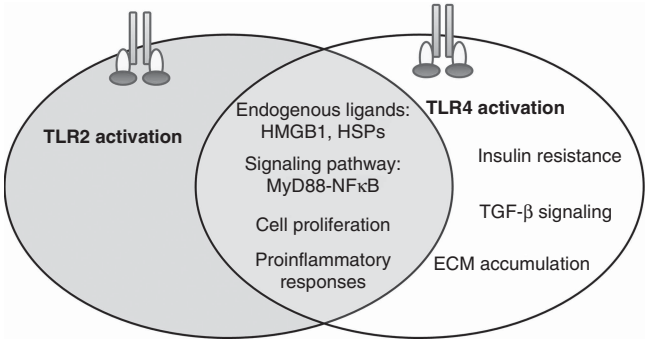

Figure 2 | Role of Toll-like receptor (TLR)2 and TLR4 in renal fibrosis. During chronic kidney injury, TLR2 initiates the proinflammatory pathway via MyD88-nuclear factor (NF)-κB signal transduction in response to endogenous ligands, while TLR4 activates both proinflammatory and pro-fibrotic pathways. ECM, extracellular matrix; HMGB1, high-mobility-group box 1; HSPs, heat shock proteins; TGF-β, transforming growth factor-β.

proinflammatory and pro-fibrotic pathways (Figure 2). Thus, targeting TLR signaling may confer a novel therapeutic paradigm for renal fibrosis and end-stage renal disease of diverse origins.

DISCLOSURE

All the authors declared no competing interests.

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