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TLR2 Signaling in Tubular Epithelial Cells Regulates NK Cell Recruitment in Kidney Ischemia–Reperfusion Injury

Hye J. Kim,*† Jong S. Lee,*+† Ahra Kim,‡ Sumi Koo,* Hee J. Cha,*†† Jae-A Han,‡ Yoonkyung Do,*† Kyung M. Kim,†‡ Byoung S. Kwon,# Robert S. Mittler,†‡**, Hong R. Cho,*+† and Byungsuk Kwon*†

Damage-associated molecular patterns released from damaged kidney cells initiate postischemic inflammation, an essential step in the progression of kidney ischemia–reperfusion injury (IRI). However, the mechanism that coordinates this highly specific process in ischemic kidneys remains to be clarified. Previously, we demonstrated that CD137 from NK cells specifically stimulates CD137 ligand (CD137L) on tubular epithelial cells (TECs) such that TECs produced the high CXCR2 chemokine levels required for neutrophil chemotaxis. We report in the present study that endogenous TLR2 ligands released from ischemic TECs induce CCR5 chemokine expression, which is critical to promoting NK cell recruitment. By implanting CD137L−/− TECs into the kidney capsule of TLR2−/− mice, we further showed that TLR2-mediated NK cell recruitment is an uncoupled event that can occur independently of CD137L signaling in TECs, which is responsible for recruiting neutrophils. Therefore, our findings identify TECs as both a target for kidney damage and also as a master regulator that actively modulates stepwise signaling, leading to the initiation and amplification of acute sterile inflammation that inflicts kidney IRI. Being clinically important, the signaling pathway of innate receptors in epithelial cells may therefore be a good target to block acute sterile inflammation resulting from tissue damage, including kidney IRI. The Journal of Immunology, 2013, 191: 2657–2664.

Inflammation is a physiological response that manages both external and internal danger signals and restores homeostasis (1). Sterile inflammation, caused by tissue damage without infection, can occur in conditions such as trauma, ischemia, ischemia and reperfusion (IR), or cellular malfunction (1–3). Sensors that recognize molecules released by damaged tissues induce inflammation through signaling that results in the production of inflammatory mediators by tissue-specific immune cells or parenchymal cells (1). TLRs are the most well-known sensors and recognize a number of endogenous molecules released from injured cells (3). Evidence of TLR involvement in kidney ischemia–reperfusion injury (IRI) is accumulating (4–8). Endogenous TLR2 and TLR4 ligands are released from the tubular epithelium after IR (9–11). Tubular epithelial cells (TECs) seem to play a central role in initiating kidney IRI, as they express TLR2 and TLR4. Additionally, a parenchymal cell deficiency of either gene dramatically limits kidney injury following IR and diminishes the associated intrarenal inflammation (4, 5). In addition to this autocrine mechanism, endogenous TLR ligands may act on other cell types such as endothelial cells and innate immune cells in a paracrine manner to promote IRI-associated acute inflammation. TLR2 or TLR4 signaling results in proinflammatory cytokine and chemokine production, but little is known regarding how the TLR inflammatory cascade is triggered by cell–cell interactions among TECs, stromal cells, endothelial cells, and infiltrating leukocytes after IR.

We have previously identified an inflammatory loop between TECs and inflammatory cells during kidney IRI (12). NK cells infiltrate the kidney early after IR and approach the tubular epithelium across the basement membrane, which is rapidly destroyed by IRI (12, 13). Inferring NK cells in turn stimulate TECs to produce CXCR2 chemokines by stimulating CD137 ligand (CD137L) signaling and inducing neutrophil migration (12). Recent studies have revealed a positive feedback among dendritic cells, NKT cells, and neutrophils to amplify acute inflammation leading to kidney IRI (14). Therefore, the events following IR and the complex network of stromal cells, endothelial cells, TECs, and immune cells should be defined to better understand IRI-associated inflammation.

Currently, the regulation of signals that recruit NK cells following IRI is not well understood. The aim of this study was to determine the mechanism of NK cell recruitment to the kidney after IR. We found that TECs produced CCR5 chemokines, which are required for NK cell chemotaxis, early after IR in a TLR2-dependent manner. Moreover, NK cell and neutrophil recruit-
ment by TECs was a sequential and independent event. These results indicate that epithelial cells actively regulate sterile tissue inflammation more than was previously recognized.

**Materials and Methods**

**Mice**

CD137<sup>−/−</sup>, CD137L<sup>−/−</sup>, CCR5<sup>−/−</sup>, and TLR2<sup>−/−</sup> C57BL/6 mice were maintained in a specific pathogen-free facility and used between 7 and 8 wk of age. Wild-type (WT) C57BL/6 mice were purchased from Orient. Animal studies were approved by the University of Ulsan Animal Care and Use Committee.

**Abs and reagents**

Cells or tissue sections were stained with fluorescent- or biotin-conjugated mAbs that specifically recognize CD3, CD11b, CD45, CCL3, CCL4, CCL5, cytokeratin, Gr-1, or NK1.1 (eBioscience). The following chemokine antagonists were purchased from Tocris Bioscience: BX513 hydrochloride (CCR1), RS504393 (CCR2), and DAPTA (CCR5). Purified rabbit anti-rat CX3CR1 was purchased from Torrey Pines Biolabs. The TLR2 ligand Pam3CSK4 and anti-TLR2 mAb were purchased from InvivoGen. Purified anti–high mobility group box 1 (HMGB1) mAb was purchased from BioLegend.

**Induction of kidney IRI**

Kidney IRI was induced as previously described (12). In brief, following anesthesia, bilateral flank incisions were performed, both kidney arteries were exposed and cross-clamped for 35 min, and reperfusion was allowed for 24 h through clamp release. A sham operation was performed using the same surgical procedure without clamping the renal artery. During the surgery, mice were placed on a 37˚C heat pad.

**Preparation of kidney cells**

Kidneys were perfused, minced, and placed in DMEM containing 1 mg/ml collagenase IA (Sigma-Aldrich) at 37˚C for 30 min. Digested kidney tissues were passed through a 40-μm cell strainer (BD Falcon) using the rubber end of a 1-ml syringe plunger, and a cell suspension was obtained via centrifugation at 1200 rpm for 10 min. Cells were washed in PBS containing 2% BSA and suspended in 36% Percoll (Amersham Pharmacia Biotech). The suspension was gently overlaid onto 72% Percoll and centrifuged at 2000 rpm at room temperature for 30 min. Cells were retrieved from the Percoll interface and washed twice in DMEM medium and once with staining buffer (PBS containing 0.2% BSA and 0.1% sodium azide).

**Flow cytometry**

Prepared cells were preincubated in blocking buffer (PBS containing anti-CD16/CD32 [2.4G2] mAbs, 0.2% BSA, 0.01% sodium azide) for 20 min at 4˚C. After washing twice with staining buffer, cells were incubated with the relevant mAbs for 30 min at 4˚C. They were then washed twice with staining buffer and analyzed using FACS Canto II (BD Biosciences).

**Analysis of renal function**

Kidney function was determined by measuring creatinine and blood urea nitrogen (BUN) concentrations in sera obtained at various time points after kidney IRI. Creatinine concentrations were measured enzymatically using either a creatinine colorimetric/fluorometric assay kit (BioVision) or the IDTox creatinine assay kit (ID Labs). BUN concentrations were measured colorimetrically using the QuantiChrom urea assay kit (Bioassay Systems) according to the manufacturer’s instructions.

**Pathological scoring**

Kidneys were fixed in 10% formalin, embedded in paraffin, and sectioned (5 μm). Paraffin sections were stained with H&E and analyzed. Kidney injury was scored by a single pathologist (H.J.C.) as the percentage of damaged tubules in the corticomedullary junction. Criteria for kidney injury included tubular necrosis, cast formation, loss of brush border, tubular dilatation, and immune cell infiltration. Scoring for each category was as follows: 0, no change; 1, < 10%; 2, 11–25%; 3, 25–45%; 4, 46–75%; 5, >76% area change. Scores for all the categories were added for the final injury scoring.
Isolation of NK cells

Single-cell suspensions in PBS were prepared from the spleen, filtered through a sterile mesh (BD Falcon), and washed. After erythrocytes were lysed in hemolysis buffer (144 mM NH₄Cl and 17 mM Tris-HCl [pH 7.2]), the remaining cells were resuspended in MACS buffer (1× PBS containing EDTA and 3% calf serum) and incubated with anti-CD3 microbeads for 20 min on ice. CD3⁺ cells were depleted using MACS (Miltenyi Biotec). The remaining cells were stained with anti-NK1.1 mAbs. Labeled cells were sorted using the MoFlo XDP cell sorter (Beckman Coulter). The purities of the remaining cells were stained with anti-NK1.1 mAbs. Labeled cells were then implanted beneath the kidney capsule.

Culture of TECs

Primary TECs were obtained from kidneys after being digested with collagenase IA for 30 min. All TECs were grown in complete DMEM/Ham’s F12 (50:50) culture medium (Invitrogen), supplemented with 5% FBS, 100 μg/ml streptomycin, 100 U/ml penicillin, hormone mix (5 μg/ml insulin, 5 μg/ml transferrin, 1.25 ng/ml PGE1, 1.73 ng/ml sodium selenite), and 25 ng/ml epidermal growth factor. Cells were trypsinized before each passage. Third-passage TECs were used for experiments. To stimulate TEC TLR2, 100 ng/ml anti-TLR2 or 20 ng/ml epidermal growth factor. Cells were trypsinized before each passage. Third-passage TECs were harvested and resuspended in methylcellulose medium (StemCell Technologies). TECs (2 × 10⁵ cells in 10 μl medium) were then implanted beneath the kidney capsule.

ELISA

The CCL3, CCL4, and CCL5 concentrations in the culture supernatant or medium (StemCell Technologies) according to the manufacturer’s instructions.

Implantation of TECs

Third-passage TECs were harvested and resuspended in methylcellulose medium (StemCell Technologies). TECs (2 × 10⁵ cells in 10 μl medium) were then implanted beneath the kidney capsule.

Chemotaxis assay

Purified NK cells were plated (1 × 10⁵/well) in transwell inserts containing 5-μm pores. Conditioned medium was added to the lower chamber and the plates were incubated at 37°C for 12 h. DAPTA (30 μg/ml) was used to neutralize the NK cell surface CCR5.

Immunohistochemistry

Kidneys were embedded in OCT (Sakura Finetek) and frozen in liquid nitrogen. Frozen sections (8 μm thick) were permeabilized with 0.3% Triton X-100, and nonspecific binding was blocked with 10% donkey serum and anti-mouse CD16/CD32 mAbs (10 μg/ml). Sections were labeled with the relevant Abs for 1 h at room temperature. All specimens were mounted with ProLong Antifade reagent (Molecular Probes). Slides were examined under a laser-scanning confocal microscope (Olympus).

Statistics

GraphPad Prism 5 (GraphPad Software) was used to analyze and present data. Differences between groups were analyzed using a two-tailed t test or one- or two-way ANOVA with posthoc analysis, as appropriate. Values are expressed as the means ± SEM. A p value < 0.05 was considered statistically significant.

Results

Impaired NK cell recruitment after IR in TLR2⁻/⁻ mice

NK cells are rapidly recruited to the kidney after IR (12, 13). Moreover, NK cells are indispensable in recruiting neutrophils, which are major effectors of secondary kidney damage during IR (12). We examined the possibility that TLR2 signaling might recruit NK cells to the kidney during IR. TLR2⁻/⁻ mice had significantly fewer NK cells in the kidney at the peak of NK cell infiltration (4 h after IR) compared with WT mice (Fig. 1A). This impaired NK cell recruitment in TLR2⁻/⁻ mice correlated with

![FIGURE 2.](http://www.jimmunol.org/content/2659/5/2659/F2.large.jpg)

TLR2 signaling in TECs is required to recruit NK cells in kidney IRI. TECs (2 × 10⁵) from WT or TLR2⁻/⁻ mice were implanted under the kidney capsules of WT or TLR2⁻/⁻ mice. Immediately thereafter, kidney IRI was induced. (A) Percentage of NK cells in kidneys 4 h after kidney IR. (B) Percentage of neutrophils in kidneys 24 h after kidney IR. (C) Serum creatinine and BUN levels 24 h after kidney IR. (D) Tubular injury scores. Data are the means ± SEM (n = 5–6/group) and represent two independent experiments. *p < 0.05, **p < 0.01.
poor CD45<sup>+</sup>CD11b<sup>+</sup>Gr-1<sup>hi</sup> neutrophil infiltration (Fig. 1B). After 24 h of IR, TLR2<sup>−/−</sup> mice had significant decreases in kidney dysfunction and damage, as reflected by lower plasma creatinine and BUN levels, less tubular necrosis, and less overall acute tubular injury compared with WT mice (Fig. 1C–F).

Because TLR2 signaling in TECs is involved in kidney IRI (4, 6, 11), we asked whether TLR2 expression on TECs could contribute to NK cell recruitment. To test this possibility, we used an in vivo experiment in which normal TECs implanted into the kidney capsule induce kidney IRI (12). We implanted WT TECs under the kidney capsule of TLR2<sup>−/−</sup> mice and induced IRI immediately after implantation. We implanted WT TECs into WT mice as a positive control and TLR2<sup>−/−</sup> TECs into TLR2<sup>−/−</sup> mice as a negative control. Comparable numbers of NK cells were

**FIGURE 3.** TLR2-mediated NK cell recruitment occurs independently of CD137L-mediated neutrophil recruitment in TECs. TECs (2 × 10<sup>5</sup>) from WT or CD137L<sup>−/−</sup> mice were implanted under the kidney capsules of WT or TLR2<sup>−/−</sup> mice. Immediately thereafter, kidney IRI was induced. (A) Diagram describing a complementation test. (B) Percentage of NK cells in kidneys 4 h after kidney IR. (C) Percentage of neutrophils in kidneys 24 h after kidney IR. (D and E) Serum creatinine and BUN levels 24 h after kidney IR. (F) Photomicrographs of representative sections from the three groups at ×200 (top) or ×400 (bottom) magnification (H&E staining). Outer medullary areas are shown in upper panels, and cortex and subcapsular areas containing implanted TECs are shown in lower panels. (G) Tubular injury scores. Data are the means ± SEM (n = 4/group) and represent two independent experiments. *p < 0.05, **p < 0.01.

**FIGURE 4.** CCR5 is involved in recruiting NK cells during kidney IRI. CD3<sup>−</sup>NK1.1<sup>+</sup> NK cells were purified from WT or CCR5<sup>−/−</sup> spleens using the MoFlo cell sorter and adoptively transferred into WT and CCR5<sup>−/−</sup> or CCR5<sup>−/−</sup> mice, respectively, after labeling with CFSE (A) or without labeling (B–F) 1 h before kidney IR. Samples were obtained 4 (A) or 24 h after IR (B–F). (A) Percentage of CFSE<sup>+</sup>CD45<sup>+</sup>CD3<sup>+</sup>NK1.1<sup>+</sup> NK cells. (B) Percentage of neutrophils. (C and D) Levels of serum creatinine and BUN. (E and F) Histology (H&E staining) and injury scores. Data are the means ± SEM (n = 4–5/group) and are representative of two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
observed in kidneys of WT→TLR2−/− and WT→WT mice 4 h after IR whereas TLR2−/−→TLR2−/− mice recruited fewer NK cells (Fig. 2A). Consistent with this finding, counting neutrophils indicated that TLR2−/−→TLR2−/− mice had significantly fewer neutrophil infiltrates in the kidney than did WT→TLR2−/− and WT→WT mice (Fig. 2B). TLR2−/−→TLR2−/− mice had significantly lower serum creatinine and BUN levels than did WT→TLR2−/− and WT→WT mice (Fig. 2C, 2D). Histological analysis showed broader tissue inflammation, ranging from the subcapsular space through the renal outer medulla and cortex, in WT→TLR2−/− and WT→WT mice, with tissue damage being evident in these areas (Fig. 2E, 2F). In particular, heavy neutrophil infiltration and tissue damage in the outer cortex beneath implantation sites were characteristic of these mice compared with TLR2−/−→TLR2−/− mice (Fig. 2E, lower panels). Implanting TLR2−/− TECs into TLR2−/− mice did not induce the neutrophil migration or tissue damage seen in the other two groups (Fig. 2E, 2F). Taken together, these results clearly demonstrate that TLR2 expression on TECs was required to recruit NK cells during kidney IRI.

TECs control sequential NK cell and neutrophil recruitment through TLR2 and CD137L signaling during kidney IRI, respectively

The results presented in Figs. 1 and 2 suggest that TLR2−/− mice might be resistant to kidney IRI owing to defects in NK cell recruitment regulated by TLR2 signaling in TECs. To test this hypothesis, we assumed that NK cell and neutrophil recruitment are induced by two temporally separate signaling events that are delivered through TLR2 and CD137L, respectively, in TECs. Based on this assumption, we hypothesized that CD137L−/− TECs (wherein TLR2 signaling is intact) and TLR2−/− TECs (wherein CD137L signaling is intact) in the same kidney could result in kidney IRI, because CD137L−/− TECs would complement TLR2−/− TECs by recruiting NK cells through TLR2 signaling and TLR2−/− TECs could complement CD137L−/− TECs by recruiting neutrophils through CD137L signaling (Fig. 3A). Implanting CD137L−/− TECs into the kidney capsule of TLR2−/− mice completely restored NK cell and neutrophil recruitment to the extent found in WT→WT mice (Fig. 3B, 3C). Analyzing creatinine and BUN levels and inspecting kidney sections indicated severe kidney damage and loss of kidney function in these two groups (Fig. 3D–G). TLR2−/−→TLR2−/− mice had significantly impaired NK cell and neutrophil recruitment and milder kidney IRI than did the other two groups (Fig. 3B–G). Overall, this complementation assay demonstrated that NK cell and neutrophil recruitment can be mediated by two uncoupled signaling events that occur as a result of TLR2 and CD137L signaling in TECs, respectively.

CCR5 is critical in recruiting NK cells

We hypothesized that TLR2 signaling in TECs would produce chemokines that can recruit NK cells to the kidney during kidney IRI. To test this hypothesis, we screened chemokine receptors that could be involved in NK cell recruitment in kidney IRI. Among CCR1, CCR2, CCR5, and CX3CR1 antagonists, a CCR5 antagonist blocked NK cell recruitment most potently at 4 h after IR (Supplemental Fig. 1). Consistently, the CCR5 antagonist inhibited neutrophil recruitment, kidney damage, and loss of kidney function (Supplemental Fig. 2). To obtain more concrete evidence for the involvement of NK cells expressing CCR5 in kidney IRI, we adoptively transferred WT NK cells into CCR5−/− mice. We first

**FIGURE 6.** Model summarizing the role of the TEC/NK cell/neutrophil axis. Injury to TECs following IRI (step 1) promotes release of HMGB1 (step 2). This molecule stimulates TECs to produce CCR5 chemokines through TLR2 activation (step 3) in an autocrine fashion. CCR5 chemokines in turn induce NK cell recruitment (step 4). Infiltrated NK cells use their cell-surface molecule CD137L to stimulate CD137L on the surface of TECs (step 5). CD137L signaling results in the production of additional signaling molecules, CXCL1 and CXCL2, in TECs (step 7). Once infiltrated (step 8), neutrophils participate in active tissue destruction (step 9).
confirmed that a significantly higher number of CFSE-labeled WT NK cells were recruited to the kidney of CCR5−/− mice, compared with CCR5+/− NK cells, 4 h after IR (Fig. 4A). WT→CCR5−/− mice had significantly restored neutrophil infiltration and kidney damage and loss of function compared with WT→WT (positive control) and CCR5−/−→CCR5−/− (negative control) mice (Fig. 4B−F). Taken together, these data suggest that CCR5 plays a critical role in NK cell recruitment during kidney IRI.

TECs secrete CCR5 chemokines in response to HMGB1, an endogenous TLR2 ligand

We examined whether CCR5 chemokines are produced by TECs during kidney IRI. Immunohistochemical analysis showed expression of the CCR5 chemokines, CCL3, CCL4, and CCL5, in WT TECs 2 h after IR compared with TLR2−/− TECs (Supplemental Fig. 3). ELISA of 2 h IRI mouse kidney homogenates also demonstrated that TLR2−/− mice had significantly lower levels of CCL3, CCL4, and CCL5 compared with WT mice (Fig. 5A). These data suggest that TLR2 signaling should signal TECs to produce CCR5 chemokines in an autocrine manner. We next examined the ability of isolated TECs to produce CCR5 chemokines in response to Pam3CSK5, a potent TLR2 ligand. WT, but not TLR2−/−, TECs secreted high levels of CCL3, CCL4, and CCL5 within 4 h after treatment with Pam3CSK5 (Fig. 5B). Conditioned medium from cultured TECs stimulated with Pam3CSK4 induced NK cell migration in a chemotaxis assay compared with conditioned medium from cultured TLR2−/− TECs (Fig. 5C). Blocking CCR5 with CCR5 antagonist or deleting the CCR5 gene completely abrogated the increased chemotaxis of NK cells in response to conditioned medium from cultured WT TECs stimulated with Pam3CSK4 (Fig. 5D). Next, we tested whether an endogenous TLR2 ligand stimulates TECs to produce CCR5 chemokines under hypoxic conditions. We detected significantly higher levels of CCL3, CCL4, and CCL5 in culture medium of WT TECs than in that of TLR2−/− TECs, and neutralization of HMGB1, a well known endogenous ligand of TLR2 released during kidney IRI (reviewed in Ref. 15), markedly decreased levels of CCL3, CCL4, and CCL5 (Fig. 5E). Blocking TLR2 had a similar effect in this culture condition (Fig. 5E). Moreover, we found that levels of CCL3, CCL4, and CCL5 contained in conditioned medium from this set of experiments positively correlated with its ability to induce NK cell migration in a chemotaxis assay (Fig. 5F). In sum, our results suggest that after ischemic damage, TECs release HMGB1 that induces CCR5 chemokine production through TLR2 signaling.

Discussion

In kidney IRI, hypoxic and anoxic cell injuries occur mainly in the microvasculature and tubular epithelium during the ischemic phase (16). These injured cells undergo necrosis and are thought to produce endogenous TLR ligands (15). TLR2 signaling in TECs is a major inducer of acute inflammation following kidney IRI (4, 6, 11). However, the elaborate mechanism through which TLR2 signaling controls inflammation in TECs remains unclear, largely due to technical difficulties in characterizing this class of signal in vivo. Implanting TECs into the kidney capsule of recipient mice is a useful tool to dissect the signaling events in TECs that result in kidney IRI (12). In this study, we used this technique to develop another in vivo technique that allowed us to analyze two temporally separate signaling events in the same axis leading to kidney IRI. Using this new technique, we demonstrated that TECs are a master regulator of kidney IRI by releasing inducers and mediators of inflammation (HMGB1 and CXCR2 chemokines) for kidney IRI. Once damage in TECs is beyond repair, they may induce inflammation to remove damaged cells. Therefore, TECs seem to be able to survey their stress status and make a spontaneous decision regarding whether damaged cells should be removed. The amazing ability of TECs to use immune cells for their own purpose may ultimately allow the kidney to restore its function and homeostasis (1, 17).

Renal TECs produce proinflammatory cytokines and chemokines, including IL-6, CCL2, CCL5, CXCL1, and CXCL2, upon exposure to exogenous or endogenous TLR2 ligands (4, 18). These results indicate that TLR2 signaling can trigger recruitment of other leukocytes, such as neutrophils and monocytes. However, an influx of neutrophils due to TLR2 signaling in TECs is not sufficient to induce fulminant kidney IRI. Instead, TECs initiate signaling through CD137L, with help from NK cells to induce a second wave of neutrophil influx. Therefore, NK cells are required to recruit the full wave of neutrophils needed for clinical kidney IRI. Even though NK cells interact with various immune system components (19), our results show that NK cell–parenchymal cell interactions control acute tissue inflammation.

Epithelial cells are exposed to a variety of microbial and nonmicrobial challenges. To manage these stressful conditions (17), they have numerous sensors, many of which converge on inflammation. Well-known examples are the TLR and complement systems (4–8, 20). Individual signaling through TLR2, TLR4, and complement receptors results in proinflammatory cytokine and chemokine production (20). This result, however, does not mean that they have redundant roles in kidney IRI (6, 20). Rather, there seems to be a coordinated regulatory system where each signaling pathway crosstalks to control the progression of kidney IRI. For example, targeted deletion of TLR2 and factor B or MyD88 exacerbated kidney IR compared with deletion of factor B or TLR2 alone (6, 20).

Various chemokines play a role in kidney IRI by recruiting leukocytes such as neutrophils, macrophages, and Th1 T cells (21–25). In this study, we added CCR5 chemokines to the list of chemokines involved in kidney IRI. TLR2 signaling resulted in CCL3, CCL4, and CCL5 production in TECs, and blocking of CCR5 completely inhibited NK cell migration in vitro and in vivo. Although CCL3 and CCL5 are versatile chemokines acting on two chemokine receptors (26), our results clearly showed that CCR5 functioned as the major receptor for NK cells during kidney IRI. Moreover, our observation that blocking CCR5 had a similar effect on NK cell recruitment and kidney IRI as depleting NK cells (12) provides strong evidence that prior NK cell infiltration is needed for neutrophil migration during kidney IRI. In summary, our results demonstrate that TECs play a central role in the pathogenic axis consisting of TECs, NK cells, and neutrophils in kidney IRI. TECs have an autoamplification loop where two important signaling events occur through TLR2 and CCL3, CCL4, and CCL5 (21–25). In this study, we added CCR5 chemokines to the list of chemokines, including IL-6, CCL2, CCL5, CXCL1, and CXCL2, upon exposure to exogenous or endogenous TLR2 ligands (4, 18). These results indicate that TLR2 signaling can trigger recruitment of other leukocytes, such as neutrophils and monocytes. However, an influx of neutrophils due to TLR2 signaling in TECs is not sufficient to induce fulminant kidney IRI. Instead, TECs initiate signaling through CD137L, with help from NK cells to induce a second wave of neutrophil influx. Therefore, NK cells are required to recruit the full wave of neutrophils needed for clinical kidney IRI. Even though NK cells interact with various immune system components (19), our results show that NK cell–parenchymal cell interactions control acute tissue inflammation.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental information

Figure legends

**Fig. S1. Screening chemokine receptors that potentially recruit NK cells after kidney IR.** Mice were administered BX513 hydrochloride (CCR1 antagonist, 10 mg/kg), RS504393 (CCR2 antagonist, 2 mg/kg), DAPTA (CCR5 antagonist, 1 mg/kg), or purified rabbit anti-rat CX3CR1 (25 ug/mouse) 1 h before kidney IR. (A and B) Serum creatinine and BUN levels 4 h after kidney IR. (C and D) Percentage of NK cells and neutrophils in kidneys 4 h after kidney IR. (E and F) Histology and injury scores. Data are the mean ± SEM (n = 5 per group). *, P < 0.05; **, P < 0.01; *** P < 0.001.

**Fig. S2. CCR5 is involved in recruiting NK cells during kidney IRI.** (A) Percentage of NK cells in kidneys 4 h after kidney IR. (B) Percentage of neutrophils in kidneys 4 h after kidney IR. (C and D) Serum creatinine and BUN levels 24 h after kidney IR. (E and F) Histology and injury scores 24 h after kidney IR. Data are the mean ± SEM (n = 6-8 per group) and represent two independent experiments. *, P < 0.05; **, P < 0.01; *** P < 0.001.
Fig. S3. Expression of CCL3, CCL4 and CCL5 in TECs during kidney IR.

Immunohistochemical analysis of CCL3/4/5 expression was performed using kidneys harvested 2 h after IR. Green: CCL3/4/5; red: Cytokeratin.