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Kidney proximal tubular TLR9 exacerbates ischemic acute kidney injury

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Abstract

The role for kidney TLR9 in ischemic acute kidney injury (AKI) remains unclear. Here, we tested the hypothesis that renal proximal tubular TLR9 activation exacerbates ischemic AKI by promoting renal tubular epithelial apoptosis and inflammation. To test this hypothesis, we generated mice lacking TLR9 in renal proximal tubules ($TLR9^{fl/fl}$ PEPCK Cre mice). Contrasting previous studies in global TLR9KO mice, mice lacking renal proximal tubular TLR9 were protected against renal ischemia reperfusion (IR) injury with reduced renal tubular necrosis, inflammation (decreased pro-inflammatory cytokine synthesis and neutrophil infiltration) and apoptosis (decreased DNA fragmentation and caspase activation) when compared to wild type ($TLR9^{fl/fl}$) mice. Consistent with this, a selective TLR9 agonist ODN-1668 exacerbated renal IR injury in $TLR9^{fl/fl}$ mice but not in renal proximal tubular TLR9 null mice. Furthermore, in cultured human and mouse proximal tubule cells, TLR9 selective ligands induced NFκB activation, pro-inflammatory cytokine mRNA synthesis as well as caspase activation. We further confirm here that global TLR9 deficiency had no impact on murine ischemic AKI. Taken together, our studies show that renal proximal tubular TLR9 activation exacerbates ischemic AKI by promoting renal tubular inflammation, apoptosis as well as necrosis after IR via NFκB and caspase activation. Our studies further suggest complex nature of TLR9 activation as renal tubular epithelial TLR9 promote cell injury and death whereas TLR9 signaling in other cell types may promote cytoprotective effects.

Keywords

Apoptosis; inflammation; ischemia and reperfusion injury; necrosis; NFκB; neutrophil

Introduction

Acute kidney injury (AKI) is a major clinical problem (1). Renal ischemia and reperfusion (IR) injury is a leading cause of AKI and patients undergoing major surgical procedures

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(cardiac, vascular or liver transplantation) have ~50–80% chance of developing ischemic AKI (2, 3). Renal IR results in proximal tubular necrosis and apoptosis with rapid upregulation of pro-inflammatory cytokines and chemokines that causes influx of inflammatory leukocytes into the renal parenchyma (4–6). Unfortunately, despite nearly 7 decades of research, there is no effective preventive measures or therapy for ischemic AKI (7, 8).

Toll-like receptors (TLRs) are pattern recognition receptors critical for regulating innate as well as adaptive immunity and play important roles in protecting against microbial invasion (9–11). Of 13 identified TLRs for mice and 11 for humans (9, 10), TLRs can be classified into cell surface TLRs (TLR1, 2, 4, and 6 that recognize bacterial or fungal products) and intracellular TLRs (TLR 3, 7, 8 and 9 which recognize DNA and RNA products) (12). Numerous endogenous ligands can also activate TLRs including histones, high mobility group box 1, heat shock proteins and mitochondrial DNA – all of these endogenous damage associated pattern ligands (DAMPs) are rapidly released after IR injury. Indeed, previous studies showed that cell surface TLR4 and TLR2 play important roles in liver and kidney IR injury (12–15).

TLR9 is a cytosolic receptor for un-methylated cytosine-phosphate-guanosine (CpG) deoxyribonucleic acid found in microbial DNA and DNA viruses (12, 16). Directly relevant to ischemic tissue injury, TLR9 also recognizes endogenous mitochondrial DNA products released from injured cells to trigger MyD88-dependent NFκB-mediated gene transcription leading to inflammation and apoptosis (16–19). Indeed, TLR9 activation plays a critical role in hepatic IR injury (16, 18). Furthermore, mitochondrial DNA released after trauma causes neutrophil extracellular trap (NET) formation (20). Recent studies suggest that NET formed by DAMPs exacerbates sterile liver IR injury in mice (21).

However, the role for kidney TLR9 in ischemic AKI remains unclear. Previous studies suggest TLR9 does not play a role in ischemic AKI as mice deficient in TLR9 mice were not protected against ischemic AKI (22, 23). However, TLR9 has diverse effects depending on cell types and organs studied (12). Indeed, TLR9 activation induces inflammation in hepatic IR and plays a role in septic AKI (17, 18, 24, 25). In contrast, TLR9 induces cytoprotective signaling in immune, cardiac and neuronal cells (26–28). In addition, global TLR9 deficiency may lead to compensatory changes in multiple non-renal tissue and cell types (29). Taken together, global TLR9 deficiency may not allow for direct examination of TLR9 in ischemic AKI. Therefore, we generated mice with proximal specific deletion of TLR9 and tested the hypothesis that selective renal proximal tubular TLR9 activation exacerbates ischemic AKI by promoting renal tubular epithelial apoptosis and inflammation.

Methods

Generation of mice with renal proximal tubule cell specific TLR9 deficiency

We bred mice with floxed TLR9 gene ($TLR9^{fl/fl}$ mice on a C57BL/6 background (30)) with mice that express Cre-recombinase selectively in proximal tubular epithelia (Cre recombinase under the control of the phosphoenolpyruvate carboxykinase promoter or PEPCK Cre – generated by Dr. Volker Haase, Vanderbilt University) (31). PEPCK Cre mice

were obtained through the laboratory of Dr. Holger Eltzschig (University of Colorado School of Medicine) after they were backcrossed with C57BL/6 strain for several generations. This approach allowed us to generate sibling mice with proximal tubule-specific deletion of TLR9 ($TLR9^{fl/fl}$ PEPCK Cre mice) or wild type ($TLR9^{fl/fl}$) mice. At least 4 generations of sibling breeding in our laboratory resulted in mice used in this study. Tail PCR with PEPCK Cre and TLR9 loxP-specific primers (Table 1) confirmed the genotypes of proximal tubule cell specific TLR9 null mice and the control wild type mice ($TLR9^{fl/fl}$) generated from breeding.

Confirmation of renal proximal tubular TLR9 deletion in $TLR9^{fl/fl}$ PEPCK Cre mice

Paraffin embedded kidney tissues were cut at 5 μm, deparaffinized, and rehydrated in graded ethanol series. Endogenous peroxidase was inhibited using 3% hydrogen peroxide in methanol for 30 min, and then the sections were autoclaved in sodium citrate buffer (10 mM, pH 6) at 120 °C for 10 min. The sections were incubated with 3% BSA in PBS for 60 min and then incubated overnight at 4°C with rabbit monoclonal anti-TLR9 antibody (1:100; 1% BSA in PBS; Abcam, Cambridge, MA). After washing 3 times with PBS buffer, the kidney sections were incubated with Alexa 594-conjugated goat anti-rabbit secondary antibody (1:100; 1% BSA in PBS; Abcam, Cambridge, MA). To stain brush border of proximal tubules, kidney sections were also stained using *Phaseolus vulgaris leucoagglutinin* (PHA-L) lectin conjugated to a green fluorescent dye according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA) as described previously (32). PHA-L lectin binds avidly to the brush border of proximal tubules (33).

We also confirmed selective deletion of renal proximal tubular TLR9 in $TLR9^{fl/fl}$ PEPCK Cre mice by measuring TLR9 mRNA in isolated proximal tubule cells (34) as well as bone-marrow cells, small intestine and spleen with RT-PCR as described previously (35, 36). Primer design was based on published GenBank sequences. To control for RNA loading, GAPDH mRNA expression was also measured.

Renal IR injury in mice

After Columbia University Institutional Animal Care and Use Committee approval, 8–10 week old male TLR9 proximal tubule deficient ($TLR9^{fl/fl}$ PEPCK Cre) mice or control wild type ($TLR9^{fl/fl}$) mice weighing 20–25g were anesthetized with pentobarbital i.p. (Sigma, St Louis, MO: 50 mg/kg body weight or to effect). Mice were then subjected to right nephrectomy and 30 min left renal ischemia as described previously (37, 38). A separate cohort of $TLR9^{fl/fl}$ mice or renal tubular TLR9 null mice were pretreated with a selective TLR9 agonist (1 mg/kg ODN-1668 or control ODN, Invivogen, San Diego, CA) and subjected to 20 min renal IR injury. Some mice were treated with vehicle for ODN (saline) before 20 min renal ischemia. To determine the effects of global TLR9 deletion in ischemic AKI, we also subjected TLR9 knockout mice (TLR9KO, $C57BL/6J^{Tlr9M7Btlr/Mmjax}$) and their wild type controls (C57BL/6J mice (B6)) to 30 min renal IR injury (Jackson Labs, Bar Harbor, ME). For pain management, all mice received 0.5–1 mg/kg s.c. buprenorphine SR prior to surgery. Sham-operated animals underwent anesthesia followed by laparotomy, right nephrectomy, bowel manipulations and wound closure without renal ischemia. Body

temperature of all mice were sustained at ~37°C using a surgical heating pad during surgery as well as during recovery from anesthesia.

Kidney TLR9 protein expression after sham-surgery and renal IR injury

Paraffin embedded kidney sections were stained rabbit monoclonal anti-TLR9 antibody as described above. The sections were subsequently incubated with anti-rabbit secondary antibody (1:100, 2% goat serum in PBS; Vector Laboratories) for 1 h, washed, and incubated with avidin-biotin complex (ABC kit; Vector Laboratories). The sections were developed with 3, 3′-diaminobenzidine (DAB). Negative controls were performed on serial sections by omitting primary antibody and by using non-specific isotype control primary antibody. Kidney TLR9 expression was quantified from 3–5 randomly chosen 200× microscope image fields surrounding corticomedullary junction as described by Ruifrok et al. (39).

Measurement of renal injury after IR injury

Twenty-four hrs after renal IR injury, we measured plasma BUN and creatinine using an enzymatic creatinine reagent kit (Thermo Fisher Scientific, Waltham, MA). This creatinine measurement method limits the interferences from mouse plasma chromagens known to occur in the Jaffe method. We also performed qRT-PCR for kidney NGAL mRNA from mice subjected to sham-surgery or to renal IR injury. NGAL is an early and sensitive marker of renal tubular injury (40).

Histological detection of kidney injury

Twenty-four hrs after renal IR injury, kidney hematoxylin-eosin (H&E). Kidney H&E sections post renal IR surgery or sham surgery were blindly assessed using a grading scale of kidney necrotic IR injury to the proximal tubules (0–4, Renal Injury Score) as outlined by Khalid *et al.* (41). In brief, no damage = 0; loss of brush border in less than 25% of tubular cells = 1; loss of brush border in more than 25% of tubular cells = 2; necrosis up to 60% of tubular cells = 3 and necrosis in more than 60% of tubular cells = 4.

Detection of kidney tubular apoptosis

We utilized 2 independent assays to assess kidney apoptosis after IR –with TUNEL staining as well as detection of caspase 3 and caspase 8 fragmentation by immunoblotting. Twenty four hrs after sham surgery or renal IR injury, TUNEL staining detected fragmented DNA as described (42) using a commercially available kit (Roche, Indianapolis, IN). Apoptotic terminal deoxynucleotidyl transferase 2′-deoxyuridine-5′-triphosphate nick end labeling positive cells were quantified in 5–7 randomly chosen 100× microscope images fields in the corticomedullary junction and results were expressed as apoptotic cells counted per 100× field. Kidney caspase 3 and caspase 8 immunoblotting were performed as described previously (43). Primary antibodies for cleaved mouse caspase 3 and caspase 8 were from Cell Signaling Technology (Danvers, MA).

Detection of kidney neutrophil infiltration

Kidney neutrophil infiltration after IR injury was detected with immunohistochemistry using rat anti-mouse Ly6B monoclonal antibody (AbD Serotec, Raleigh, NC) as described (36,

44). Primary IgG_{2a} antibody (MCA1212, AbD Serotec, Raleigh, NC) was utilized as a negative isotype control. Quantification of kidney infiltrating neutrophils was performed using 5–7 randomly chosen 200× microscope image fields (corticomedullary junction for kidney neutrophils) and results were expressed as neutrophils counted per 200× field.

Q-RTPCR for pro-inflammatory cytokine and chemokine mRNA expression

Renal inflammation after IR was also assessed by measuring pro-inflammatory mRNA markers including IL-6, IL-8, intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractive protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), and tumor necrosis factor-α (TNF-α) quantitative RT-PCR as described previously with primers listed in Table 2 (35, 36). Primer design was based on published GenBank sequences. To confirm equal RNA loading, GAPDH mRNA expression was also measured.

Proximal tubule cell culture and TLR9 agonist treatment

Immortalized human proximal tubular cells (HK-2; ATCC, Manassas, VA) were grown as described (45, 46). Mouse kidney proximal tubules were isolated and grown using Percoll density gradient separation as described previously (34). Confluent cells were treated with control oligonucleotides or with selective 1–5 μM TLR9 ligands [ODN2006 (human specific), ODNBW006 (human and mouse specific) or ODN1668 (mouse specific)] for 3 days as described (47). Proximal tubule cells were subjected to quantitative RTPCR for pro-inflammatory genes with primers listed in Table 2. We also performed caspase 3 and caspase 8 immunoblotting in HK-2 cells (43). Finally, HK-2 cell nuclear and cytosolic fractions were prepared and subjected to p65 and phospho-p65 subunit of NFκB with antibodies from Santa Cruz Biotechnologies (Santa Cruz, CA) as described (48).

Statistical analysis

Data were analyzed with Student's *t*-test, one-way ANOVA plus Tukey's *post hoc* multiple comparison test or Mann–Whitney nonparametric U test to analyze renal injury scores. All data are expressed throughout the text as means ± SEM.

Results

Genotyping and confirmation of renal proximal tubular TLR9 deletion

Tail PCR confirmed the genotypes of proximal tubule-specific TLR9 null mice ($TLR9^{fl/fl}$ PEPCK Cre) and the control wild type mice ($TLR9^{fl/fl}$) generated (Figure 1A). PEPCK Cre primers generated a 466-bp fragment. TLR9 flox primers amplified a 507-bp fragment for the TLR9 floxed allele and a 433-bp fragment for the TLR9 wild type allele.

We simultaneously stained kidney sections for TLR9 immunofluorescence (red) as well as *Phaseolus vulgaris* leucoagglutinin lectin to identify the brush border of proximal tubules (green) (Figure 1B, representative of 4 experiments). Merged images demonstrate that cytosolic TLR9 staining was visible in lectin-positive proximal tubule cells as well as lectin-negative renal cells in $TLR9^{fl/fl}$ mice (left panel). In contrast, TLR9 staining was nearly absent in lectin-positive proximal tubule cells but was visible in lectin-negative renal cells in $TLR9^{fl/fl}$ PEPCK Cre mice (right panel).

To further confirm renal proximal tubule cell specific deletion of TLR9 in $TLR9^{fl/fl}$ PEPCK Cre mice, we measured the TLR9 mRNA expression in isolated proximal tubules (Figure 1C). TLR9 mRNA expression in renal proximal tubules from $TLR9^{fl/fl}$ PEPCK Cre mice were decreased by >95% compared to $TLR9^{fl/fl}$ mice. However, TLR9 mRNA expressions in isolated bone marrow cells, intestine and spleen were equivalent between in $TLR9^{fl/fl}$ PEPCK Cre mice and in $TLR9^{fl/fl}$ mice (Figure 1D) again demonstrating renal proximal tubule specific deletion of TLR9 in $TLR9^{fl/fl}$ PEPCK Cre mice.

Kidney IR injury induces renal tubular TLR9 expression

Figure 2 shows a representative TLR9 staining in $TLR9^{fl/fl}$ mice subjected to sham operation or to 30 min renal IR injury. Faint cytosolic TLR9 staining is visible in renal tubules from sham-operated mice. Kidneys from mice subjected to Renal IR injury show increased cytosolic TLR9 staining compared with sham-operated mice (200X, representative of 6 immunohistochemistry experiments).

Renal proximal tubular TLR9 plays a critical role in ischemic AKI

Plasma creatinine values were similar between $TLR9^{fl/fl}$ mice and renal proximal tubular TLR9 null mice subjected to sham-operation (Figure 3A). $TLR9^{fl/fl}$ mice subjected to renal IR had significantly increased plasma BUN and creatinine as well as kidney NGAL mRNA (N=6–8) compared to sham-operated mice (N=4–5). We show here that mice lacking renal proximal tubular TLR9 were protected against ischemic AKI compared to $TLR9^{fl/fl}$ mice as demonstrated by reduced plasma BUN and creatinine as well as kidney NGAL mRNA expression (N=6–8). In contrast, global TLR9KO mice are not protected against renal IR injury and had similar increases in plasma BUN and creatinine and kidney NGAL mRNA expression compared to B6 mice (N=5–7) (Figure 3B).

Reduced renal tubular necrosis in proximal tubular TLR9 deficient mice after ischemic AKI

Figure 4A shows representative H&E images of renal proximal tubular TLR9 null mice and $TLR9^{fl/fl}$ mice subjected to sham surgery or 30 min renal IR and 24 hr reperfusion (magnification 200X, N=6). $TLR9^{fl/fl}$ mice subjected to renal IR showed severe tubular necrosis and proteinaceous casts as well as increased tubular dilatation and congestion. Renal proximal tubular TLR9 null mice had decreased renal tubular necrosis, congestion and cast formation compared to $TLR9^{fl/fl}$ mice subjected to renal IR. Kidneys from renal proximal tubular TLR9 null mice had slightly but significantly reduced renal tubular injury score compared to control $TLR9^{fl/fl}$ mice after IR (Figure 4B). Unlike reduced necrosis observed in renal proximal tubule TLR9 null mice, global TLR9KO mice and control B6 wild type mice showed similarly severe renal tubular necrotic injury after IR (Figure 4C and 4D).

Renal proximal tubular TLR9 regulates pro-inflammatory chemokine and cytokine induction after ischemic AKI

Figure 5A shows fold increases in pro-inflammatory mRNAs normalized to GAPDH for each indicated mRNA (N=4–6). Ischemic AKI increased all of the pro-inflammatory genes measured in $TLR9^{fl/fl}$ mice. Consistent with the critical role of renal proximal tubular TLR9

for the induction of neutrophil and macrophage attracting chemokines, we show that MIP-2 and MCP-1 expression was significantly attenuated in renal proximal tubular TLR9 null mice. Moreover, IL-6 as well as ICAM-1 induction was attenuated by renal proximal tubular TLR9 deficiency. In contrast, we determined that global TLR9KO mice had similar degree of induction in KC, TNF-α, MIP-2 and MCP-1 after ischemic AKI when compared TLR9 competent mice (Figure 5B). Interestingly, global TLR9KO mice had significantly reduced kidney IL-6 mRNA expression but had higher kidney ICAM-1 mRNA expression. These data again suggest fundamental differences in renal inflammatory response between renal proximal tubular TLR9 null mice and global TLR9KO mice.

Renal proximal tubule TLR9 deficient mice have reduced kidney neutrophil infiltration after ischemic AKI

Figure 6A shows representative immunohistochemistry images and Figure 6B shows counts of infiltrating kidney neutrophils (N=6) in the kidneys of $TLR9^{fl/fl}$ mice and $TLR9^{fl/fl}$ PEPCK Cre subjected to sham surgery or renal IR (magnification 200X). Kidney neutrophil infiltration was significantly higher in the $TLR9^{fl/fl}$ wild type mice compared to $TLR9^{fl/fl}$ PEPCK Cre mice after renal IR. In contrast, kidney neutrophil infiltration was similar between B6 mice and global TLR9KO mice after ischemic AKI (Figure 6C, D).

Reduced kidney apoptosis in renal proximal tubule TLR9 deficient mice after ischemic AKI

Figure 7A shows representative TUNEL staining images indicative of renal apoptosis and Figure 7B shows counts of TUNEL positive kidney cells (B, N=5) from $TLR9^{fl/fl}$ mice and $TLR9^{fl/fl}$ PEPCK Cre subjected to sham surgery or to renal IR (magnification 200X). Many TUNEL (fragmented DNA) positive cells were detected suggestive of renal tubular apoptosis in the kidneys from $TLR9^{fl/fl}$ mice subjected to renal IR injury. TUNEL positive kidney cell counts were significantly reduced in proximal tubular TLR9 null mice compared to $TLR9^{fl/fl}$ mice. We also detected caspase 3 and caspase 8 activation by measuring cleaved caspase 3 and caspase 8 in the kidney lysates from sham-operated mice and mice subjected to renal IR injury (Figure 7C). Caspase 3 and caspase 8 cleavage was increased in $TLR9^{fl/fl}$ mice subjected to renal IR that were significantly reduced in proximal tubular TLR9 null mice. In contrast, TUNEL positive kidney cells were equivalent between B6 mice and global TLR9KO mice after ischemic AKI (Figure 7D, E).

TLR9 activation exacerbates ischemic AKI and increases renal tubular necrosis, inflammation and apoptosis

There were no differences of plasma creatinine, kidney NGAL mRNA as well as renal tubular necrosis between vehicle (saline)-treated mice and control ODN-treated mice (Figure 8A, B). $TLR9^{fl/fl}$ mice subjected to 20 min. renal IR after ODN-1668 treatment had significantly increased plasma creatinine as well as kidney NGAL mRNA compared to control ODN-treated mice (N=5–6, Figure 8A). In contrast, not only mice lacking renal proximal tubular TLR9 had reduced renal injury after 20 min renal IR, ODN-1668 treatment failed to exacerbate ischemic AKI in these mice (N=5–6). Moreover, selective TLR9 activation with ODN-1668 increased renal tubular necrosis (Figure 8B), neutrophil infiltration (Figure 8C, E), pro-inflammatory cytokine mRNA induction (Figure 8G) and TUNEL positive cells (Figure 8D, F) in $TLR9^{fl/fl}$ mice compared to control ODN-treated

TLR9$^{fl/fl}$ mice. However, ODN-1668 failed to increases these markers of renal injury and inflammation in renal proximal tubular TLR9 null mice. Furthermore, mice lacking renal proximal tubular TLR9 had reduced renal injury scores, neutrophil infiltration, pro-inflammatory cytokine induction and TUNEL staining compared to TLR9$^{fl/fl}$ mice after 20 min renal IR injury.

TLR9 agonists induce renal proximal tubular NFκB activation, caspase cleavage and pro-inflammatory mRNA induction

Figure 9A shows a representative immunoblotting for nuclear p65 NFκB subunit and band intensity quantifications normalized to PARP-1 in HK-2 cells (N=4). TLR9 activation with ODN2006 increased nuclear translocation of p65 NFκB subunit in HK-2 cells. Moreover, TLR9 activation with another TLR9 ligand (ODNBW006) in HK-2 cells also increased the phosphorylated p65 NFκB subunit in the cytosol as shown by the representative immunoblotting images for cytosolic p65 subunit (top) and band intensity quantifications normalized to p65 subunit or β-actin (N=4, bottom) (Figure 9B). Figure 9C shows a representative immunoblotting experiment for cleaved caspase 3 and caspase 8 (top) and band intensity quantifications normalized to β-actin (N=4, bottom) demonstrating increased TLR9 ligand-mediated cleavage of caspase 3 and caspase 8 in HK-2 cells.

Quantitative RTPCR revealed that a selective TLR9 ligand ODNBW006 treatment significantly induced the expression of IL-8, TNF-α and ICAM-1 mRNAs measured without changing the MIP-2 expression (Figure 9D, N=4) in HK-2 cells. In addition, we show that TLR9 agonist ODN1668 treatment significantly induced the expression of pro-inflammatory mRNAs (IL-8, TNF-α, ICAM-1, MCP-1 and MIP-2) in renal proximal tubules from TLR9$^{fl/fl}$ mice but not in renal proximal tubules isolated from renal proximal tubule TLR9 null mice (Figure 9E, N=4).

Discussion

TLR9 activation mediates IR injury in several organs including the heart, and liver but plays no role in intestinal IR injury (16, 18, 49). In cardiac and hepatic IR injury, TLR9 activation by endogenous DAMPs plays a critical role in cardiomyocyte and hepatocyte death (18, 21, 30, 50). In particular, mitochondrial DNA with high un-methylated CpG motif that can strongly stimulate TLR9 (30, 51). Previous studies show that circulating and locally released mitochondrial DNA can activate TLR9 to promote liver injury after trauma or IR (21, 30).

However, the role for TLR9 in ischemic AKI remains unclear. Suggesting a detrimental role of TLR9 in the kidney, mitochondrial DNA-induced TLR9 activation appears to play a critical role in septic AKI as TLR9 signaling inhibition or genetic TLR9 deletion attenuates CLP-induced sepsis in mice (17, 24). Moreover, siRNA-mediated TLR9 deletion also protects against CLP-sepsis (25). In addition, TLR9 activation by endogenous DNA induces kidney podocyte apoptosis (51). These findings in various kidney pathologies suggest kidney TLR9 may play a role in ischemic AKI. However, 2 previous studies using global TLR9 null mice failed to show a detrimental role for TLR9 in ischemic AKI (22, 23).

We demonstrate here that renal proximal tubule TLR9 plays a critical role in ischemic AKI as renal tubular TLR9 null mice had significantly reduced renal tubular necrosis and apoptosis after IR. Necrotic renal cells after IR release mitochondrial DNA that can target intracellular renal tubular TLR9 (24). In particular, we demonstrate that TLR9 signaling regulates renal proximal tubular apoptosis after kidney IR injury. Mice deficient in renal tubular TLR9 had reduced caspase 3 and 8 activation after renal IR compared to control $TLR9^{fl/fl}$ mice. Moreover, TLR9 selective ligand induced proximal tubule apoptosis in culture. Our data is consistent with the hypothesis that renal IR injury causes renal proximal tubular TLR9 activation to induce kidney apoptosis via caspase 3/8 activation.

Renal proximal tubule TLR9 null mice had significantly attenuated kidney IL-6, MCP-1, MIP-2 and ICAM-1 synthesis expression after ischemic AKI when compared to $TLR9^{fl/fl}$ mice (Figure 5). Consistent with reduced pro-inflammatory cytokines and chemokines, renal proximal tubule TLR9 null mice had reduced neutrophil infiltration after IR. Moreover, TLR9 selective ligand induced pro-inflammatory cytokines and chemokines in cultured human and mouse renal proximal tubule cells. Finally, TLR9 specific agonist ligand only induced inflammatory cytokines in $TLR9^{fl/fl}$ mice but not in TLR9 null renal proximal tubules. Taken together, our studies suggest that renal proximal tubules are the major source of pro-inflammatory cytokines and chemokines and renal tubular TLR9 is a key regulator for several of these critical inflammatory mediators.

Neutrophils infiltrating the kidney after renal IR are major contributor to additional renal injury after reperfusion and are able to recruit other inflammatory leukocytes including natural killer cells, monocytes and macrophages (52–54). Reduced kidney neutrophil infiltration most likely attenuated additional cytokine generation in renal proximal tubule TLR9 null mice that may have contributed to improved renal function after ischemic AKI. Consistent with this, renal proximal tubule cell TLR9 null mice also had significantly reduced MCP-1 expression in the kidney after IR. MCP-1 (CCL2) is the major chemokine that promote macrophage infiltration and migration after IR (55, 56).

Although our findings suggest that renal IR-induced TLR9 activation directly promotes inflammation and apoptosis in renal proximal tubular cells and exacerbates ischemic AKI, it is interesting that mice globally deficient in TLR9 were not protected against ischemic AKI as shown previously by other investigators (22, 23). These discrepancies in renal injury between tissue specific TLR9 null mice and global TLR9KO mice point out major differences between these mice and suggest caution in interpreting studies with global genetic deletions studies. These differences also suggest divergent effects of TLR9 activation depending on the cell and tissue types studied. Although renal proximal tubular TLR9 activation causes kidney injury by inducing apoptosis and inflammation after IR, other cell types in the kidney may benefit from TLR9 signaling. Indeed, a renal protective role for TLR9 in cisplatin-induced AKI model has been described as TLR9 deficient mice had exacerbated renal injury with higher BUN, tubular injury and inflammatory cytokine induction (57). In cisplatin-induced AKI as well as in murine lupus model, TLR9 may promote tissue protection by promoting accumulation of beneficial regulatory T-cells (57, 58). Directly relevant to our studies, global TLR9 deficiency leads to enhanced renal inflammation in several murine models of lupus (59, 60). Therefore, it is possible that global

TLR9KO mice were not protected against ischemic AKI as they may lack the tissue protective regulatory T-cells modulated by TLR9 signaling. Furthermore, not all TLR9 signaling results in exacerbation of IR injury as TLR9 activation protects against cerebral as well as cardiac IR injury via activation of PI3K/Akt signaling (26, 27). Finally, TLR9 activation may mediate cardiac and neuronal protection by modulating energy metabolism (61). Therefore, our study as well as previous studies suggest that kidney TLR9 signaling results in complex and perhaps divergent effects depending on the cells targeted. In addition, our findings also suggest that these variations in TLR9 signaling suggest caution in interpreting studies with global TLR9 gene deletion.

In summary, we demonstrate in this study that renal proximal tubular TLR9 plays a critical role in murine ischemic AKI by exacerbating necrosis, apoptosis and inflammation after IR. Our study suggest caution when interpreting data from global gene deletion studies as there were critical differences between proximal tubule TLR9 deficient mice and global TLR9 deficient mice subjected to ischemic AKI. Figure 10 shows a schematic of proposed mechanisms for renal proximal tubular TLR9-mediated exacerbation of ischemic AKI. After renal IR, damaged renal cells release endogenous TLR9 activators (presumably mitochondrial DNA products) leading to NFκB-mediated induction of pro-inflammatory chemokines and cytokines as well as Caspase 3/8-mediated induction of renal tubular apoptosis.

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Abbreviations

AKI	acute kidney injury
IR	ischemia reperfusion
TLR	Toll-like receptor
IL-6	Interleukin-6
ICAM-1	intercellular adhesion molecule-1
MCP-1	monocyte chemoattractive protein-1
MIP-2	macrophage inflammatory protein-2
TNF-α	tumor necrosis factor-α
KC	Keratinocyte chemoattractant
NGAL	Neutrophil gelatinase-associated lipocalin
HK-2	Immortalized human proximal tubular cells

ODN oligonucleotide

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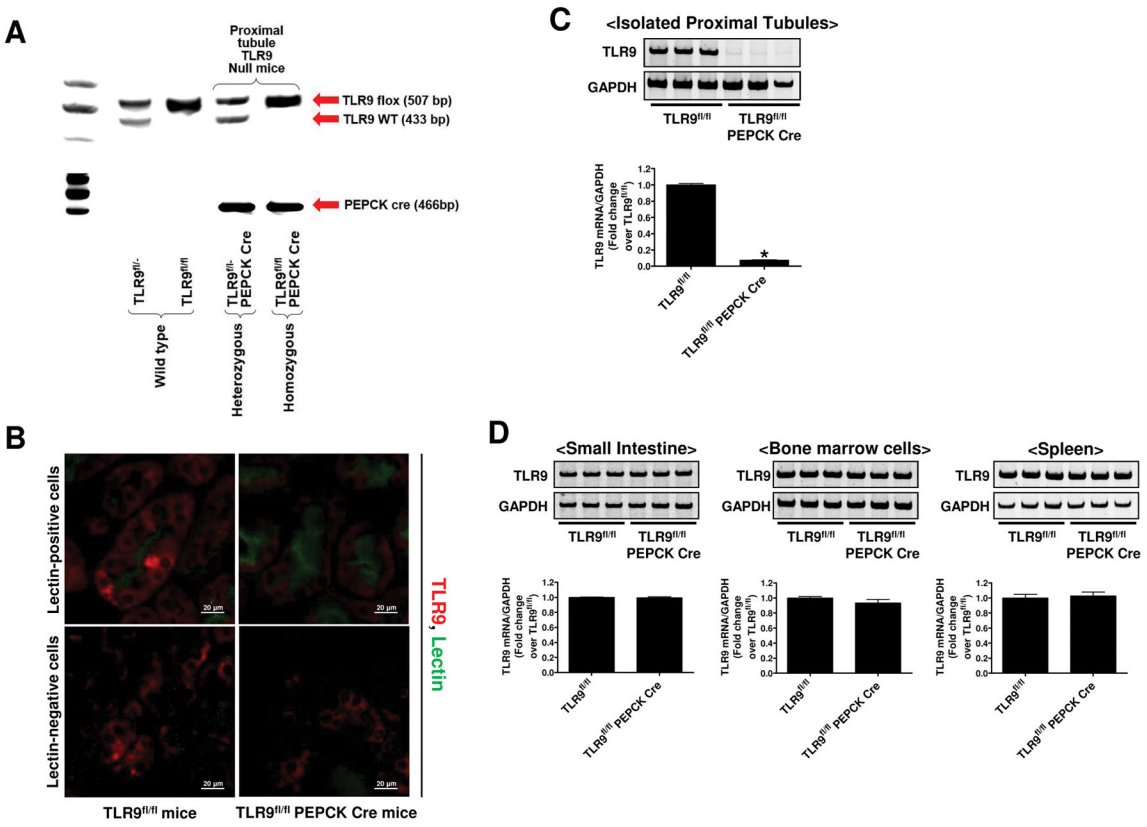

Figure 1. Genotyping and Confirmation of renal proximal tubule cell TLR9 deletion in proximal tubule cell TLR9 null mice

A) Tail PCR confirming the genotypes of renal proximal tubule-specific TLR9 null mice (TLR9$^{fl/fl}$ PEPCK Cre) and the control transgenic mice (TLR9$^{fl/fl}$). Bands were cut from multiple genotype gel images to create this figure to illustrate how we were able to differentiate TLR9 null mice and control mice. B) To confirm deletion of renal proximal tubular TLR9 in TLR9$^{fl/fl}$ PEPCK Cre mice, we simultaneously stained kidney sections for TLR9 immunofluorescence (red) as well as *Phaseolus vulgaris* leucoagglutinin lectin to identify the brush border of proximal tubules (green). C) and D) Representative gel images and band intensity quantifications of TLR9 mRNA normalized to GAPDH from RT-PCR reactions in isolated proximal tubule cells, small intestine, bone-marrow cells, and spleen of TLR9$^{fl/fl}$ and TLR9$^{fl/fl}$ PEPCK Cre mice. For statistical analysis, the student's t-test was used to detect significant changes. *P<0.05 vs. TLR9$^{fl/fl}$ mice (N=3). Error bars represent 1 SEM.

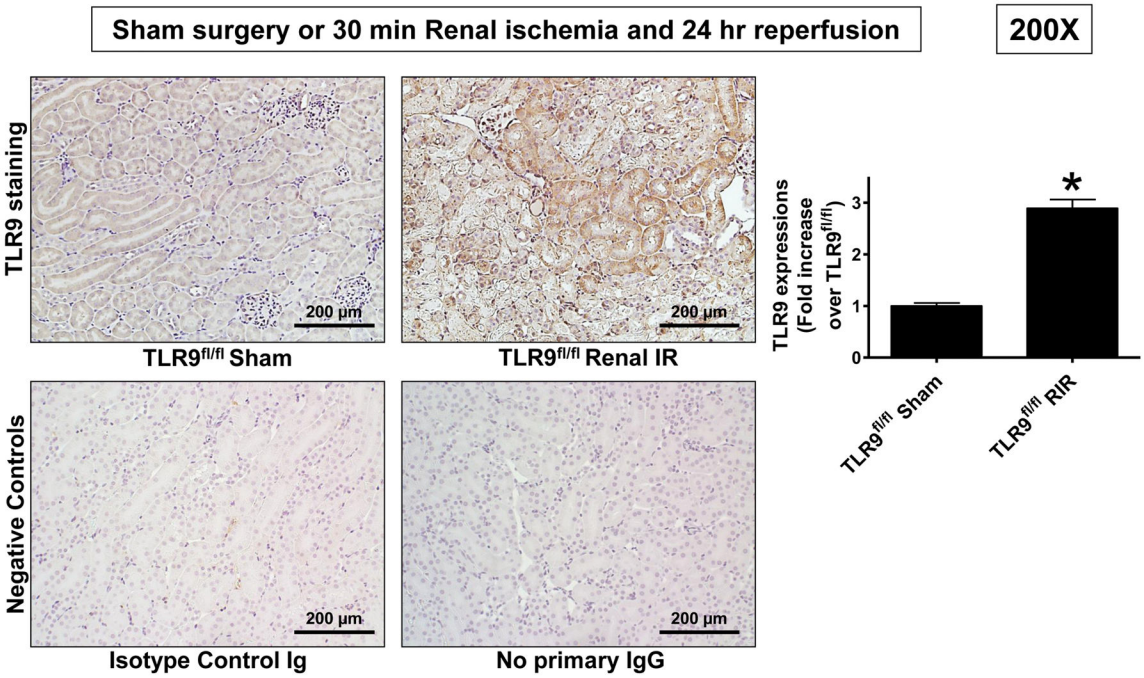

Figure 2. Ischemic AKI induces renal tubular TLR9 expression

Representative of 6 immunohistochemistry experiments for TLR9 protein staining in $TLR9^{fl/fl}$ mice subjected to sham operation or to 30 min renal IR injury. Two negative control slides show that staining is specific for TLR9. TLR9 expression was quantified using the imageJ software as described in Methods (N=6). For statistical analysis, the student's t-test was used to detect significant changes. *P<0.05 vs. $TLR9^{fl/fl}$ mice subjected to sham surgery. Error bars represent 1 SEM.

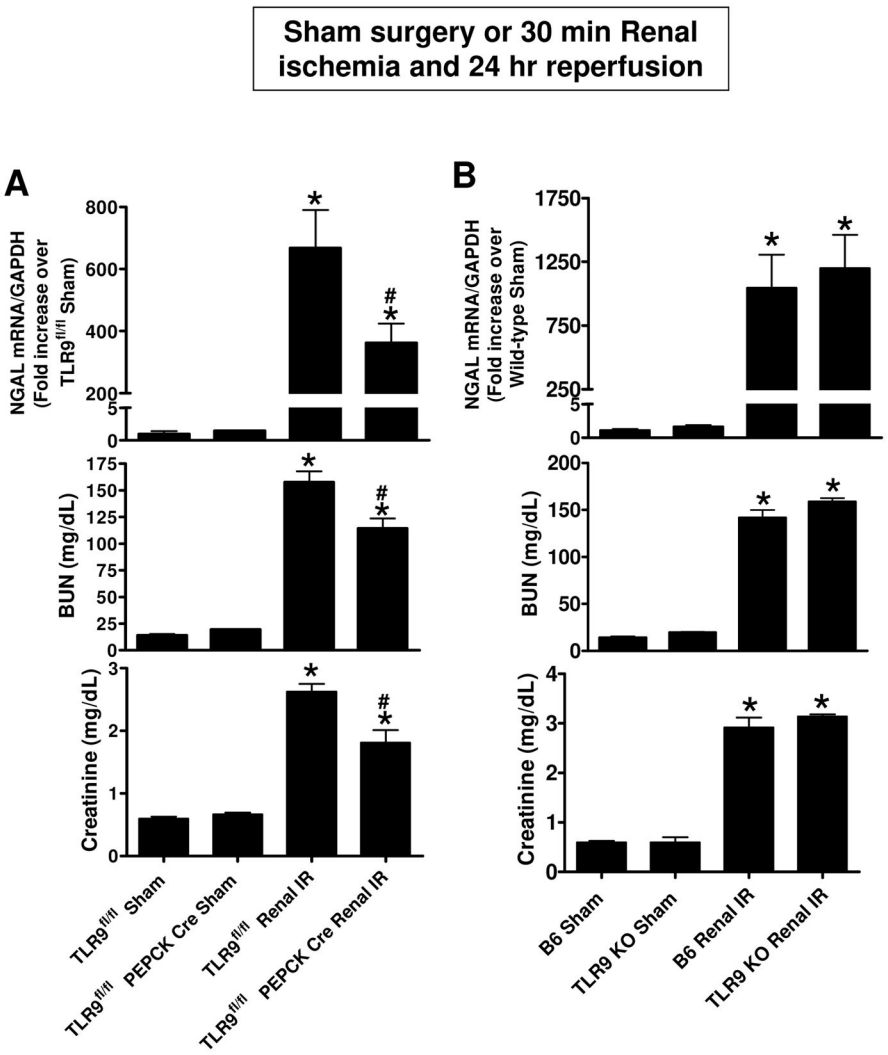

Figure 3. Renal proximal tubular TLR9 plays a critical role in ischemic AKI

A) TLR9$^{fl/fl}$ PEPCK Cre mice and TLR9$^{fl/fl}$ mice were subjected to sham-surgery or to 30 min renal ischemia and 24 hr reperfusion (IR). Plasma BUN and creatinine as well as kidney NGAL mRNA (N=6–8) were measured. B) Global TLR9KO mice had similar increases in plasma BUN and creatinine and kidney NGAL mRNA expression when compared to B6 wild type mice (N=5–7). For statistical analysis, the one-way ANOVA plus Tukey's *post hoc* multiple comparison test were used to detect significant changes. *P<0.05 vs. TLR9$^{fl/fl}$ mice subjected to sham surgery. #P<0.05 vs. TLR9$^{fl/fl}$ mice subjected to renal IR. Error bars represent 1 SEM.

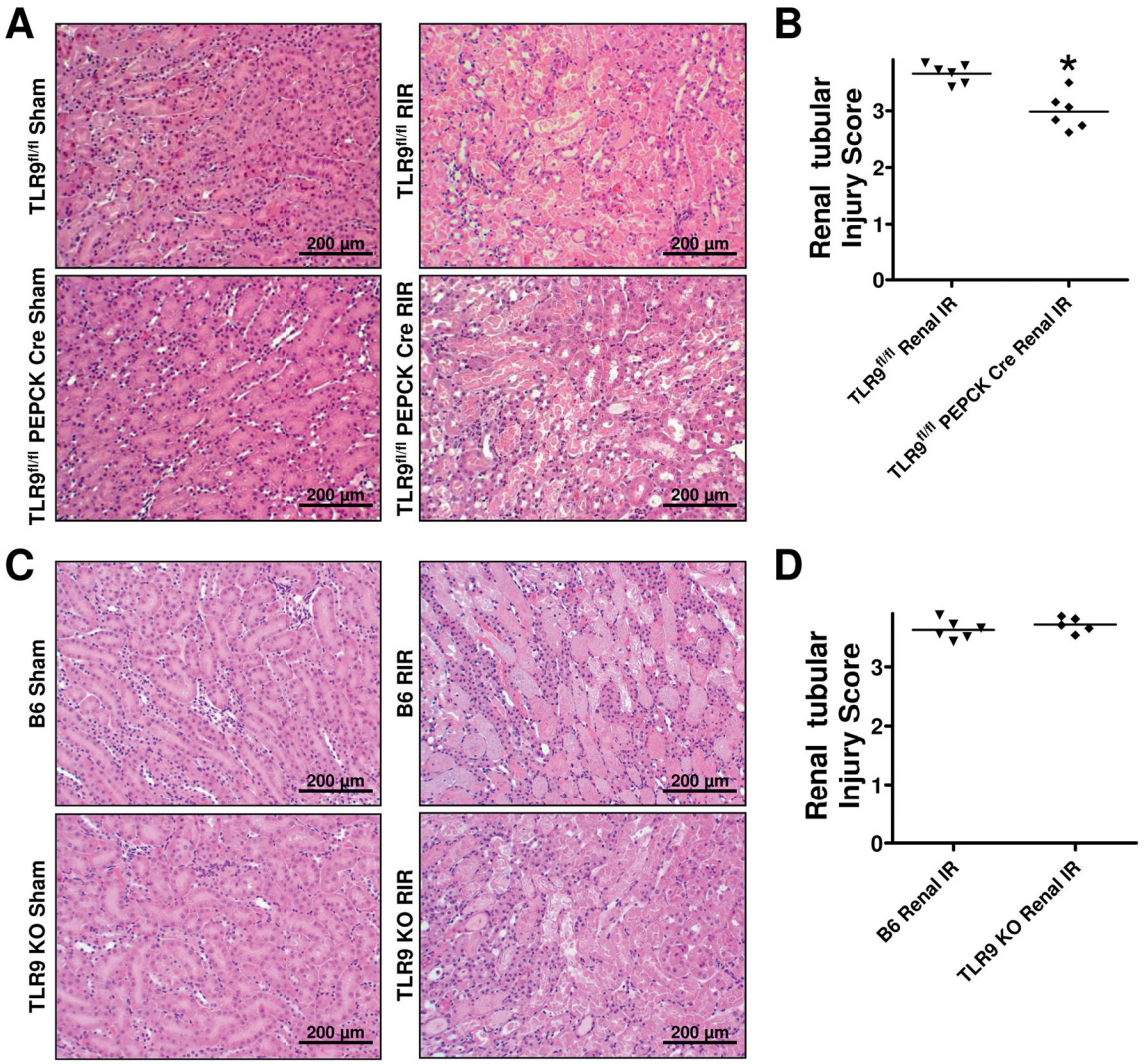

Figure 4. Reduced renal tubular necrosis in proximal tubule TLR9 deficient mice after ischemic AKI

A) Representative H&E images (from 5–6 experiments) of renal proximal tubular TLR9 null mice and $TLR9^{fl/fl}$ mice subjected to 30 min renal ischemia and 24 hrs reperfusion (magnification 200X). B) Renal injury scores assessing the degree of renal tubular necrosis (scale: 0–4,(41)) 24 hrs after renal IR (N=6). *P<0.05 vs. $TLR9^{fl/fl}$ mice subjected to renal IR injury. Representative H&E images (C) and renal injury scores (D) of global TLR9KO mice and B6 wild type mice subjected to sham-surgery or to 30 min renal ischemia and 24 hr reperfusion (magnification 200X, N=5–6). For statistical analysis, the Mann–Whitney nonparametric test was used to detect significant changes.

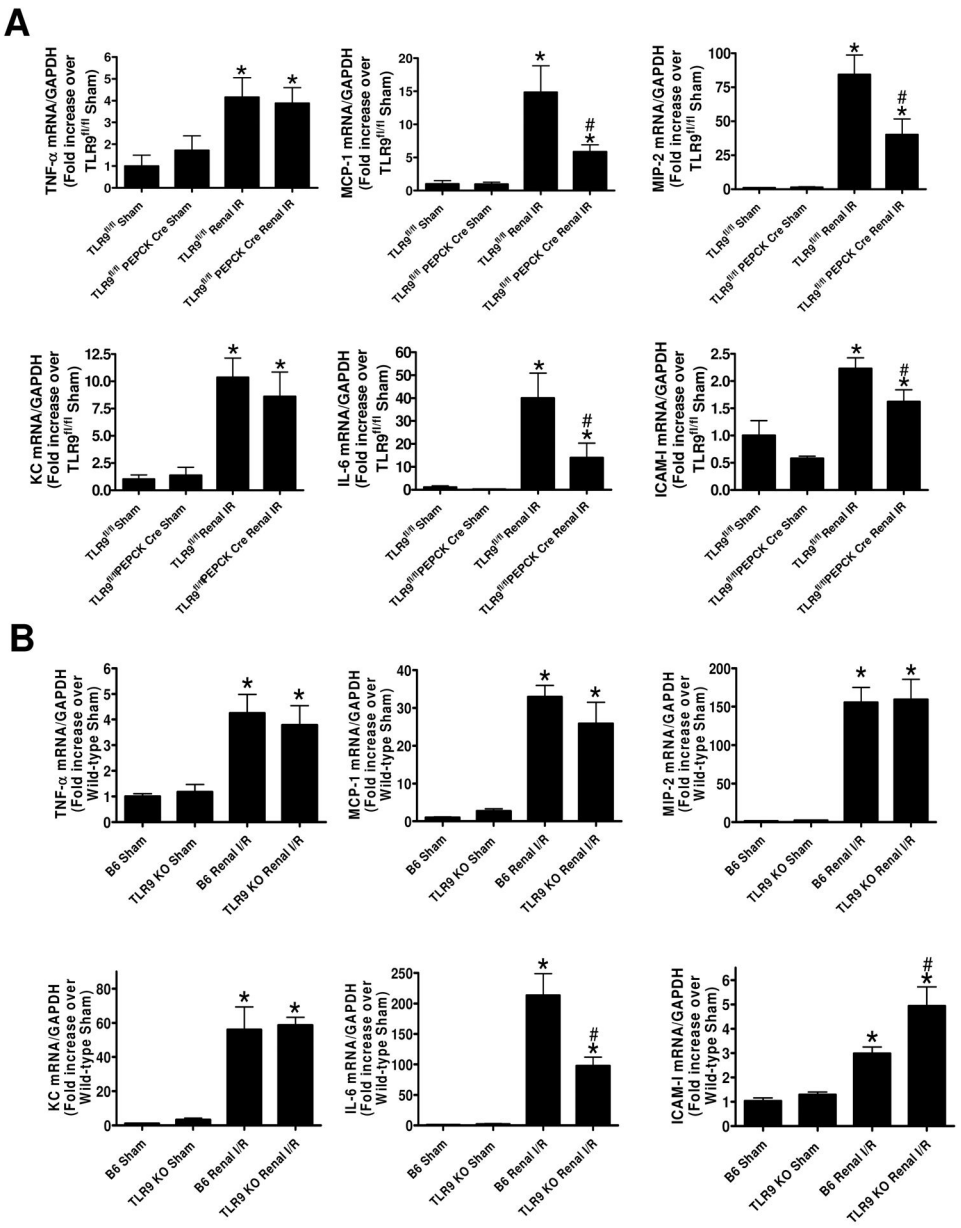

Figure 5. Renal proximal tubular TLR9 regulates kidney pro-inflammatory chemokine/cytokine induction and neutrophil infiltration after ischemic AKI

With quantitative RT-PCR, we measured the expression of pro-inflammatory cytokine and chemokine mRNAs in the kidney [keratinocyte-derived cytokine (KC), monocyte chemoattractive protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and intercellular adhesion molecule-1 (ICAM-1)] 24 hr after sham-surgery or 30 min renal ischemia. Fold increases in pro-inflammatory mRNAs normalized to GAPDH from quantitative RT-PCR reactions for each indicated mRNA (N=4–6) are shown. For statistical analysis, the one-way ANOVA plus Tukey's *post hoc* multiple comparison test were used to detect significant changes. *P < 0.05

vs. sham-operated mice. #P < 0.05 vs. $TLR9^{fl/fl}$ or B6 wild-type mice subjected to renal IR injury. Error bars represent 1 SEM.

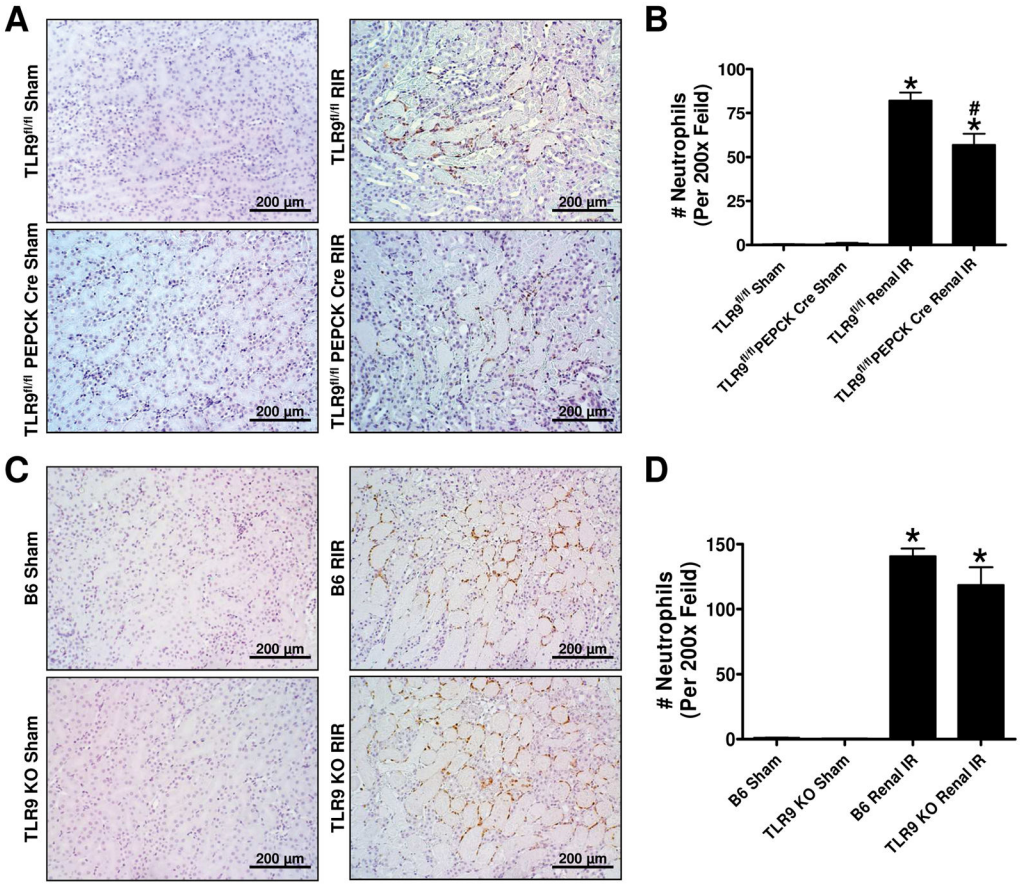

Figure 6. Reduced kidney neutrophil infiltration in renal proximal tubule TLR9 deficient mice after ischemic AKI

Representative images of immunohistochemistry for neutrophils (dark brown, A) and counts of infiltrating kidney neutrophils (B, N=6) in the kidneys of $TLR9^{fl/fl}$ mice and renal proximal tubule TLR9 null mice subjected to sham-surgery or to 30 min renal ischemia and 24 hr reperfusion (magnification 200X). Representative images of immunohistochemistry for neutrophils (C) and counts of infiltrating kidney neutrophils (D, N=5–6) in the kidneys of B6 wild type mice and global TLR9KO mice subjected to 30 min renal ischemia and 24 hr reperfusion (magnification 200X). For statistical analysis, the one-way ANOVA plus Tukey's *post hoc* multiple comparison test were used to detect significant changes. *P<0.05 vs. $TLR9^{fl/fl}$ mice subjected to sham surgery. #P<0.05 vs. $TLR9^{fl/fl}$ mice subjected to renal IR. Error bars represent 1 SEM.

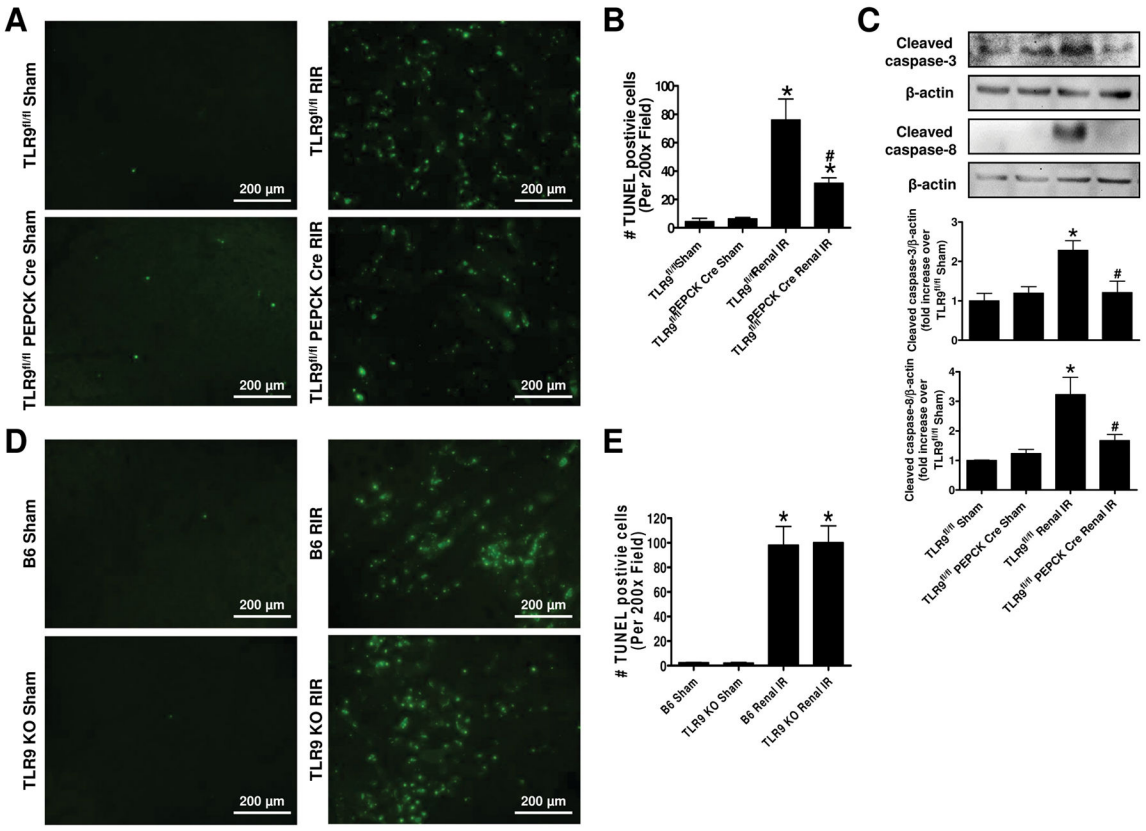

Figure 7. Reduced kidney apoptosis in renal proximal tubule TLR9 deficient mice after ischemic AKI

Representative images of terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining indicative of renal tubular apoptosis (A) and counts of TUNEL positive kidney cells (B, N=5) from $TLR9^{fl/fl}$ wild type mice and renal proximal tubule TLR9 null mice subjected to sham-surgery or to 30 min renal ischemia and 24 hr reperfusion (magnification 200X). We also detected caspase 3 and caspase 8 activation by measuring cleaved caspase 3 and caspase 8 in the kidney lysates from sham-operated mice and mice subjected to renal IR injury (C). Representative images of TUNEL staining (D) and counts of TUNEL positive kidney cells (E, N=5) from B6 mice and global TLR9KO mice subjected to sham-surgery or to 30 min renal ischemia and 24 hr reperfusion (magnification 200X). For statistical analysis, the one-way ANOVA plus Tukey's *post hoc* multiple comparison test were used to detect significant changes. *P<0.05 vs. $TLR9^{fl/fl}$ mice subjected to sham surgery. #P<0.05 vs. $TLR9^{fl/fl}$ mice subjected to renal IR. Error bars represent 1 SEM.

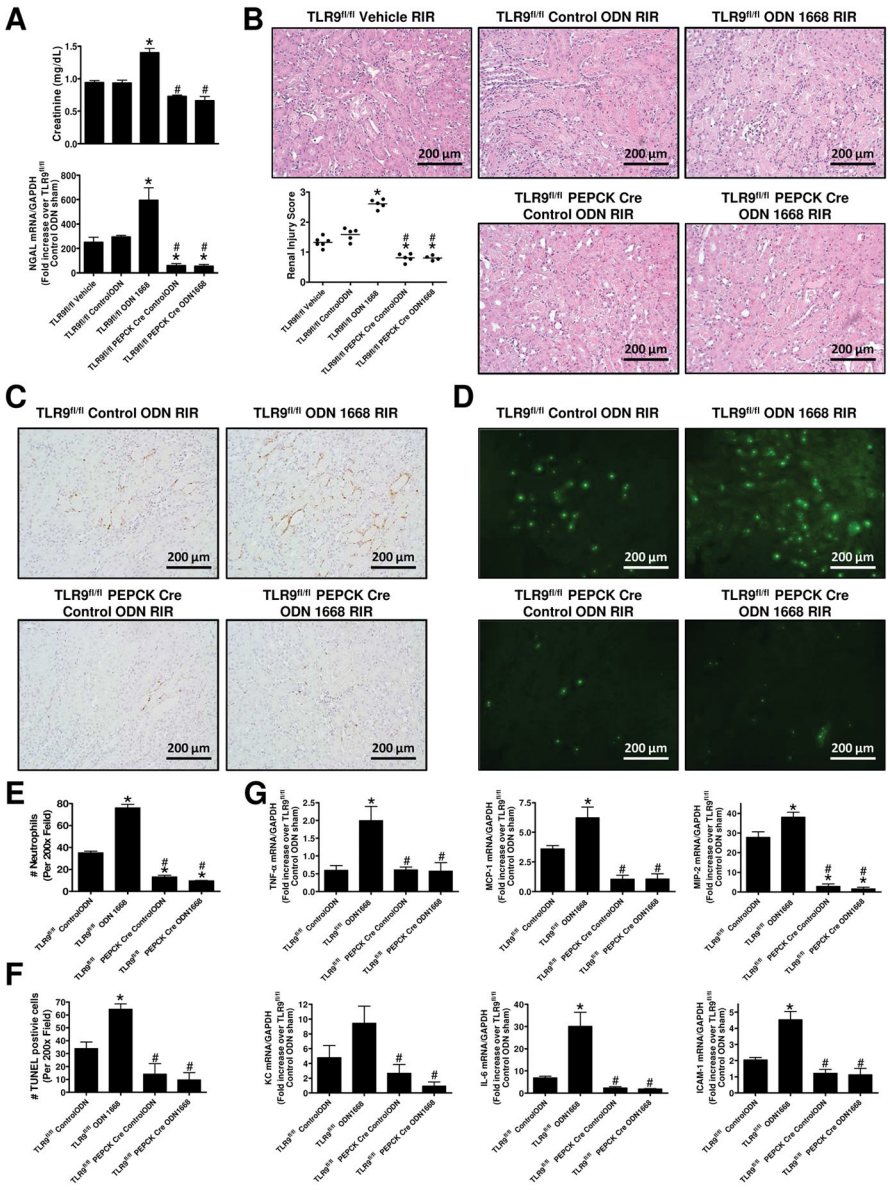

Figure 8. Renal proximal tubular TLR9 activation exacerbates ischemic AKI

$TLR9^{fl/fl}$ mice or renal proximal tubular TLR9 null mice were pretreated with saline (vehicle), control oligonucleotide (control-ODN) or with 1 mg/kg ODN-1668 (a selective TLR9 agonist) and subjected to 20 min renal ischemia and 24 hr reperfusion (IR). A) $TLR9^{fl/fl}$ mice subjected to 20 min. renal IR after ODN-1668 treatment had significantly increased plasma creatinine as well as kidney NGAL mRNA compared to control ODN-treated mice (N=5–6). In contrast, not only mice lacking renal proximal tubular TLR9 had reduced renal injury after 20 min renal IR, ODN-1668 treatment failed to exacerbate ischemic AKI in these mice (N=5–6). B) Representative H&E images (magnification 200X) and scatter plot renal injury scores (N=5–6). Representative images of immunohistochemistry for neutrophils (C, 200X) and counts of infiltrating kidney neutrophils (E) (N=5–6). Representative images TUNEL staining (D, 200X) and counts of

TUNEL positive kidney cells (F) (N=5–6). G) Quantitative RT-PCR measured the expression of pro-inflammatory cytokine and chemokine mRNAs in the kidney [KC, MCP-1, MIP-2, TNF-α, IL-6 and ICAM-1] Fold increases in pro-inflammatory mRNAs normalized to GAPDH from quantitative RT-PCR reactions of control ODN-treated sham-operated mice for each indicated mRNA (N=5–6) are shown. For statistical analysis, the one-way ANOVA plus Tukey's *post hoc* multiple comparison test were used to detect significant changes. *$P < 0.05$ vs. control ODN-treated $TLR9^{fl/fl}$ mice subjected to renal IR injury. #$P < 0.05$ vs. ODN-1668-treated $TLR9^{fl/fl}$ mice subjected to renal IR injury. Error bars represent 1 SEM.

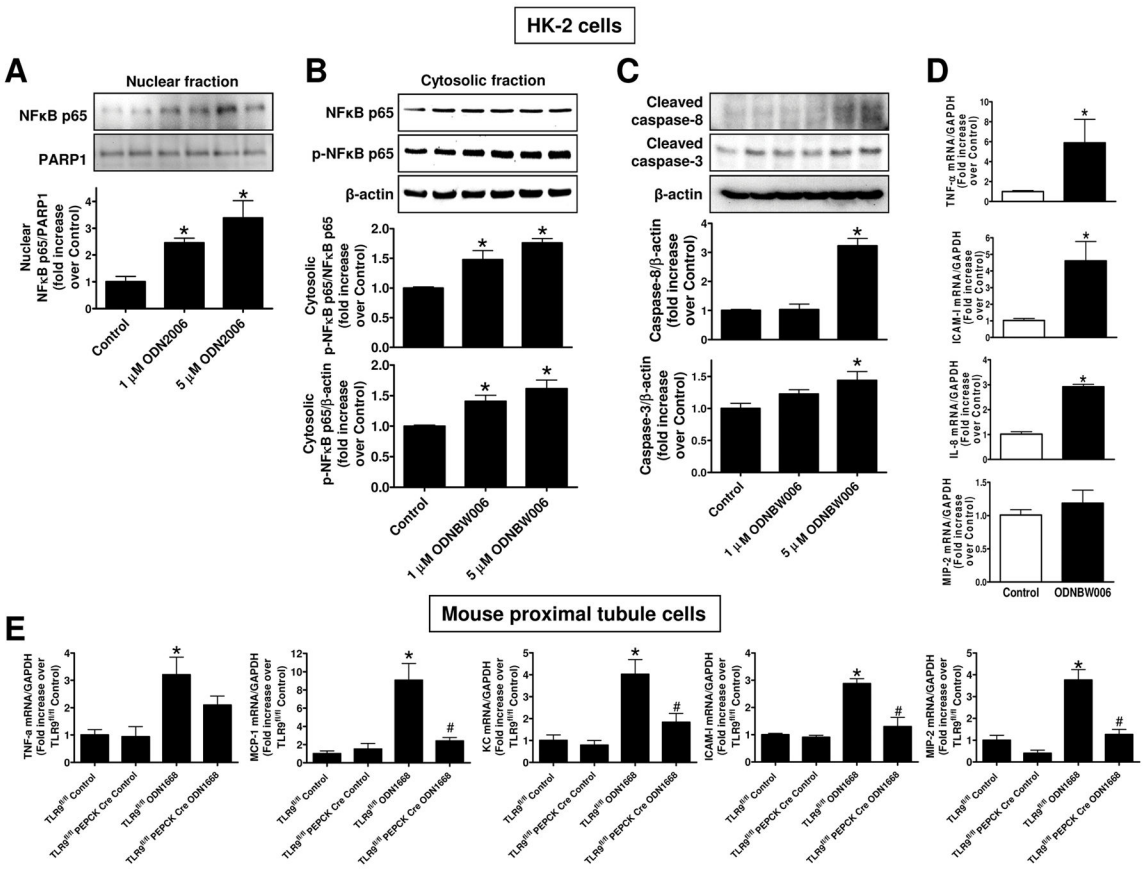

Figure 9. TLR9 agonists induce NFκB activation, caspase cleavage and pro-inflammatory mRNA induction in renal proximal tubule cells

A–D. HK-2 human renal proximal tubule cells were treated with 1–5 μM TLR9 agonist (ODN2006 or ODNBW006) or with respective control oligonucleotide (ODN2137 or ODN BW007) for 3 days. A. A representative immunoblotting experiment for nuclear p65 NFκB subunit (top) and band intensity quantifications normalized to PARP-1 (N=4, bottom). B. A representative immunoblotting experiment for cytosolic p65 subunit (top) and band intensity quantifications normalized to p65 subunit or β-actin (N=4, bottom) are shown. C. A representative immunoblotting experiment for cleaved caspase 3 and caspase 8 (top) and band intensity quantifications normalized to β-actin (N=4, bottom) are shown. D. With quantitative RTPCR, we measured the expression of pro-inflammatory IL-8, TNF-α, ICAM-1 and MIP-2 mRNA expression in HK-2 cells. Fold increases in pro-inflammatory mRNAs normalized to GAPDH from quantitative RTPCR reactions for each indicated mRNA (N=4) are shown. E. Finally, primary cultures of mouse renal proximal tubules from wild type $TLR9^{fl/fl}$ mice and renal proximal tubule TLR9 null mice were treated with a mouse TLR9 selective oligonucleotide ODN1688 for 3 days. With quantitative RTPCR, we measured the expression of pro-inflammatory IL-8, TNF-α, ICAM-1, MCP-1 and MIP-2 mRNA expression in primary cultures of mouse renal proximal tubules. Fold increases in pro-inflammatory mRNAs normalized to GAPDH from quantitative RTPCR reactions for each indicated mRNA (N=4) are shown. For statistical analysis, the one-way ANOVA plus Tukey's *post hoc* multiple comparison test were used to detect significant changes. *P<0.05

vs control oligonucleotide-treated cells. #P<0.05 vs. $TLR9^{fl/fl}$ renal proximal tubule cells treated with ODN1668. Error bars represent 1 SEM.

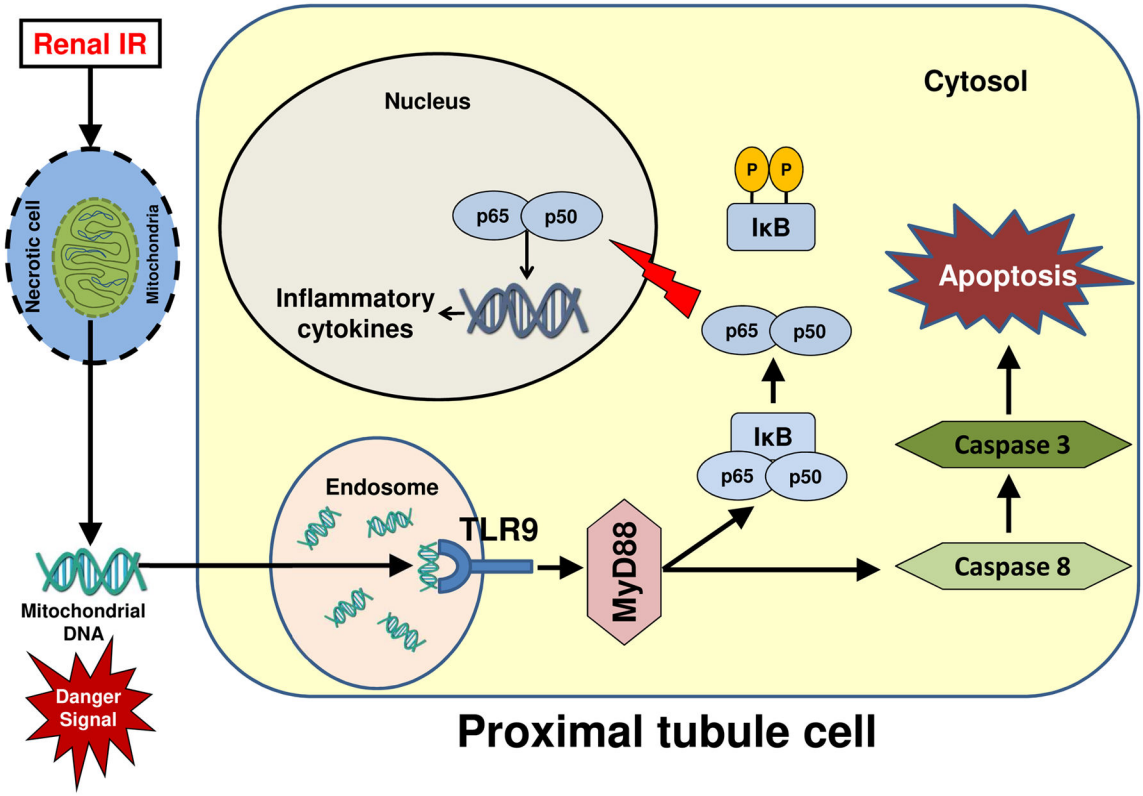

Figure 10. Schematic of proposed mechanisms for renal proximal tubular TLR9-mediated exacerbation of ischemic AKI

After renal ischemia and reperfusion, damaged renal cells release endogenous TLR9 activators (presumably mitochondrial DNA products). Renal proximal tubular TLR9 activation would then lead to NFκB-mediated induction of pro-inflammatory chemokines and cytokines as well as Caspase 3/8-mediated induction of renal tubular apoptosis.

Table 1

Primers used in genotyping to amplify mouse genomic DNAs based on published GenBank sequences for mouse. Annealing temperatures used for each primer are also provided.

Primers	Sequence (Sense/Antisense)	Annealing Temp (°C)
TLR9 FLOX	5′-CAAGGAGAATCCAGGAGGCTAGTG-3′ 5′-GGAGAACCTGTGAGAGCCAGG-3′	59 °C
PEPCK Cre	5′-TGGGCGGCATGGTGCAAGTT-3′ 5′-CGGTGCTAACCAGCGTTTTC-3′	60 °C

Table 2

Primers used in quantitative reverse transcription polymerase chain reactions to amplify mouse cDNAs based on published GenBank sequences. Annealing temperatures used for each primer are also provided.

Primers	Sequence (Sense/Antisense)	Annealing Temp (°C)
mouse TLR9	5′-CCAGTTTGTCAGAGGGAGCC-3′ 5′-GGACAGGTGGACGAAGTCAG-3′	66 °C
mouse TNF-α	5′-TACTGAACTTCGGGGTGATTGGTCC-3′ 5′-CAGCCTTGTCCCTTGAAGAGAACC-3′	65 °C
mouse MCP-1	5′-ACCTGCTGCTACTCATTCAC-3′ 5′-TTGAGGTGGTTGTGGAAAAG-3′	60 °C
mouse MIP-2	5′-CCAAGGGTTGACTTCAAGAAC-3′ 5′-AGCGAGGCACATCAGGTACG-3′	60 °C
mouse IL-8	5′-CAATGAGCTGCGCTGTCAGTG-3′ 5′-CTTGGGGACACCTTTTAGCATC-3′	60 °C
mouse IL-6	5′-CCGGAGAGGAGACTTCACAG-3′ 5′-GGAAATTGGGGTAGGAAGGA-3′	62 °C
mouse NGAL	5′-CACCACGGACTACAACCAGTTCGC-3′ 5′-TCAGTTGTCAATGCATTGGTCGGTG-3′	66 °C
mouse ICAM-1	5′-TGTTTCCTGCCTCTGAAGC-3′ 5′-CTTCGTTTGTGATCCTCCG-3′	60 °C
human TNF-α	5′-CGGGACGTGGAGCTGGCCGAGGAG-3′ 5′-CACCAGCTGGTTATCTCTCAGCTC-3′	68 °C
human IL-8	5′-TCTGCAGCTCTGTGTGAAGG-3′ 5′-ATTGCATCTGGCAACCCTAC-3′	64 °C
human ICAM-I	5′-GCAGACAGTGACCATCTACAGC-3′ 5′-GCCATCCTTTAGACACTTGAGC-3′	60 °C
human MIP-2	5′-CTTGCCAGCTCTCCTCCTC-3′ 5′-GCTTTCTGCCCATTCTTGAG-3′	64 °C
GAPDH	5′-ACCACAGTCCATGCCATCAC-3′ 5′-CACCACCCTGTTGCTGTAGCC-3′	65 °C