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Complement Activation and Toll-Like Receptor-2 Signaling Contribute to Cytokine Production After Renal Ischemia/Reperfusion

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Abstract

The innate immune system causes tissue inflammation and injury after renal ischemia/reperfusion (I/R). The complement system is activated on ischemic tubular epithelial cells (TECs) and induces the cells to produce pro-inflammatory chemokines. TECs also express toll-like receptors (TLRs) -2 and -4. Signaling through the TLRs induces TECs to produce a variety of chemokines, some of which can also be induced by complement activation fragments. We sought to determine whether the effects of complement activation and TLR signaling in TECs are redundant, or whether additive protection can be achieved by blocking both of these innate immune systems. To confirm that the complement system, TLR-2 signaling, and TLR-4 signaling induce production of a similar repertoire of inflammatory chemokines, we stimulated TECs with complement sufficient serum or with TLR-2 and TLR-4 ligands *in vitro*. We found that all three of these stimuli induce TECs to produce KC, MIP-2, IL-6, and TNF- α , and that there was a trend towards greater production of KC in cells exposed to two stimuli. Based upon these results, we hypothesized that mice deficient in both complement activation and TLR-2 signaling would demonstrate greater protection from I/R than mice deficient only in the complement system. To test this hypothesis we induced ischemic acute kidney injury (AKI) in wild-type mice, mice with targeted deletion of complement factor B (*fB*^{-/-} mice), or mice with targeted deletion of factor B and TLR-2 (*fB*^{-/-} *TLR2*^{-/-} mice). Surprisingly, we found that *fB*^{-/-} *TLR2*^{-/-} mice developed more severe injury than those with single deficiency of factor B. Our results indicate that blockade of the complement system may be more protective than simultaneous blockade of both the complement system and TLR-2 in ischemic AKI.

Keywords

acute kidney injury; innate immunity; complement; toll-like receptor

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1. Introduction

AKI is a common clinical condition that is associated with increased mortality (Hoste and Kellum, 2006; Levy et al., 1996). Numerous innate immune factors have been demonstrated to contribute to renal injury after I/R, including the alternative pathway of complement (Thurman et al., 2003), TLR-2 (Leemans et al., 2005), TLR-4 (Wu et al., 2007), various cytokines and chemokines (Hoke et al., 2007; Thurman et al., 2007), as well as neutrophils and macrophages (Kelly et al., 1996; Lee et al., 2011). These immune factors can cause direct cytotoxic injury of the TECs, and they may indirectly exacerbate renal injury by inducing the TECs to elaborate other downstream inflammatory signals (Daha and van Kooten, 2000; Gerritsma et al., 1996; Leemans et al., 2005; Pulskens et al., 2008; Thurman et al., 2007). In addition to their effects upon the kidney, the numerous inflammatory mediators generated or activated within the post-ischemic kidney may also mediate remote organ injury, potentially explaining the link between AKI and increased mortality (Hoke et al., 2007).

The TLRs and the complement system represent two important arms of the innate immune system. Complement and the TLRs are rapidly activated in response to conserved molecular patterns on pathogens, and both systems generate pro-inflammatory signals important for the early immune defense against these microorganisms (Trinchieri and Sher, 2007; Walport, 2001). Damage to host cells can also trigger activation of both of these immune systems. Impaired expression of complement regulatory proteins on the surface of tubular epithelial cells is sufficient to activate the alternative pathway of complement on the surface of TECs (Renner et al., 2010; Thurman et al., 2006a). Endogenous ligands released by damaged cells can function as ligands for the TLRs. High-mobility group box 1 (HMGB1), for example, is released by necrotic cells and was recently shown to cause TLR-4-mediated injury after renal I/R (Wu et al., 2010). Given their important role in the early recognition of invasive pathogens and injured cells, the complement system and the TLRs are likely to be proximal initiators of the inflammatory response. Consequently, both systems may be effective therapeutic targets for ischemic AKI.

It is also noteworthy that the complement system, TLR-2, and TLR-4 can each trigger TECs to produce the same pro-inflammatory chemokines. We recently showed that signaling through C3a induces TECs to produce keratinocyte-derived chemokine (KC or CXCL1) and MIP-2 (CXCL2) (Thurman et al., 2007). TLR-2 and -4 ligands also induce TECs to produce KC and TNF- α (Chowdhury et al., 2006), and mice deficient in either TLR-2 or TLR-4 mice produce less KC, MIP-2, MCP-1, TNF- α and IL-6 in the kidney after renal I/R (Leemans et al., 2005; Wu et al., 2007). These findings support the concept that the complement system and TLRs are proximal sensors of tissue injury, and that activation of these systems occurs early in the inflammatory response generated by renal I/R.

In spite of the redundancy of these immune systems in relation to the downstream cytokines and chemokines they trigger, blockade of each of these systems in isolation is protective in animal models of AKI (Leemans et al., 2005; Thurman et al., 2003; Thurman et al., 2006b; Wu et al., 2007). This suggests that these pathways may be additive and that activation of multiple systems is required for the full inflammatory response. We have previously found that mice deficient in the alternative pathway of complement are partially protected in a model of ischemic AKI (Thurman et al., 2003). We hypothesized that complement activation and TLR signaling each independently contribute to the inflammatory response within the renal tubulointerstitium, and that mice deficient in both systems would show greater protection from ischemic AKI than mice with isolated deficiency of the alternative pathway of complement. To test this hypothesis, we grew TECs from wild-type and *TLR2*^{-/-} mice in order to examine the *in vitro* production of cytokines by the cells in

response to TLR ligands and complement sufficient serum. We also bred double knockout mice that lack both TLR-2 and the alternative pathway of complement ($fB^{-/-}$ $TLR2^{-/-}$ mice) and compared them to $fB^{-/-}$ mice in a model of ischemic AKI.

2. Materials and Methods

2.1 Mice

Factor B-deficient mice were generated as previously described (Matsumoto et al., 1997). $TLR2^{-/-}$ mice and C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). The $fB^{-/-}$ and $TLR2^{-/-}$ mice were crossed to generate $fB^{-/-}$ $TLR2^{-/-}$ double knockout mice. The mice were housed and maintained in the University of Colorado Center for Laboratory Animal Care in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and all animal procedures were in adherence to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

2.2 Protocol for induction of renal I/R

Renal I/R was induced as previously described (Thurman et al., 2003). Briefly, eight to ten week old male mice were anesthetized with 300 μ l of 2,2,2-tribromoethanol (Sigma-Aldrich, St. Louis, MO) injected intra-peritoneally. Mice were placed on a heating pad to maintain their body temperature during surgery. Laparotomies were then performed and the renal pedicles were isolated and clamped with surgical clips (Miltex Instrument, Bethpage, NY). Ischemia of the kidneys was confirmed by visual inspection of the kidneys. The vessels were occluded for 24 minutes and then released. The kidneys were observed for approximately one minute to ensure blood re-flow, then fascia and skin were sutured with 4-0 silk (United States Surgical, Norwalk, CT). Sham surgery was performed in an identical fashion, except that the renal pedicles were not clamped. The mice were volume resuscitated with 0.5 ml of normal saline injected subcutaneously, and the mice were kept in an incubator at 29°C to maintain body temperature for two hours after the surgery. After 24 hours the mice were anesthetized, and blood was obtained by cardiac puncture. Laparotomy was performed and the kidneys were harvested and snap frozen in liquid nitrogen.

2.3 Renal function

Serum was collected after 24 hours of reperfusion and stored at -80°C. Renal function was assessed by measurement of the serum urea nitrogen (SUN) using a Beckman Autoanalyzer (Beckman, Fullerton, CA).

2.4 Renal histopathology and immunofluorescence

Kidneys were cut in half sagittally and fixed in 4% formaldehyde. Five μ m sections were cut from paraffin-embedded blocks and stained with Periodic Acid Schiff (PAS). For immunofluorescence, kidney halves cut sagittally were snap frozen optimum cutting temperature compound (OTC, Sakura Finetek, Torrance, CA). Five μ m kidney sections were air dried and stored at -80°C until use. For immunostaining, the slides were warmed to room temperature, fixed in ice-cold acetone, washed with PBS, and blocked with 10% goat serum. The sections were then incubated with Alexa-Fluor 488 conjugated rat anti mouse CD11b (Invitrogen-Molecular Probes, Eugene, OR) diluted 1:100 for detection of polymorphonuclear leukocytes. Sections were counterstained with hematoxylin and mounted using Vecta Shield media with DAPI (Vector Laboratories, Burlingame, CA). Sections were examined and scored under high power in a blinded fashion to evaluate PMN infiltration in 10 high-powered fields in the outer-medulla using a Nikon T2000 inverted fluorescent microscope and Slidebook software (Intelligent Imaging Innovations). The number of CD11b positive cells was expressed as an average per high power field (400X).

2.5 Cell culture

Tubular epithelial cells were isolated from wild-type and *TLR2*^{-/-} mice as previously described (Renner et al., 2010). Briefly, blocks of cortical tissue were finely minced under sterile conditions using sterile blades and suspended into 6 mL of 1 mg/mL Type 1A Collagenase (Sigma) in PBS. The suspensions were incubated at 37° C for 10 minutes and then gently mixed for 1 minute. This procedure was repeated three times. The cell suspension was then passed sequentially over 100 µm, 70 µm, and 40 µm filters (BD Biosciences). The filtered cells were centrifuged at 1000 rpm for 5 minutes and were then resuspended in epithelial growth medium [DMEM/F12 (Invitrogen), 2% heat-inactivated fetal calf serum (Hyclone), Insulin 5 mg/L (Gibco), Transferrin 5 mg/L (Invitrogen), 10⁻¹²M T3 (Sigma), 1% Penicillin-Streptomycin (Invitrogen), and Hydrocortisone 40 ng/L (Sigma)]. Monolayers of mouse TECs were cultured until they achieved a transepithelial resistance (TER) >1,000Ω cm² (EVOM volt-ohmmeter from World Precision Instruments). Immortalized murine TECs (BUMPT) were cultured as described elsewhere (Doctor et al., 2005).

2.6 Reporter activity assays

A construct containing a tandem repeat of the cis region for the NF-κB binding site and firefly luciferase was kindly given to us by Dr. David Riches (National Jewish Medical Center, Denver). Plasmids were transfected into BUMPT cells using Lipofectamine 2000 (Life Technologies/Invitrogen; Gaithersburg, MD), as directed by the manufacturer. As a control for transfection efficiency, a plasmid containing a renilla luciferase reporter gene under the TK promoter was co-transfected into the cells (Amura et al., 2008). After 18–24 h following transfection, media was changed to serum-free media and cells were incubated for an additional 24 hrs. Subsequently, cells were stimulated as indicated and cell extracts were then prepared in 500 µl of Luciferase Passive Lysis Buffer (Promega; Madison, WI). Both firefly and renilla luciferase activities were assayed in 60 µl lysate aliquots using the Dual Luciferase Kit (Promega; Madison, WI) and detected in a 96 well-plate luminometer (Luminoskan Ascent, Thermo Electron Corporation; Franklin, MA). Firefly luciferase was normalized to renilla luciferase activity in each group.

2.7 Western blot analysis

Analysis of phosphorylated IκBα in mouse TEC cell lysates was performed by Western blot analysis. Samples were solubilized in RIPA buffer, and separated by SDS-Page on a 10% polyacrylamide gel (Invitrogen). The proteins were transferred to a nitrocellulose membrane. Phospho-IκBα was detected using a peroxidase-conjugated polyclonal goat anti-mouse antibody. The antibody was then detected by enhanced chemiluminescence (Pierce, Rockford, IL).

2.8 Determination of cytokine production

To evaluate the secretion of KC, MIP-2, IL-6, and TNF-α from mouse TECs, culture supernatants were collected from treated TECs derived from C57BL/6 or *TLR2*^{-/-} mice. The media was changed to serum-free media 24 hours prior to the addition of complement sufficient serum, Pam3Cys, or LPS. Supernatants were collected 8 hours after adding the stimuli. The concentrations of the various cytokines were measured using commercially available ELISA kits (ELISATech, Aurora, CO) according to the manufacturer's protocol. Absorbance at 405 nm was measured using a microplate reader and cytokine levels were correlated to an internal standard curve.

Real time-quantitative PCR (qPCR) was performed to analyze chemokine mRNA synthesis in stimulated cells and in mouse kidneys. Total RNA (1 µg) isolated from tissues using

Trizol (Invitrogen) was reverse transcribed using iScript (Roche) according to the manufacturer's protocol. Aliquots (2.5 μ l) of 1:10 dilutions of reverse transcription reactions were subjected to PCR in 25- μ l reaction mixtures using 2X Power Sybr Green PCR Master Mix (Applied Biosystems, Foster City CA) and the primers listed in Table 1 using a Roche 480 Thermal Cycler (Roche, CA). Primers were designed using a Beacon Designer Program (Premier Biosoft International). Cyclophilin mRNA levels were used as a control housekeeping gene for normalization of the different mRNA expression levels, and the corrected mRNA levels are presented in arbitrary units. The level of expression of each gene for each animal is reported as fold-increase compared to the level in a sham treated wild-type mouse that was arbitrarily set at one.

2.9 Statistical analyses

Comparison between multiple groups was performed with ANOVA. Comparison between two groups was performed using unpaired T-tests. The analysis and graphing were performed using GraphPad PRISM 5 software (San Diego, CA). A *P* value of <0.05 was considered statistically significant. Results are reported as means $\pm$ SEM.

3. Results

3.1 Complement-sufficient serum, TLR-2, and TLR-4 induce production of cytokines by TECs in culture

We grew TECs from wild-type and *TLR2*^{-/-} mice in primary culture. We stained the cells for cytokeratin to confirm their epithelial cell phenotype (Figure 1A). We also confirmed that TECs in primary culture synthesize KC after stimulation with complement sufficient serum, Pam3Cys, or LPS as has been previously reported (Figure 1C) (Leemans et al., 2005; Thurman et al., 2007; Wu et al., 2007). Next, we treated the cells with complement-sufficient serum, Pam3Cys (a TLR-2 agonist), LPS (a TLR-4 agonist), serum and Pam3Cys together, or serum and LPS together. We then measured the levels of KC, MIP-2, IL-6, and TNF- α in the supernatants of treated cells (Figure 2). The cells responded to all of the stimuli by producing these cytokines. There was a trend towards greater KC production in the cells treated with serum and LPS compared to cells receiving either treatment alone, suggesting that these stimuli may have an additive effect, although this did not reach statistical significance. Interestingly, the production of KC in response to serum, LPS, and serum with LPS was attenuated in the *TLR2*^{-/-} cells, suggesting that autocrine or paracrine signaling through TLR-2 may contribute to full KC production in response to complement activation or TLR-4 stimulation. Furthermore, *TLR2*^{-/-} cells treated with serum and LPS produced less KC than wild-type cells treated with serum and LPS. The *TLR2*^{-/-} cells were isolated from *TLR2*^{-/-} mice using the same method as was used for TECs isolated from wild-type mice. Nevertheless, these cells were isolated from different mice and were isolated during a separate procedure. Compared to control, treatment of wild-type cells with complement-sufficient serum, Pam3Cys, or LPS all induced production of MIP-2 (Figure 2B). There were no differences between the groups, however, when all of the treatment groups were compared by ANOVA, and the same was true for TNF- α and IL-6 (Figures 2C and 2D). As with KC, the production of TNF- α in response to LPS appeared attenuated in cells derived from and *TLR2*^{-/-} mice (Figure 2D).

3.2 Complement activation, TLR-2 stimulation, and TLR-4 stimulation activate NF- κ B in renal tubular epithelial cells

We have previously demonstrated that complement activation induces MIP-2 and KC production by TECs in culture, and that inhibition of NF- κ B attenuates this response (Thurman et al., 2007). We grew TECs to confluence and stimulated them with complement-sufficient serum, Pam3Cys, or LPS. We found that stimulation with either

Pam3Cys or LPS caused translocation of the NF- κ B P65 subunit to the nucleus (Figures 3C and 3D), but this effect was not seen in cells treated with complement sufficient serum (Figure 3B). In immortalized cells transfected with an NF- κ B reporter element, however, all three stimuli elicited NF- κ B activation (Figure 3E). Furthermore, stimulation of the cells with complement sufficient serum induced phosphorylation of I κ B, but phosphorylation of this protein was attenuated if alternative pathway activation in the serum was blocked with an inhibitory antibody (Figure 3F), confirming that complement activation induces NF- κ B activation in TECs. P65 is part of the “canonical” pathway of NF- κ B signaling (Beinke and Ley, 2004). Given that complement activation induces NF- κ B activation in TECs without translocation of P65 to the nucleus, it is possible that it activates this signaling cascade through one of the alternative activation pathways (Beinke and Ley, 2004). Interestingly, stimulation of the cells with LPS or Pam3Cys did not cause detectable phosphorylation of I κ B at the same time-point (24 hours), and degradation of I κ B was not observed in response to any of the stimuli.

3.3 Mice with targeted genetic deletion of both factor B and TLR2 ($fB^{-/-}$ TLR2 $^{-/-}$ mice) are not protected from ischemic AKI

We have previously found that mice deficient in factor B are protected from ischemic AKI (Thurman et al., 2003). Two other groups have independently found that mice deficient in TLR-2 are protected from ischemic AKI in models similar to the one that we used (Leemans et al., 2005; Shigeoka et al., 2007). We have also subjected TLR2 $^{-/-}$ single knockout mice to renal I/R. We found that these mice are not protected from injury in our model. The SUNs in these mice after 24 hours of reperfusion were similar to that seen in wild-type mice (187.5 ± 7.8 mg/dL in TLR2 $^{-/-}$ mice versus 168.6 ± 13.0 in wild-type mice, $P = \text{NS}$). The difference in results between our experiment and the protective effect seen by other investigators may be explained by subtle experimental differences or by differences in the local conditions.

We next crossed these two strains to generate $fB^{-/-}$ TLR2 $^{-/-}$ double knockout mice, and we subjected these mice to renal I/R. Histologic examination of the kidneys demonstrated severe tubular necrosis in the outer medullas of wild-type (Figure 4A) and $fB^{-/-}$ TLR2 $^{-/-}$ mice (Figure 4C). Less extensive necrosis was seen in the kidneys of $fB^{-/-}$ mice (Figure 4B). We measured SUN as a measure of renal function. As we have previously found, the SUNs in $fB^{-/-}$ mice after 24 hours of reperfusion were lower than those of wild-type mice. The $fB^{-/-}$ TLR2 $^{-/-}$ mice, however, had SUN values similar to those seen in wild-type mice.

We stained kidney sections from the different strains of mice for CD11b as a marker of leukocyte infiltration, and we found that both strains of the knockout mice ($fB^{-/-}$ mice and $fB^{-/-}$ TLR2 $^{-/-}$ mice) had reduced infiltration of the kidneys with CD11b leukocytes relative to wild-type controls. These surprising results suggest that mice lacking both the complement system and TLR-2 signaling develop more severe ischemic AKI than mice with isolated deficiency of the complement system, even though infiltration of the kidneys with leukocytes is still attenuated. It is possible that some of the positively stained cells are intrinsic renal myeloid cells (e.g. dendritic cells), and represent differences in these cell types between the mouse strains.

3.4 Targeted genetic deletion of both factor B and TLR2 exacerbates the production of inflammatory cytokines and chemokines after renal I/R

Mice lacking TLR-2 generate lower levels of KC and IL-6 within the kidneys after I/R than do wild-type mice (Leemans et al., 2005; Shigeoka et al., 2007). TLR-2 may also mediate MIP-2 and TNF- α production in the kidneys after I/R (Leemans et al., 2005). We performed RT-PCR to investigate the production of these inflammatory mediators in the kidneys of

fB^{-/-} *TLR2*^{-/-} mice (Figure 5). A trend towards lower mRNA levels for all of the genes was observed in sham treated *fB*^{-/-} and *fB*^{-/-} *TLR2*^{-/-} mice relative to sham treated wild-type mice, although the differences did not achieve statistical significance. We found a reduction in the production of these cytokines in *fB*^{-/-} mice compared to wild-type controls after I/R. However, we found higher levels of mRNA for KC, MIP-2, and TNF- α in the kidneys of *fB*^{-/-} *TLR2*^{-/-} mice compared to *fB*^{-/-} single knockout mice, and the values were similar to those seen in wild-type mice subjected to I/R. These findings indicate that the production of these cytokines is higher in *fB*^{-/-} *TLR2*^{-/-} mice than they are in *fB*^{-/-} mice. This finding indicates that complement and/or TLR-2 may also have a counter-regulatory role in production of these inflammatory mediators. It also indicates that production of these cytokines in the *fB*^{-/-} *TLR2*^{-/-} mice is not sufficient to cause infiltration of the kidneys by leukocytes.

4. Discussion

The complement system and TLR-2 signaling contribute to the pathogenesis of ischemic AKI. These distinct innate immune systems have independently been demonstrated to trigger TECs to produce cytokines and chemokines such as KC (Chowdhury et al., 2006; Leemans et al., 2005; Thurman et al., 2007). Surprisingly, we found that mice with deficiency of both of these factors (*fB*^{-/-} *TLR2*^{-/-} mice) are not protected from ischemic AKI, and do not demonstrate reduced production of KC, MIP-2, IL-6, or TNF- α in the post-ischemic kidney. These results suggest that although complement and TLR-2 induce pro-inflammatory signals, they may also have counter-regulatory effects on other inflammatory pathways. Thus, blockade of both pathways does not result in additive protection, and it appears to be less effective at isolated blockade of the alternative pathway of complement.

We also used TECs in culture to examine the direct effects of complement activation and TLR-2 stimulation on chemokine production by TECs. We found that complement sufficient serum, TLR-2 stimulation, and TLR-4 stimulation all induced NF- κ B activation. The TLR agonists caused translocation of P65 to the nucleus of the cells but complement activation did not, suggesting that complement-induced NF- κ B activation occurs through a different pathway. Nevertheless, all three pathways induced the production of an overlapping repertoire of cytokines. The production of KC by TECs after stimulation with serum and TLR agonists appeared to be additive, although this did not reach statistical significance. This could potentially explain why deficiency of one factor is sufficient to reduce the production of KC *in vivo*, even if the other pathways are intact. We also found that TECs that lack TLR-2 produce less KC and TNF- α after stimulation with serum or with LPS, suggesting that some of the effects of these stimuli may be mediated by signaling through TLR-2. Of note, it has previously been shown that TLR-4 agonists can induce signaling through TLR-2 (Fan et al., 2003).

This study has several limitations. The cell culture experiments do not provide a clear answer as to why production of these downstream targets is higher in the *fB*^{-/-} *TLR2*^{-/-} mice than in the *fB*^{-/-} mice. The production of pro-inflammatory cytokines and chemokines in response to LPS indicates that signaling pathways other than the complement or the TLR-2 pathway are likely to be involved *in vivo*, and the attenuated production of these same cytokines in TLR-4 deficient mice supports this hypothesis (Wu et al., 2007). Several signals have been demonstrated to negatively regulate the inflammatory of TLRs, including modulation by other TLRs (Bagchi et al., 2007; Lee and Kim, 2007). Although the net effect of complement activation and TLR-2 signaling within the kidney after I/R is pro-inflammatory, it is possible that these systems also engage anti-inflammatory mechanisms. Studies are underway to identify potential mechanisms of this cross-talk, as strategies to

block the pro-inflammatory mediators of injury while leaving the counter-regulatory systems intact may lead to more effective therapies.

Another question raised by these studies is that even though the levels of the chemokines KC and MIP-2 in *fB*^{-/-} *TLR2*^{-/-} mice were comparable to those seen in wild-type mice, there were still fewer CD11b leukocytes in the kidneys of *fB*^{-/-} *TLR2*^{-/-} mice than in wild-type mice after I/R. Although KC and MIP-2 have strong chemotactic activity, they can exert pathologic effects on other cell types such as endothelial cells. It is possible, therefore, that these chemokines contribute to renal injury through their actions on non-hematopoietic targets such as renal endothelial cells, or that injury is mediated by other downstream effectors. Furthermore, these results demonstrate that even in the presence of KC and MIP-2, other chemotactic factors can cause leukocyte infiltration of the kidneys.

In conclusion, we have found that complement activation, TLR-2, and TLR4 signaling can each induce TECs to produce an overlapping array of pro-inflammatory cytokines. We also found that complement-induced production of some cytokines by TECs *in vitro* seems to involve autocrine/paracrine signaling through TLR-2. Mice that lack both of these systems, however, demonstrate increased cytokine production in the kidney after renal I/R, and develop more severe renal injury than mice deficient in the complement system only. These results indicate an interaction of these two innate immune systems, and raise the possibility that counter-regulatory systems dampen the pro-inflammatory effects of simultaneous signaling through complement activation fragments, TLR-2, and TLR-4 at the level of the TEC. Thus, inhibition of the alternative pathway of complement may be more protective than simultaneous inhibition of this pathway and TLR2 signaling. However, identification of the signals that mediate cross-talk between these systems may enable the development of new therapies that more efficiently block the pro-inflammatory effects of these systems while leaving the anti-inflammatory effects intact.

Acknowledgments

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Abbreviations

TLR	toll-like receptor
I/R	ischemia-reperfusion
KC	keratinocyte-derived chemokine,

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Highlights

- Mice deficient in either the alternative pathway of complement, TLR-2, or TLR-4 have previously been shown to be protected from ischemic acute kidney injury.
- The complement system, TLR-2, and TLR-4 all induce renal tubular epithelial cells to produce pro-inflammatory cytokines, including KC, MIP-2, IL-6, and TNF- α .
- The complement system, TLR-2, and TLR-4 all induce activation of NF- κ B in renal tubular epithelial cells.
- Mice deficient in the alternative pathway of complement are protected from ischemic acute kidney injury, but mice deficient in both the alternative pathway of complement and TLR-2 are not.
- Mice deficient in the alternative pathway of complement generate reduced levels of KC, MIP-2, IL-6, and TNF- α in the kidneys after renal ischemia, but mice deficient in both the alternative pathway of complement and TLR-2 do not.

Figure 1. Tubular epithelial cells grown in primary culture produce KC after stimulation with complement sufficient serum or LPS

Tubular epithelial cells were isolated from C57BL/6 mice and grown in primary culture. Cells were stained with an antibody for (A) cytokeratin or with (B) an isotype control, and greater than 95% of the cells demonstrated staining for cytokeratin. Cells were stimulated with serum, Pam3Cys, or LPS. RT-PCR was performed to measure the levels of mRNA for KC. The level of mRNA for KC was significantly increased in cells treated with serum or LPS. $N = 4-6$ per experiment. $**P < 0.01$, $***P < 0.001$.

Figure 2. Cytokine production in tubular epithelial cells after stimulation with complement or toll-like receptor ligands

Tubular epithelial cells were isolated from C57BL/6 or *TLR2*^{-/-} mice and grown in primary culture. The cells were then treated with complement sufficient serum, 10μg/mL Pam3Cys, or 10ng/mL LPS. Cells were also treated with a combination of serum and Pam3Cys or serum and LPS. After 8 hours supernatants were collected and the levels of (A) KC, (B) MIP-2, (C) IL-6, and (D) TNF-α were measured. *P<0.05, **P < 0.01, and ***P<0.001 compared to control treated cells.

Figure 3. Complement sufficient serum, TLR-2 stimulation, and TLR-4 stimulation all cause activation of NF-κB in tubular epithelial cells

Tubular epithelial cells were grown to confluence and changed to serum free media for 48 hours. The cells were then treated for 8 hours with (A) vehicle control, (B) complement-sufficient serum, (C) 10 μg/mL Pam3Cys, or 10 ng/mL LPS. The cells were then fixed, permeabilized, and stained for P65 (red). Nuclei were stained with DAPI (blue). Treatment with Pam3Cys or LPS caused nuclear translocation of the P65. This experiment was performed three times, and representative images are shown. Original magnification x400. (E) TECs were transfected with a NF-κB luciferase reporter construct and were then exposed to serum, Pam3Cys, or LPS. After 8 hours the luciferase activity was measured and normalized for transfection efficiency. All three stimuli caused an increase in NF-κB activity. (F) TECs were stimulated with LPS, TNF-α, serum, serum with an inhibitory monoclonal antibody to factor B (mAb 1379), Pam3Cys, or control. Phospho-IκBα was measured by Western blot analysis. Exposure of the cells to serum increased phosphorylation of IκBα compared to untreated cells, and this increase was attenuated when complement activity was inhibited. The results shown are from the same Western blot, but the unmanipulated samples was placed next to the serum treated samples for direct comparison.

Figure 4. AKI is attenuated in *fB*^{-/-} mice but not *fB*^{-/-}*TLR2*^{-/-} mice after renal I/R
 Mice were subjected to ischemia and 24 hours of reperfusion. PAS stained kidney sections were examined. (A) wild-type mice demonstrated areas of tubular necrosis. (B) Kidneys from *fB*^{-/-} mice demonstrated tubular injury, although less severe than in wild-type mice. (C) Kidneys from *fB*^{-/-}*TLR2*^{-/-} mice demonstrated tubular necrosis. Original magnification ×200. (D) SUN values were measured. The SUNs in *fB*^{-/-} mice were lower than those of wild type mice, but levels in the serum of *fB*^{-/-}*TLR2*^{-/-} mice were significantly higher than those in *fB*^{-/-} mice and were equivalent to those in wild-type mice. Kidney sections were stained for CD11b leukocytes (E and F). Infiltration of the kidneys of *fB*^{-/-} and *fB*^{-/-}*TLR2*^{-/-} mice was lower than that of wild-type mice. *P<0.05, **P<0.01, ***P<0.001.

Figure 5. Cytokine production is attenuated in the kidneys of *fB*^{-/-} mice after renal I/R, but not in the kidneys of *fB*^{-/-}*TLR2*^{-/-} mice. RT-PCR was performed to measure the levels of mRNA for (A) KC, (B) MIP-2, (C) IL-6, and (D) TNF-α in the kidneys of *fB*^{-/-} and *fB*^{-/-}*TLR2*^{-/-} mice after ischemia and 8 hours of reperfusion. Expression of all 4 factors was increased in all four strains compared to sham treated animals. The levels of KC, MIP-2, and TNF-α were significantly higher in kidneys from *fB*^{-/-}*TLR2*^{-/-} mice compared to *fB*^{-/-} mice. N = 4–6 per group. *P<0.05.

Table 1

PRC primers used for qPCR.

Product	Primer	Sequence
KC	Forward	5'-GGC TGG GAT TCA CCT CAA GAA C-3'
	Reverse	5'-TGT GGC TAT GAC TTC GGT TTG G-3'
MIP-2	Forward	5' ACC AAC CAC CAG GCT ACA G-3'
	Reverse	5'-GCG TCA CAC TCA AGC TCT G-3'
IL-6	Forward	5'-ACCGCTATGAAGRRCCTCTC
	Reverse	5-CCTCTGTGAAGCTCCTCTC
TNF- α	Forward	5-CGTGGAAGTGGCAGAAGAG
	Reverse	5-GGAATGAGAAGAGGCTGAAGC
Cyclo	Forward	5'TGG AGA GCA CCA AGA CAG ACA-3'