Role of Toll-like receptor-4 in renal graft ischemia-reperfusion injury

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TOLL-LIKE RECEPTORS (TLRs) are transmembrane proteins with a key role in innate immunity (61). They are members of the interleukin-1 receptor (IL-1R) superfamily and structurally related to Drosophila Toll. In general, TLRs function as pattern recognition receptors in response to infection and detect pathogen-associated molecular patterns (PAMPs), which in turn lead to the activation of innate immune defenses via intracellular signaling pathways that culminate in the release of proinflammatory cytokines and chemokines. Toll-like receptors (TLRs) are produced constitutively in renal cells and play a key role in innate immunity against invading pathogens (26, 61). In addition to recognizing pathogens, TLRs can also recognize molecules which are released by stressed and necrotic cells, as well as degraded products of endogenous macromolecules. Upregulation of TLR-4 is found in tubular epithelial cells, vascular endothelial cells, and infiltrating leukocytes during renal ischemia-reperfusion injury, which is induced by massive release of endogenous damage-associated molecular pattern molecules such as high-mobility group box chromosomal protein 1. Activation of TLR-4 promotes the release of proinflammatory mediators, facilitates leukocyte migration and infiltration, activates the innate and adaptive immune system, and potentiates renal fibrosis. TLR-4 inhibition serves as the target of pharmacological agents, which could attenuate ischemia-reperfusion injury and associated delayed graft function and allograft rejection. There is evidence in the literature showing that targeting TLR-4 could improve long-term transplantation outcomes. Given the pivotal role of TLR-4 in ischemia-reperfusion injury and associated delayed graft function and allograft rejection, inhibition of TLR-4 using pharmacological agents could be beneficial for long-term graft survival.

renal transplantation; ischemia-reperfusion injury; Toll-like receptor 4; delayed graft function; renal graft failure

TLR-4 in renal ischemia-reperfusion injury (IRI) of renal grafts. This review provides a detailed overview of the new understanding of the involvement of TLR-4 in ischemia-reperfusion injury of renal grafts and its clinical significance in renal transplantation. TLR-4 not only responds to exogenous microbial motifs but can also recognize molecules which are released by stressed and necrotic cells, as well as degraded products of endogenous macromolecules. Upregulation of TLR-4 is found in tubular epithelial cells, vascular endothelial cells, and infiltrating leukocytes during renal ischemia-reperfusion injury, which is induced by massive release of endogenous damage-associated molecular pattern molecules such as high-mobility group box chromosomal protein 1. Activation of TLR-4 promotes the release of proinflammatory mediators, facilitates leukocyte migration and infiltration, activates the innate and adaptive immune system, and potentiates renal fibrosis. TLR-4 inhibition serves as the target of pharmacological agents, which could attenuate ischemia-reperfusion injury and associated delayed graft function and allograft rejection. There is evidence in the literature showing that targeting TLR-4 could improve long-term transplantation outcomes. Given the pivotal role of TLR-4 in ischemia-reperfusion injury and associated delayed graft function and allograft rejection, inhibition of TLR-4 using pharmacological agents could be beneficial for long-term graft survival.

Expression of TLR-4 in the Kidney After IRI

One of the most detrimental factors contributing to early graft injury in renal transplantation is ischemia injury during lengthy hypothermic storage and its subsequent reperfusion injury (19, 71). Hypothermic storage of kidneys from cadaveric donors is necessary for the performance of tissue matching to optimize graft-recipient immunocompatibility and also enables the sharing and transporting of organs between transplant centers (31). During ischemia, the cytoskeleton is disrupted and polarity of tubular epithelial cells is lost. ATP exhaustion rapidly drives the conversion of monomeric G-actin to F-actin, which is an inevitable episode during kidney transplantation.

Kidney transplantation is the leading transplant type worldwide and the treatment of choice for patients with end-stage renal disease, shown to be associated with increased life expectancy, decreased morbidity, higher quality of life, and greater cost effectiveness compared with dialysis (28). However, the effects of IRI on renal transplantation, such as delayed graft function (DGF), continue to present a significant barrier to improving clinical outcomes for patients (63).

Herein, we review the evidence of the role of TLR-4 in renal IRI and its possible clinical impact on renal transplantation.

Zhao H, Perez JS, Lu K, George AJ, Ma D. Role of Toll-like receptor-4 in renal graft ischemia-reperfusion injury. Am J Physiol Renal Physiol 306: F801–F811, 2014. First published February 12, 2014; doi:10.1152/ajprenal.00469.2013.—Toll-like receptor-4 (TLR-4) has been increasingly recognized as playing a critical role in the pathogenesis of ischemia-reperfusion injury (IRI) of renal grafts. This review provides a detailed overview of the new understanding of the involvement of TLR-4 in ischemia-reperfusion injury of renal grafts and its clinical significance in renal transplantation. TLR-4 not only responds to exogenous microbial motifs but can also recognize molecules which are released by stressed and necrotic cells, as well as degraded products of endogenous macromolecules. Upregulation of TLR-4 is found in tubular epithelial cells, vascular endothelial cells, and infiltrating leukocytes during renal ischemia-reperfusion injury, which is induced by massive release of endogenous damage-associated molecular pattern molecules such as high-mobility group box chromosomal protein 1. Activation of TLR-4 promotes the release of proinflammatory mediators, facilitates leukocyte migration and infiltration, activates the innate and adaptive immune system, and potentiates renal fibrosis. TLR-4 inhibition serves as the target of pharmacological agents, which could attenuate ischemia-reperfusion injury and associated delayed graft function and allograft rejection. There is evidence in the literature showing that targeting TLR-4 could improve long-term transplantation outcomes. Given the pivotal role of TLR-4 in ischemia-reperfusion injury and associated delayed graft function and allograft rejection, inhibition of TLR-4 using pharmacological agents could be beneficial for long-term graft survival.
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Basolateral Na⁺-K⁺-ATPase pumps dissociate from the actin cytoskeleton and are redistributed (56). Anchorage of epithelial cells to the basement membrane is lost due to redistribution of integrins to the apical surface, induced by oxidative stress (24). A high level of TLR-4 expression was found in kidneys with ischemia-reperfusion, and induction of its upregulation may be caused by infiltrating macrophages as well as intrinsic renal cells (8, 97). A study by Bergler (8) demonstrated that significantly high TLR-4 expression occurs in rat allogeneic renal transplantation, which is strongly correlated with renal function of the graft.

The studies regarding TLR-4 on animal models are limited due to the technically demanding surgical procedure. However, the warm ischemia-reperfusion model provides valuable evidence for the molecular role of TLR-4 in renal graft IRI. Renal tubular epithelial cells and vascular endothelial cells are the key cell types for the biological action of TLR-4.

Tubular epithelial cells. Enhanced TLR-4 expression was observed in tubular epithelial cells in ischemic rat renal grafts 24 h after transplant surgery (97) (Fig. 1). TLR-4 is constitutively expressed by proximal tubular cells, but 24 h after reperfusion it is upregulated in the cortex and outer medulla while TLR-4 can be detected in the medullary thick ascending limb and distal tubules 3–5 days following reperfusion (11). Pulskens et al. (66) used bone marrow transplantation to create chimeric mice, and they found that wild-type mice reconstituted with TLR-4-deficient bone marrow had a similar degree of dysfunction compared with mice reconstituted with wild-type bone marrow. This suggested that TLR-4 expression on parenchymal cells and on leukocytes may equally contribute to injury. However, this is not supported by the work reported by Wu et al. (88), in which TLR-4 mRNA levels are upregulated in tubular epithelial cells subjected to ischemia compared with nonischemic controls. They performed a more rigorous bone marrow chimera experiment but concluded that functional TLR-4 on kidney parenchymal cells made the more significant contribution to renal IRI compared with that on leukocytes. The discrepancies in the results of these two studies require further in-depth investigation with more vigorous molecular approaches to elucidate the potential role of TLR-4 on renal parenchymal and infiltrating leukocytes.

Vascular endothelial cells. It has been shown that the high-mobility group box chromosomal protein 1 (HMGB-1), a ligand of TLR-4, was released from injured renal cells and then bound to endothelial TLR-4, and this increased the expression of proinflammatory adhesion molecules (11, 12). The absence of endothelial TLR-4 inhibited upregulation of adhesion molecules and ameliorated inflammation and injury. This indicated that TLR-4 triggered the endothelial activation which is necessary for inflammation during ischemic injury (13). In addition, TLR-4 expression on renal endothelial cells was affected by reactive oxygen species and cellular stress with the insult of hydrogen peroxide (11).

Endogenous Ligands for TLR-4 During Renal Ischemia-Reperfusion

TLR-4 was first recognized for its specific binding to LPS, a major cell wall component of gram-negative bacteria (26). Now, it is accepted that TLR-4 not only responds to exogenous microbial motifs but can also recognize molecules which are released by stressed and necrotic cells, as well as degraded products of endogenous macromolecules (78). The “surveillance model” proposes that, apart from its role in pathogen recognition, TLR-4 can act as a monitoring receptor implicated in the detection of tissue injury (37). Proposed endogenous ligands for TLR-4 that are upregulated during ischemia-reperfusion include HMGB-1, extracellular matrix (ECM) components, and heat-shock proteins (HSPs) (Table 1). However, there was concern that studies of the endogenous ligands could be confounded by contamination with LPS in these animal models. Transgenic techniques have been proven to be a useful tool in resolving this issue; for example, animals or cells deficient in these TLR-4 ligands have shown altered progression of injury and provided the direct evidence supporting their crucial association with the TLR-4 pathway (38, 42, 92).

HMGB-1. HMGB-1 is a highly conserved nuclear factor with functions in nucleosome stabilization and promotion of DNA transcription. It contains three domains: box A, box B (both homologous DNA-binding motifs), and a negatively charged C terminus (2). HMGB-1 is predominantly located in the nuclei of most cells. HMGB-1 can also be released from the nucleus into the cytoplasm and extracellular milieu by both passive release from injured cells and active secretion by immune cells (30, 53). Extracellular HMGB-1 serves a different function, acting as a proinflammatory cytokine that mediates the inflammatory response to injury via various activations of receptors, including TLR-4 (30, 54).

Fig. 1. Ischemia-reperfusion induced Toll-like receptor 4 (TLR-4) expression on renal cortex in transplanted grafts. Left: schematic diagram of TLR-4 pathway. Right: a Lewis rat kidney was stored in 4°C Soltran preserving solution for 24 h (cold ischemia 24 h) and then transplanted into a Lewis rat recipient; the graft was harvested 24 h after transplantation (warm reperfusion 24 h). The normal kidney serves as a naive control (NC). TLR-4 was expressed on the tubular surface (red fluorescence; cell nuclei were counterstained with DAPI). In NC kidney, TLR-4 expression was detectable through an immunofluorescence technique but weak. In the transplanted renal graft after renal ischemia-reperfusion injury (rIRI), the expression of TLR-4 was significantly upregulated. Scale bar = 50 μm. HMGB-1, high-mobility group box chromosomal protein 1; HSPs, heat shock proteins ECM, extracellular matrix.
Table 1. Recent publications of TLR-4 and its ligands in renal transplantation

<table>
<thead>
<tr>
<th>TLR 4 and Ligands</th>
<th>Reference</th>
<th>Year</th>
<th>Model</th>
<th>Major Findings</th>
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<tr>
<td>TLR-4</td>
<td>Andrade-Oliveira et al. (3)</td>
<td>2012</td>
<td>Human renal transplantation</td>
<td>Expression levels of TLR-4 and MYD88 were higher in kidneys from deceased donors than from living donors.</td>
</tr>
<tr>
<td>TLR-4</td>
<td>Kwon et al. (43)</td>
<td>2008</td>
<td>Human renal transplantation</td>
<td>TLR-4 mRNA expression was increased in renal allograft patients with chronic allograft dysfunction.</td>
</tr>
<tr>
<td>TLR-4</td>
<td>Lim et al. (50)</td>
<td>2005</td>
<td>Rat renal transplantation</td>
<td>Enhanced TLR-4 mRNA and protein expression on renal tubular cells in long-term rat renal grafts.</td>
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<td>HMGB-1</td>
<td>Zhao et al. (97)</td>
<td>2013</td>
<td>Rat renal transplantation</td>
<td>Suppression of cytoplasmic translocation of HMGB-1 protected renal grafts against ischemia-reperfusion injury.</td>
</tr>
<tr>
<td>HMGB-1</td>
<td>Zhao et al. (96)</td>
<td>2013</td>
<td>Rat renal transplantation</td>
<td>Suppression of the release of HMGB-1 protected the renal grafts against ischemia-reperfusion injury.</td>
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<td>HMGB-1</td>
<td>Kruger et al. (41)</td>
<td>2013</td>
<td>Human renal transplantation</td>
<td>Enhanced expression of HMGB-1 in ischemic renal grafts.</td>
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<td>HSP70</td>
<td>Kim et al. (38)</td>
<td>2005</td>
<td>Rat renal transplantation</td>
<td>Ischemia-reperfusion injury increased TLR-2 and TLR-4 mRNA and protein expression. Production of endogenous TLR ligand HSP70 on renal tubular cells was enhanced.</td>
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<tr>
<td>HSP70</td>
<td>Zhao et al. (98)</td>
<td>2013</td>
<td>Rat renal transplantation</td>
<td>Enhanced expression of HSP70 protected kidney grafts against ischemia-reperfusion injury.</td>
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<tr>
<td>Biglycan</td>
<td>Kruger et al. (41)</td>
<td>2013</td>
<td>Human renal transplantation</td>
<td>Enhanced expression of biglycan in ischemic renal grafts.</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>Snoeij s et al. (73)</td>
<td>2010</td>
<td>Human renal transplantation</td>
<td>Enhanced production of heparan sulfate, which could mediate acute ischemic injury to the renal microvasculature.</td>
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<tr>
<td>Heparan sulfate</td>
<td>Ali et al. (1)</td>
<td>2005</td>
<td>Human renal transplantation</td>
<td>Expression of heparan sulfate was increased significantly during allosimmune response.</td>
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<tr>
<td>Hyaluronan/hyaluronic acid</td>
<td>Tuuminen et al. (79)</td>
<td>2013</td>
<td>Rat renal transplantation</td>
<td>Inhibition of hyaluronan induction prevented activation of innate and adaptive immune responses and protected the renal grafts.</td>
</tr>
<tr>
<td>Hyaluronan/hyaluronic acid</td>
<td>Zhang et al. (94)</td>
<td>2000</td>
<td>Rat renal transplantation</td>
<td>Blocking cell-cell interaction through hyaluronic acid interaction significantly prolonged rat allograft survival.</td>
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</tbody>
</table>

TLR, Toll-like receptor; HMGB-1, high-mobility group box chromosomal protein 1; HSP, heat shock protein.

Wu et al. (89) confirmed that HMGB-1 expression is upregulated after kidney IRI: it is secreted by necrotic or damaged tubular epithelial cells and acts as a DAMP. Apoptotic cells fail to activate monocytes due to HMGB-1 binding to chromatin. However, if this is suppressed by trichostatin A, HMGB-1 is able to act as an extracellular potent proinflammatory cytokine (6). This role has been confirmed in human kidney (HK)-2 cells. Treatment with recombinant HMGB-1 (rHMGB-1) leads to increased mRNA levels of cytokines involved in the pathology of IRI (41). HMGB1-induced cytokine upregulation reaches a similar level to that provoked by LPS treatment, suggesting the involvement of TLR-4 signaling. Administration of anti-HMGB1 antibody or rHMGB-1 to tubular epithelial cells from TLR-4-deficient mice, which block or augment HMGB-1 activity, respectively, does not result in further protection or damage in cells (89). It was therefore concluded that HMGB-1 mediates renal injury via TLR-4. Similarly, when HK-2 cells are stimulated with rHMGB-1, cytokine production is markedly blunted in cells with TLR-4 knockdown by transfection with TLR-4-specific small interfering (si) RNA compared with those transfected with nonspecific siRNA (41). Rat renal grafts have increased HMGB-1 release from nuclei and enhanced TLR-4 expression on renal tubules (97).

ECM components. Biglycan is a small leucine-rich proteoglycan that consists of a core protein and two glycosaminoglycan (GAG) chains, chondroitin and dermatan sulfate. From day 1 to day 5 after reperfusion, biglycan mRNA levels are significantly increased in mice tubular epithelial cells (88). The role of biglycan as a TLR-4 endogenous ligand was investigated by Schaefer et al. (72), who showed that biglycan was unable to promote the expression of TNF-α in TLR-4-deficient mutant macrophages in contrast to wild type.

Heparan sulfate is a polysaccharide that has been proposed to activate TLR-4. Johnson et al. (36) concluded that it promotes dendritic cell (DC) maturation in a TLR-4-dependent manner, since this process is blocked in C57BL/10ScNcr mice that have a deletion in chromosome 4 containing the TLR-4 locus.

Intracellular enzymes released during cellular rupture and tissue injury can cleave proteoglycans into smaller soluble polysaccharides. Using human embryonic kidney (HEK) 293 cells transfected with TLR-4 expression plasmids, Okamura et al. (60) elucidated that fibronectin fragments containing the alternatively spliced exon coding for the extra domain A (EDA) can bind and activate TLR-4. EDA is capable of interacting with TLR-4 while other recombinant fragments or alternatively spliced exon coding for the extra domain B (EDB) fail to trigger cytokine and metalloproteinase secretion. Like LPS, EDA requires an accessory protein (MD-2) to elicit a maximal response.

Hyaluronan is a GAG that can be rapidly degraded in sites of inflammation to produce low-molecular-weight fragments of soluble hyaluronan (sHA). These are able to activate macrophages and DCs. Termeer et al. (76) deduced that this process is TLR-4 dependent since the administration of anti-TLR-4 antibodies blocked DC maturation and TNF-α production.
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HSPs. HSPs are molecular chaperones that facilitate the folding of proteins into their precise functional conformation and can be released from necrotic cells in the kidney. Their contribution to IRI has been observed in vital organs including the kidney: HSP60 and HSP70 activate TLR-4 on cardiomyocytes that are still viable in the ischemic area after reperfusion as well as on other immune cells (14). Endogenous HSP60 can be recognized by TLR-4 expressed on antigen-presenting cells (APCs) after being internalized via receptor-mediated endocytosis to activate APC function (80). TLR-4 is required for responsiveness to HSP70, as shown using HEK fibroblasts 293T (81). Interestingly, Wu et al. (88) found however that HSP70 mRNA levels in mice kidneys after ischemia-reperfusion remain no different from those in the sham-operated controls. This is different from HMGB-1, which showed that both active production and passive release were enhanced during renal IRI (88, 89). This indicated that HMGB-1 and HSPs might behave differently during ischemia-reperfusion injury. This certainly warrants more studies to further clarify the underlying molecular mechanisms.

The exact role of HSP70 in renal cell injury is still being debated. On the one hand, it is a TLR-4 ligand that initiates the inflammatory pathway which could lead to cell injury. Ben et al. (7) showed that heat shock protein-70 gp96 interacted with TLR-4 on renal tubular epithelial cells and this interaction was associated with hypoxia-induced apoptosis. On the other hand, overexpression of HSP70 has also been shown to be protective against renal injury through activating prompt repair and survival mechanisms. A study by Wang et al. (86) demonstrated that HSP70 promoted proximal tubule epithelial cell survival after acute ischemia caused by bilateral renal pedicle occlusion. HSP70 regulates the activity of Akt and GSK3β and reduces Bax activation after ischemia. Consistent with these findings, our recent work (98) demonstrated that exposure of xenon, an anaesthetic gas, greatly enhanced the expression of HSP70 and conferred significant protection to renal allografts against ischemia-reperfusion injury and associated acute rejection.

**Tamm-Horsfall protein.** Tamm-Horsfall protein (THP) is a glycoprotein which can modify TLR-4 expression and is found exclusively in the thick ascending limbs (18). It aggregates in casts formed during acute kidney injury, suggesting that it is responsible for tubular cast formation and tubular obstruction in acute kidney injury (87). It was found that THP could activate TLR-4 and enhance TNF-α production (70), indicating that THP might promote inflammation and enhance renal injury. However, contradictory evidence rendered the exact role of THP to be elusive. THP has been shown to have a renoprotective role: THP knockout mice kidneys manifested greater cellular necrosis, histological damage, and renal dysfuction after ischemic and reperfusion compared with wild-type mice (18). THP may regulate TLR-4 localization in tubular epithelial cells, perhaps promoting a more apical and less basolateral distribution, thereby reducing the interaction of TLR-4 with proinflammatory interstitial ligands (18).

**TLR-4 Signaling Pathways**

Ligand binding activates TLR-4, leading to downstream signaling via the Myd88-dependent and Myd88-independent signaling cascades. TLR-4 signaling triggered by different ligands can involve multiple components of these pathways. For example, biglycan activates p38 (72), while HSP60s activate p38, Jun N-terminal kinases (JNK1, JNK2), and the IκB (inhibitor of NF-κB) kinase (IKK) complex (80). sHA utilizes the p38/p42–44 pathway (76). Generally speaking, TLR-4 pathways could be divided into a Myd88-dependent pathway and Myd88-independent pathway (Fig. 2).

**Myd88-dependent pathway.** Myd88 and an additional TIR domain-containing adaptor protein (TIRAP or MAL) recruit and activate members of the IL-1 receptor-associated kinase (IRAK) family (90). Wu et al. (88) consider this to be the dominant pathway mediating TLR-4-associated with kidney IRI since MyD88−/− mice were protected against kidney dysfunction and therefore targeting the Myd88-dependent pathway would serve as an effective strategy against renal IRI. Li et al. (48) observed that Myd88 knockout mice exhibit not only less tubular damage but also reduced TLR-4 mRNA levels, implying a feedback relationship between Myd88 and TLR-4.

**Myd88-independent pathway.** This pathway is mediated by TIR domain-containing adaptor-inducing interferon-β (TRIF) (78). Another adaptor molecule, TRIF-related adaptor molecule (TRAM), has been identified to provide the specificity for TLR-4 signaling. TRAM-deficient mice did not express the gene encoding IFN-β, so it was concluded that this molecule is required for the Myd88-independent pathway (91). Fitzgerald et al. (23) reported that two noncanonical IKK homologs downstream of TRIF, IKKe and TANK-binding kinase-1 (TBK1), are important for this pathway.

**Effects of TLR-4 Activation in Ischemia-Reperfusion of the Renal Graft**

Activation of TLR-4 results in profound effects on renal grafts, including promoting injury to the tubular cells and sustaining the robust inflammation and fibrosis (Fig. 3).

**Promoting release of proinflammatory mediators.** The TLR-4-mediated cytokine cascade is triggered by the transcription of inflammatory genes. Cytokine release begins during the ischemic phase and is amplified upon reperfusion. Wu et al. (88) assert that the main proinflammatory cytokines upregulated following IRI are IL-6, IL-1β, and TNF-α. This is accompanied by increased expression of macrophage inflammatory protein-2 (MIP-2) and monocyte chemoattractant protein-1 (MCP-1), chemokines involved in the recruitment of neutrophils and macrophages, respectively. It has been demonstrated in experiments with TLR-4-deficient animal IRI models that this upregulation is attenuated, TNF-α is absent, and that there is limited increase in IL-6, IL-1β and MCP-1 protein levels compared with in wide-type mice (88).

**Facilitating leukocyte migration and infiltration.** Vascular endothelial activation is marked by the expression of adhesion molecules, which facilitate leukocyte migration and infiltration. Leukocyte rolling is mediated by three transmembrane receptors: E-selectin, P-selectin, and L-selectin. Firm adhesion following rolling is mediated by binding of ICAM-1 on endothelial cells to lymphocyte function-associated antigen-1 (LFA-1 or CD11a/CD18) present on leukocytes. This facilitates diapedesis and leukocyte trafficking into the renal interstitium (44, 46).

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Chen et al. (11) showed that TLR-4 signaling is a requirement for the expression of ICAM-1, VCAM-1, and E-selectin. They demonstrated increased expression of adhesion molecules during ischemic kidney injury was absent in TLR-4 knockout endothelia, and transgenic knockout of TLR-4 ameliorates ischemic injury and inflammation.

**Activating innate and adaptive immune system.** TLR-4 was widely expressed on immune cells in the innate immune system, and ligand binding to TLR-4 expressed on infiltrating leukocytes leads to cellular activation. Neutrophils release additional reactive oxygen species, secrete proteases, and can obstruct the renal microvasculature; hence they are regarded as the primary mediators of tissue injury (33). Infiltrating monocyte/macrophages release proteolytic enzymes and inflammatory mediators including TNF-α, IL-1β, and IFN-γ (35). Local TLR-4 activation by endogenous ligands connected graft damage and subsequent cytokine/chemokine release, leading to activation of innate immunity (8, 41). It has been shown that neutrophil and macrophage infiltration was greatly reduced in TLR-4 knockout mice with renal IRI (88). Moreover, the innate immune system induces adaptive immune responses via antigen presentation and enhances neutrophil infiltration. It is naturally accepted that suppressing innate immunity through inhibiting TLR-4 might agitate the adaptive immune system to a less extent and promote the graft acceptance or tolerance.

**Dendritic cells (DCs)** are APCs known to link the innate and adaptive immune responses. In kidney transplantation, donor renal DCs (rDCs) present graft peptides, migrate to draining lymph nodes, and present the antigen to T cells, leading to activation of T cells. Activated T cells then produce cytokines and chemokines, attracting more immune cells to the graft site, leading to graft rejection. Therefore, inhibiting TLR-4 signaling in rDCs might prevent their activation and reduce graft rejection.

**Fig. 2. Myeloid differentiation primary-response protein 88 (MyD88)-dependent and MyD88-independent TLR-4 signaling pathway.** In the MyD88-dependent pathway, activation of TLR-4 through TLR-4 ligand recruits and activates MyD88 and then IL-1 receptor-associated kinase (IRAK)-4. IRAK-4 phosphorylates IRAK-1, which recruits TNF-receptor-associated factor 6 (TRAF6). TRAF6, ubiquitin-conjugating enzymes (UEV1A, UBC13) and binding proteins (TAB1, TAB2) interact to activate TGF-β-activated kinase 1 (TAK1). Activation of TAK1 leads to the activation of the inhibitor of nuclear factor-κB kinase (IKK) complex, which releases NF-κB from its inhibitor and promotes its translocation into the nucleus. NF-κB is then translocated into the nucleus and initiates gene expression. In the MyD88-independent signaling pathway, TLR-4 activation leads to TRIF activation. TRIF recruits IKKε, which possibly forms a complex with TBK1 to activate transcription factors NF-κB and IFN-regulatory factor 3 (IRF-3). Gene expression is then initiated.
lymphoid tissue, and activate alloreactive T cells. Allopeptides derived from their MHC molecules can also be presented on recipient DCs (47). Furthermore, DCs play a major role in the early pathophysiology of IRI by mediating cytokine production. CDC11c+/H11001 cells (most likely DCs) secrete TNF, CCL5, IL-6, and MCP-1, while TNF production within 24 h of IRI is predominantly driven by the F4/80+/H11001 DC population, the “first responders” in the inflammatory response (16). Moreover, TLR-4 has been reported to mediate proinflammatory dendritic cell differentiation in humans and promote induction of co-stimulatory molecule expression (40). It was also suggested that TLR-4 induces dendritic cells with fully mature phenotypes, which prime CD8+ T cells more efficiently (64).

Sustaining tubular necrosis and potentiating renal fibrosis. The generation and release of TLR-4 ligands from injured tubular cells cause activation and upregulation of TLR-4, which in turn induce a robust inflammatory responses and cause widespread tubular necrosis, loss of the brush border, cast formation, and tubular dilatation at the corticomedullary junction (88). Robust inflammation is known to potentiate tissue fibrosis (52). A recent study (39) has established that infiltrating macrophages mediated persistent inflammation and fibrosis after IRI. Constantly increased expression of profibrotic protein TGF-β1 was found in kidneys several weeks after the initiation of IRI, and monocyte-macrophage depletion suppressed the increase. Furthermore, it has been demonstrated that TLR-4 initiates an exaggerated proinflammatory response during renal IRI, and lower levels of infiltrating cells were found in TLR4−/− mice compared with wild-type mice (66).

The association between TLR-4 and fibrosis in the setting of renal graft ischemia-reperfusion might be the case, but supporting evidence is limited. It was demonstrated that TLR-
MyD88- and TRIF-deficient mice recipients showed a significant reduction in fibrosis (84) in chronic allograft nephropathy, reflected by significantly reduced α-smooth muscle actin (α-SMA) and collagen I and III accumulation. The deposition of fibrinogen (8), a fibrotic marker, in renal allografts correlated well with renal TLR-4 expression, indicating that activation of TLR-4 leads to induction of renal fibrosis.

Studies in other fibrotic renal disease models could provide further evidence supporting the potential role of TLR-4 in the fibrotic pathway. Campbell et al. (10) demonstrate a novel role for TLR-4 signaling in obstruction-induced renal fibrogenesis as such mice with intact TLR-4 signaling demonstrate a significant increase in TLR-4 expression, α-SMA expression, fibroblast and collagen accumulation, and interstitial fibrosis after unilateral ureteral obstruction. These pathological features were not found in TLR-4-deficient mice. Similarly, it was revealed that renal fibrosis is clearly attenuated in TLR-4 knockout mice after unilateral ureteral obstruction, with reduced collagen deposition in the kidney (65).

However, a final conclusion is difficult to draw since chronic inflammation is always associated with repeated repair and connective tissue accumulation in vital organs. It was demonstrated that TLR-4 enhanced inflammatory signaling and activation of fibrosis in the kidneys (8, 10), suggesting that TLR-4 could function as a molecular link between proinflammatory and profibrogenic signals in renal tissue. However, it is questionable whether TLR-4 alone could induce fibrogenesis independently of inflammation. It has been demonstrated in a model of systemic sclerosis that fibroblasts produced profibrotic chemokines such as MCP-1 in a TLR-4-dependent manner (22). Furthermore, a study by Pulskens et al. (65) showed TLR-4-deficient primary tubular epithelial cells and myofibroblasts produced significantly less type I collagen mRNA after TGF-β stimulation than did wild-type cells. This indicated that, in addition to sustaining the inflammation necessary for fibrosis, TLR-4 promoted renal fibrosis through enhancing the susceptibility and responsiveness of renal cells to TGF-β.

**Allograft Rejection**

The association of renal mRNA TLR-4 expression with allograft rejection in renal transplant recipients has been investigated by Kwon et al. (43), who found an increased TLR-4 expression in those patients with acute rejection and chronic rejection compared with control patients. A further study by Braudeau et al. (9) investigated renal transplant recipients with chronic rejection and transplant glomerulopathy. Renal grafts from patients with chronic rejection were found to express greater TLR-4 mRNA than grafts with stable function and normal histology, suggesting a detrimental effect of increased TLR-4 expression on transplant outcome.

An elegant study by Hwang et al. (32) evaluated the possible association between the TLR-4 and TLR-3 polymorphisms of donor-recipient pairs and acute rejection after living donor kidney transplantation. A significant difference was found in the genotype distributions of both recipient and donor TLR-4 between the control and acute rejection groups. These findings implied the importance of TLR-4 in the pathogenesis of acute rejection in kidney transplantation.

Several studies have investigated the role of TLR-4 in allograft rejection after renal transplantation by looking at the effects of the Asp299Gly and Thr399Ile TLR-4 gene polymorphisms. Studies by Ducloux et al. (17) and Nogueira et al. (59) have demonstrated that kidneys transplanted to recipients with TLR-4 polymorphisms have been shown to manifest fewer acute rejection episodes than those from donors with wild-type TLR-4 (although the findings of the latter study did not achieve statistical significance). Although allograft rejection was explored in these studies, the results highlighted the potential difference in the contribution of donor and recipient to renal graft IRI, since the severity of renal graft IRI correlated strongly with the occurrence of acute immune rejection and later graft failure (98). The bone marrow chimera findings (66, 88) described previously should be more supportive in providing an explanation in this case, and they implied that donor graft parenchymal TLR-4 may have a different level of contribution compared with TLR-4 on infiltrating leukocytes derived from recipient bone marrow. One study, however, demonstrated that transplants from cadaveric donors with either of the polymorphisms (not the recipients) exhibited lower rates of acute rejection (62). Interestingly, this reduction in acute rejection did not translate into increased long-term survival, perhaps due to small sample size and short-term follow-up. Another study by Fekete et al. (20) investigating long-term survival did find, however, that the Asp299Gly (D299G) TLR-4 polymorphism occurred more frequently in patients with 15-yr long-term survival after transplantation compared with those with graft loss due to acute rejection. Consistent with this finding, a recently published study (21) shed light on the mechanism of inhibition of TLR-4 signaling by the Asp299Gly (D299G) polymorphism. Human embryonic kidney cells transfected with D299G TLR-4 exhibited impaired LPS-induced activation of NF-κB, whereas other mutations of TLR-4, such as Thr(399)Ile (T399I) are not associated with significant inhibition. Contrary to wild-type TLR-4, mouse macrophages with expression of the TLR4D299G mutants are unable to elicit LPS-mediated induction of TNF-α and IFN-β mRNA.

Mutlubas et al. (58) also investigated the role of TLR-4 polymorphisms in chronic allograft nephropathy (CAN; progressive decline in renal function with proteinuria and hypertension) in a cohort of pediatric renal transplant patients. In this study, it was found that the Thr399Ile (T399I) polymorphism and Ile allele were only present in those recipients that did not manifest CAN and that patients who developed CAN did not carry this genotype, although these results were not statistically significant. Although this study primarily investigated chronic allograft nephropathy, it provides important indications for renal graft IRI since graft IRI is a critical factor for the development of CAN (95). All of these studies taken together strongly suggest that reduced TLR-4 signaling may have beneficial effects on clinical outcomes after kidney transplantation.

**Therapeutic Interventions Targeting TLR-4**

Several previous studies showed the renoprotective outcome of TLR-4 knockout. TLR-4−/− mice were protected against the effects of renal IRI; they demonstrated preserved renal function, reduced numbers of infiltrating neutrophils...
and macrophages, and less tubular damage compared with WT mice (66). It is important to note that TLR-2 has also been implicated in IRI. TLR-2-targeted deletions provide renoprotection in mice (84) due to the potent effects of TLR-2 signaling on immune system activation (84, 85). However, a previous study (69) using hypoxic tubular epithelial cells demonstrated that deletions of both the TLR-4 and TLR-2 locus did not give any further protection compared with the single deletion of either receptor. Therefore, proteins that block ligand binding to TLR-4 or target downstream signaling components may have therapeutic potential.

Mice treated with anti-HMGB-1-neutralizing antibody exhibit diminished tubular damage in kidney IRI models compared with untreated animals. Reduced levels of IL-6, TNF-α, and MCP-1 mRNA attenuated leukocyte infiltration and reduced apoptosis of TECs are observed characteristics in mice treated before and soon after ischemia, and following reperfusion (89). Recently, adenylyl cyclase-activating polypeptide (PACAP) has been shown to be promising in treating renal IRI by modulating TLRs (38). PACAP belongs to the vasoactive intestinal polypeptide (VIP)-glucagon-growth hormone releasing factor-secretin superfamily (82). It is an endogenous pleiotropic peptide with renoprotective effects through inhibiting innate immune responses (25) and oxidative stress (83). PACAP has been shown to inhibit TLR-4 through suppressing both Myd88-dependent and -independent pathways (34, 48, 75).

TLR-4 inhibitors are under development, since a direct receptor blockade would potentially offer greater protection than targeting individual ligands and downstream effectors with redundant functions (41). Eritoran, a synthetic structural analog of the lipid A portion of LPS, has recently been identified as a potent TLR-4 antagonist in rat IRI models (51). Further investigation of endogenous TLR-4 negative regulators can lead to the development of new therapeutic approaches for IRI. For example, intracellular Toll-interacting protein (TOL-LIP) can associate with TLR-4 and suppress the phosphorylation and activity of IRAK (93). The RP105-MS-1 complex specifically inhibits TLR-4-signaling in HEK 293 cells and DCs (15). Single immunoglobulin interleukin-1 receptor-related protein (SIGIRR) associates with TLR-4 and forms a complex with Myd88, IRAK4, IRAK, and TRAF6 to inhibit signaling (67). Other regulators include IRAKMK, IRAK2c, IRAK2d, soluble IRAK-4, A20, and TNF-related apoptosis-inducing ligand receptor (TRAILR) (49). A study by Gu et al. (29) showed the α2-adrenoceptor agonist dexmedetomidine protected the mice against ischemia-reperfusion induced kidney injury, dexmedetomidine reduced plasma HMGB-1 elevation and also decreased TLR-4 expression in tubular cells. Prior posttreatment with dexmedetomidine improved tubular architecture and function following renal ischemia. Recently, we have demonstrated that xenon exposure to either donor or posttreatment with dexmedetomidine improved tubular architecture and function following renal ischemia. Recently, we have demonstrated that xenon exposure to either donor or recipient led to activation of a range of protective proteins. This treatment diminished cytoplasmic translocation of HMGB-1 and suppressed TLR-4/NF-κB activation, consequently, DGF was attenuated and graft survival was enhanced (97). Furthermore, this treatment remarkably attenuated alloimmune responses associated with IRI in renal allografts and conferred optimal protection when combined with cycloporsine A (98).

**Clinical Implications for TLR-4 Expression After IRI in Renal Grafts**

The principal clinical manifestation of renal ischemia-reperfusion in kidney transplantation is DGF (77), a form of acute renal failure that leads to posttransplantation oliguria (63) and is associated with increased acute rejection episodes, graft loss (57), prolonged hospitalization, greater complexity of management, and higher cost (68). As allografts from deceased-donors are exposed to longer periods of ischemia, they are associated with a higher incidence of DGF compared with allografts from live donors (63). A study by Gok et al. (27) showed that the greatest amount of free radical generation and tissue injury at reperfusion was observed in transplants from non-heart-beating donors (NHBD) compared with those from heart-beating donors (HBD) and live donors (LD). Preclinical studies have aimed to establish the contribution of donor and recipient TLR-4 to graft injury. For example, a study by Krüger et al. (41) analyzing biopsies from kidney grafts exposed to cold ischemia showed that TLR-4 expression is notably higher in the proximal and distal tubular cells of deceased-donor kidneys compared with living-donor ones. Mutations in the TLR-4 gene leading to altered receptor function may affect the outcome of transplantation. Asp299Gly and Thr399Ile are two cosegregating missense single nucleotide polymorphisms (SNPs) in the TLR-4 gene that are associated with reduced inflammatory responses to LPS (4). DGF was less frequent in recipients of kidneys from TLR-4-mutant donors. This was possibly due to a blunted expression of MCP-1 and TNF-α, as well as an increased expression of the anti-inflammatory and protective heme oxygenase-1 gene (41). Furthermore, renal graft IRI is positively associated with development of human allograft rejection (55) and development of chronic graft failure (45). All these different types of injury are associated with TLR-4, and the results obtained from the animal models of acute ischemic injury are of vital relevance to these clinical outcomes.

**Conclusions**

There is strong evidence that TLR-4 has a detrimental role in kidney IRI as it triggers an inflammatory and maladaptive immune response that aggravates tissue injury. Following ischemia-reperfusion, endogenous ligands are released that interact with TLR-4 expressed by vascular endothelial cells, tubular epithelial cells, and leukocytes, the latter of which migrate into the graft as a result of the expression of endothelial adhesion molecules (which also results from TLR-4 signaling). Now that studies have demonstrated that the full development of kidney ischemia-reperfusion is TLR-4 dependent, treatments targeting upstream and downstream components of TLR-4 signaling should be further investigated to improve outcomes after renal transplantation. Given the pivotal role of TLR-4 in IRI and associated delayed graft function and allograft rejection, it is naturally reasoned that TLR-4 inhibition could serve as the target of pharmacological agents directed toward optimizing renal graft survival.

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AUTHOR CONTRIBUTIONS

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Toll-like receptor-4 coordinates the innate immune response of the kidney to renal ischemia/reperfusion injury.

HSP70 promotes fibrosis but attenuates tubular damage in progressive renal injury.

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