IL-18 Contributes to Renal Damage after Ischemia-Reperfusion

Huiling Wu,* Melissa L. Craft,* Peng Wang,* Kate R. Wyburn,* Gang Chen,* Jin Ma,* Brett Hambly,† and Steven J. Chadban*

*Collaborative Transplant Research Group, Renal Medicine, Royal Prince Alfred Hospital and Bosch Institute, Faculty of Medicine, University of Sydney, and †Pathology Discipline, Bosch Institute, School of Medical Science, University of Sydney, Sydney, Australia

ABSTRACT

IL-18 is a proinflammatory cytokine produced by macrophages and other cell types present in the kidney during ischemia-reperfusion injury (IRI), but its role in this injury is unknown. Here, compared with wild-type mice, IL-18−/− mice subjected to kidney IRI demonstrated better kidney function, less tubular damage, reduced accumulation of neutrophils and macrophages, and decreased expression of proinflammatory molecules that are downstream of IL-18. For determination of the relative contributions of leukocytes and parenchymal cells to IL-18 production and subsequent kidney damage during IRI, bone marrow–chimeric mice were generated. Wild-type mice engrafted with IL-18−/− hemopoietic cells showed less kidney dysfunction and tubular damage than IL-18−/− mice engrafted with wild-type bone marrow. In vitro, macrophages produced IL-18 mRNA and protein in response to ischemia. These data suggest bone marrow–derived cells are the key contributors to IL-18–mediated effects of renal IRI. Finally, similar to IL-18−/− mice, pretreatment of wild-type mice with IL-18–binding protein was renoprotective in this model of IRI. In conclusion, IL-18, derived primarily from cells of bone marrow origin, contributes to the renal damage observed during IRI. IL-18–binding protein may have potential as a renoprotective therapy.


Ischemia-reperfusion injury (IRI) is an inherent process in kidney transplantation that is associated with delayed graft function and an increased risk for acute and chronic rejection. Ischemia-reperfusion injury (IRI) has been established as a multifactorial, antigen-independent inflammatory condition that is initially mediated by innate immunity and causes variable degrees of tissue damage.

IL-18 is a proinflammatory cytokine that stimulates the production of IFN-γ by T cells and natural killer cells in synergy with IL-12. It is synthesized as a biologically inactive precursor (pro-IL-18), similar to IL-1, which requires cleavage to form an active molecule by an intracellular cysteine protease called ICE, or caspase-1. IL-18 is a highly pleiotropic, pro-Th1 cytokine that dramatically enhances both innate and acquired immunity. IL-18 enhances T cell and NK cell maturation and cytotoxicity and promotes Th1 polarization. IL-18 also plays a crucial role in inflammation and in particular modulates the activity of macrophages by activation of transcription factors including NF-kB and AP-1, inducing transcription of a cascade of inflammatory molecules including induc-

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H.W. and M.L.C. contributed equally to this work.
Correspondence: Dr. Huiling Wu, Collaborative Transplant Research Group, Room W607, Blackburn Building D06, University of Sydney, NSW 2006, Australia. Phone: +612-9351-2898; Fax: +612-9351-8771; E-mail: huilingw@med.usyd.edu.au
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ible nitric oxide synthase (iNOS),\textsuperscript{17} TNF-\(\alpha\),\textsuperscript{18} chemokines,\textsuperscript{19} and adhesion molecules\textsuperscript{16,20}; therefore, IL-18 may promote immune or non–immune-mediated tissue damage via a multitude of mechanisms. Daemen \textit{et al.}\textsuperscript{21} reported IL-18 upregulation in renal IRI; however, neither caspase-1–deficient mice nor wild-type (WT) mice treated with IL-1 receptor antagonist, anti–IL-1 receptor antibody, and anti–IL-18 antibody produced significant improvement in renal function, intrarenal TNF-\(\alpha\) production, inflammation, or apoptosis in a murine model of 45 min of renal ischemia reperfusion injury.\textsuperscript{22} Conversely, another group demonstrated a significant role for IL-18 in renal IRI by using a murine model of 22 min of renal IRI with caspase-1–deficient mice.\textsuperscript{23,24} IL-18 mice have not previously been reported in a murine model of kidney IRI.

IL-18 is primarily a macrophage-derived cytokine; however, its expression has been reported in a wide range of cells, including those of bone marrow (BM) origin (macrophages, dendritic cells, T cells, and B cells) and parenchymal kidney cells (tubular epithelial cells, podocytes, and mesangial cells).\textsuperscript{25–30} Macrophage accumulation in association with increased IL-18 expression has been described in a number of inflammatory and immune-mediated processes.\textsuperscript{29,31} We previously described IL-18 upregulation within the kidney during acute rejection in a kidney allograft model and found intragraft macrophages to be the major source.\textsuperscript{32} Intrinsic kidney cells have been found to produce IL-18 in experimental and human lupus nephritis\textsuperscript{26–28} and also ANCA-associated systemic vasculitis.\textsuperscript{29} Whether IL-18 is produced by leukocytes or parenchymal cells during kidney IRI and whether it contributes to kidney injury is unknown.

In this study, we examined the role of IL-18 in experimental renal IRI using IL-18–deficient (IL-18\textsuperscript{−/−}) mice, then generated BM-chimeric mice to explore the relative contributions of BM-derived \textit{versus} parenchymal kidney cells in IL-18 production in kidney IRI. Finally, we investigated the therapeutic potential of an anti–IL-18 strategy by using IL-18–binding protein (IL-18BP), which neutralizes the biologic activity of IL-18.

**RESULTS**

**IL-18 Expression Is Increased in the Kidney**

\textit{In vivo}, WT mice showed significant upregulation of IL-18 expression within kidney at 24 h after IRI compared with sham-operated controls (\(P < 0.05\)) compared with sham-operated controls (Figure 1A). In \textit{vitro}, resident peritoneal macrophages, primary renal tubular epithelial cells (RTEC), and cell lines of both macrophages (RAW264.7) and tubular epithelial cells (MCT) ex-
pressed IL-18 mRNA; however, macrophages produced much more \( (P < 0.001; \text{Figure 1, B and C}) \). Macrophages but not tubular cells submitted to ischemia expressed significantly higher mRNA levels of IL-18 than controls \( (P < 0.01, \text{Figure 1B, and } P < 0.001, \text{Figure 1C}) \). Immunofluorescent double staining demonstrated expression of IL-18 by macrophages (Figure 1D).

**IL-18**\(^{-/-}\) Mice Are Protected from Renal IRI

As shown in Figure 2, IRI caused kidney dysfunction in WT mice, reflected by significant elevation of serum creatinine at 24 h after IRI. Function was preserved in IL-18\(^{-/-}\) mice, with serum creatinine lower than WT mice at 24 and 72 h after IRI \( (P < 0.001) \) and not significantly different from sham-operated controls. Mortality occurred in three of 20 WT mice within 4 d of IRI, whereas no mortalities were observed in the IL-18\(^{-/-}\) and IL-18BP treatment groups.

IL-18 deficiency also afforded protection as assessed by histology. WT mice incurred severe tubular damage, as evidenced by widespread tubular necrosis, loss of the brush border, cast formation, and tubular dilation at the corticomedullary junction at 24 and 72 h after IRI, which was markedly attenuated in IL-18\(^{-/-}\) mice (Figure 3, A and B). Sham-operated mice incurred no tubular injury.

**Interstitial Infiltrates Are Reduced in IL-18**\(^{-/-}\) Mice

Substantial infiltration of neutrophils was evident in WT mice 24 and 72 h after IRI versus sham-operated controls \( (P < 0.001; \text{Figure 4B}) \), and this was moderately attenuated in IL-18\(^{-/-}\) mice (Figure 4, A and B). Similarly, significant macrophage accumulation was evident in WT after IRI compared with sham-operated controls \( (P < 0.001; \text{Figure 4, A and C}) \) but significantly prevented by IL-18 deficiency at 24 and 72 h after IRI \( (P < 0.001; \text{Figure 4C}) \).

**Generation of Proinflammatory Molecules within the Kidney after IRI Is Attenuated by IL-18 Deficiency**

To investigate the effects of IL-18 deficiency, we measured mRNA expression of IL-18 downstream effector molecules and chemokines in the kidney by real-time PCR. Sham-operated controls

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**Figure 2.** IL-18\(^{-/-}\) mice (■) were protected against renal IRI with significantly lower serum creatinine compared with WT controls (□) at 24 and 72 h after reperfusion. Sham-operated mice had normal serum creatinine (10 to 20 μmol/L). Data are means ± SD; \( n = 6 \) to 9 per group.

**Figure 3.** Tubular injury was attenuated in IL-18\(^{-/-}\) mice. (A) Representative sections of outer medulla from sham (□) WT (tubular damage grade 4.5; \( n = 9 \)), and IL-18\(^{-/-}\) (tubular damage grade 1.7) mice (■) 24 h after reperfusion (hematoxylin and eosin stained). (B) Semiquantitative analysis of tubular damage in WT (□) and IL-18\(^{-/-}\) (■) mouse kidney at 24 and 72 h after reperfusion. Data are means ± SD; \( n = 6 \) to 9 per group. Magnification, ×200.
demonstrated detectable levels of TNF-α and iNOS and very low levels of macrophage inflammatory protein 2 (MIP-2) and monocyte chemoattractant protein 1 (MCP-1). There were no significant differences between WT and IL-18−/− sham-operated controls. WT mice subjected to IRI demonstrated strong upregulation of effector molecules (TNF-α and iNOS) and chemokines (MIP-2 and MCP-1) at 24 h after IRI (TNF-α, \(P < 0.001\); iNOS, \(P < 0.01\); MIP-2 and MCP-1, \(P < 0.001\); Figure 5) compared with sham-operated controls. IFN-γ was not upregulated (data not shown). IL-18−/− mice showed a significant reduction in TNF-α, iNOS, MIP-2, and MCP-1 expression compared with WT mice at 24 h after IRI (Figure 5).

**Macrophage IL-18 Expression Is Induced by the Supernatant of Ischemic Tubular Cells**

To determine whether molecules released from ischemic tubular cells can stimulate macrophages to produce IL-18, we measured IL-18 mRNA expression by macrophages stimulated with the supernatant from tubular cells that had been rendered ischemic for 1 h. Macrophage production of IL-18 was modestly enhanced \textit{in vitro} by injured tubular cell supernatant (\(P < 0.05\); Figure 6).

**BM-Derived Cells Play a Critical Role in IL-18–Mediated Kidney IRI**

To determine whether BM-derived cells or intrinsic kidney cells play the major role in IL-18 production and subsequent kidney damage caused by IRI, we generated BM chimeric mice: WT host with IL-18−/− BM (WT/IL-18−/− BM), IL-18−/− host with WT BM (IL-18−/−/WTBM), WT host with WT BM (WT/WTBM), and IL-18−/− host with IL-18−/− BM (IL-18−/−/IL-18−/− BM). Eight weeks after BM transplantation, chimeric mice were subjected to kidney ischemia. At that time, the genotype of BM-derived cells from the chimeric mice was confirmed by PCR of DNA extracted from peripheral blood cells as shown in Figure 7A.

Both WT/IL-18−/−/BM mice and IL-18−/−/WTBM mice demonstrated significantly lower serum creatinine and less tubular damage than WT/WTBM mice at 24 h after IRI (\(P < 0.001\); Figure 7, B and C). WT/IL-18−/− BM mice showed significantly lower serum creatinine and less tubular damages than IL-18−/−/WTBM mice at 24 h after IRI (\(P < 0.05\); Figure 7, B and C). This indicates that WT/IL-18−/− BM mice were protected from kidney IRI to a greater extent than IL-18−/−/WTBM mice, although IL-18−/−/WTBM were partially protected.

**IL-18BP Pretreatment Is Protective in Renal IRI**

The protection afforded from renal IRI by IL-18−/− mice led to the investigation of pretreatment with a neutralizing agent IL-18BP as a possible therapeutic intervention in renal IRI. IL-18BP pretreatment was protective against kidney dysfunction,
tubular damage, and cellular infiltration in the kidney at 24 and 72 h after IRI. Serum creatinine levels after IL-18BP pretreatment were significantly less than that of control mice at 24 h and 72 h after IRI (P < 0.01; Figure 8A). IL-18BP pretreatment also significantly reduced tubular damage compared with control mice at 24 h (P < 0.001) and 72 h after IRI (P < 0.05; Figure 8B). Immunohistochemistry demonstrated that IL-18BP pretreatment significantly reduced neutrophil infiltration at 24 h after IRI (P < 0.01; Figure 8C) compared with control mice, but there was no significant difference at 72 h after IRI. IL-18BP pretreatment significantly reduced macrophage accumulation at 24 and 72 h after IRI (Figure 8D) compared with control mice.

**IL-18BP Pretreatment Attenuates Expression of Proinflammatory Molecules in the Kidney in Renal IRI**

To determine whether neutralizing IL-18 using IL-18BP reduces IL-18 downstream effects, we measured expression of TNF-α, iNOS, MIP-2, and MCP-1. IL-18BP pretreatment significantly reduced iNOS, MIP-2, and MCP-1 expression compared with WT mice at 24 h after IRI (P < 0.05; Figure 9); however, there was no significant difference in TNF-α expression levels.

**DISCUSSION**

In this study, we demonstrated upregulation of IL-18 within the kidney after IRI and, using two separate strategies to target IL-18 specifically, were able to demonstrate its pathogenic role in kidney IRI. These observations confirm and extend previous studies that suggest IL-18 is important in IRI. One study using a similar model of IRI suggested IL-18 was not of pathogenic importance; however, that study caused a substantially longer period of kidney ischemia at higher body temperature (45 versus 21 min and 39 versus 37°C) and may thereby have caused more severe injury that may have overwhelmed any benefit of IL-18 deficiency. The mechanisms by which IL-18 promotes kidney damage in this setting seem to involve the generation of effector molecules iNOS and TNFα and chemokines important in promoting macrophage and neutrophil infiltration. We demonstrated that IL-18 produced by BM-derived cells made the more significant contribution to kidney damage, although IL-18 in kidney parenchymal cells was also important in IRI. IL-18BP pretreatment was protective, thus indicating a potential therapeutic role.

IL-18 was upregulated in IRI kidney in our model, consistent with one previous study. IL-18 is known to promote inflammation and immunity through its key cellular targets including macrophages, T cells, and NK cells. IL-18 promotes immune deviation toward a Th1 response via IFN-γ production; however, given the absence of IFN-γ upregulation in our model of IRI, this pathway may not seem relevant to IRI at least with the time frame examined (24 and 72 h). This is not surprising given the dominance of the innate immune response in IRI, which leads to inflammation and subsequent kidney injury. IL-18 is able to stimulate macrophages to induce the gene expression and synthesis of...
effector molecules including iNOS and TNF-α, known to be mediators of tissue injury in IRI. iNOS leads to dramatic bursts of NO that mediate cell damage during inflammatory responses. Targeting of iNOS in renal IRI through a variety of techniques, including the administration of antisense oligodeoxynucleotides, the use of iNOS mice, and the use of iNOS inhibitors, has been found to be protective. TNF-α stimulates the production of reactive oxygen species, primarily from neutrophils, which may cause direct tissue injury. TNF-α upregulation also leads to an inflammatory cascade that exacerbates tissue damage and includes the production of chemokines MIP-2 and MCP-1, which recruit neutrophils and macrophages, respectively, into the kidney. In addition, IL-18 is able to induce chemokine expression; this together with the chemoattractive properties of IL-18 promote cellular infiltration, local inflammation, and tissue damage. We demonstrated increased gene expression of IL-18--triggered downstream cytokines, effector molecules, and chemokines in IRI kidney in this study, consistent with a role for IL-18 in promoting kidney injury through IFN-γ--independent pathways. We were able to confirm this proposed role of IL-18 because IL-18 deficiency or blockade abrogated products of these molecules. We found that IL-18 mice were afforded a greater degree of protection from kidney IRI than mice treated with IL-18BP, evidenced by serum creatinine and tubular damage score. IL-18-- mice may have abnormalities of other cytokines, such as relative IL-6 deficiency. Because macrophage-derived IL-6 has been identified as a mediator of kidney ischemia injury, relative IL-6 deficiency in the knockouts may be one reason for the better protection afforded by IL-18 deficiency versus IL-18BP treatment.

Macrophages are a major producer of IL-18. Macrophage accumulation in association with increased IL-18 expression has been described in a number of other inflammatory and immune-mediated processes, including biliary atresia, contact hypersensitivity, and lupus-like autoimmune syndrome. Of particular interest, in the setting of rheumatoid
arthritis, increased production of IL-18 associated with these inflammatory processes is principally from macrophages.\textsuperscript{31} We previously reported that IL-18 upregulation in the kidney during acute allograft rejection was largely derived from intragraft macrophages.\textsuperscript{32} Consistent with this, our \textit{in vitro} results in this study confirmed that macrophages expressed significantly higher levels of IL-18 than tubular cells, and this production was augmented after ischemia. Macrophages but not renal tubular cells submitted to ischemia expressed significantly higher mRNA levels of IL-18 than the controls. One study showed that molecules released from injured tubular epithelial cells activated macrophages,\textsuperscript{38} although these molecules are yet to be identified. Consistent with this, we also showed that IL-18 expression by macrophages \textit{in vitro} was increased upon exposure to the supernatant of ischemic tubular cells. These data support macrophages as the primary IL-18–producing cells in renal IRI.

Both BM-derived cells and kidney parenchymal cells are known to contribute to inflammation and injury within the kidney in various conditions. To determine the relative contribution of IL-18 produced by leukocytes, particularly macrophages, \textit{versus} kidney parenchymal cells to kidney damage in IRI, we generated BM chimeric mice. Compared with WT mice with WT BM subjected to IRI, mice with IL-18–deficient parenchymal cells but competent leukocytes were modestly protected from kidney dysfunction and damage, suggesting IL-18 produced by parenchymal kidney cells is of some importance. Urinary IL-18, potentially derived from tubular cells, has been reported as a biomarker of acute kidney injury.\textsuperscript{41,42} By comparison, mice with IL-18–deficient leukocytes but IL-18–competent parenchymal cells were protected to a significantly greater degree, demonstrating that leukocyte-derived (most likely macrophage) IL-18 makes the key contribution.

The detrimental effects of macrophages in renal IRI were first suggested in a study in chemokine C-C motif receptor 2–deficient mice, which lacked MCP-1 signaling. Blocking chemokine C-C motif receptor 2/MCP-1 signaling reduced infiltration of macrophages (F4/80-positive cells) and preserved renal function in renal IRI. Furthermore, rats with macrophage depletion by using liposomal clodronate were afforded partial protection from renal IRI as assessed by kidney function, tubular damage, and cytokine expression. Our data from BM chimeras thus support a key role for macrophages. These data do not exclude a role for neutrophil-derived IL-18 in kidney IRI; however, one previous study suggested that IL-18–mediated kidney IRI may be independent of neutrophils because IL-18 antiserum–treated neutrophil-depleted mice with IRI had a significant reduction in serum creatinine and tubular damage compared with vehicle-treated neutrophil-depleted mice.\textsuperscript{24} Together, these data support the concept of the macrophage as an important mediator in the initiation period of IRI.\textsuperscript{5}

IL-18BP is a naturally occurring, inducible, soluble protein that binds with high affinity to IL-18, thereby neutralizing its biologic effects by preventing the binding of IL-18 to its recep-

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**Figure 8.** IL-18BP pretreatment is protective in kidney IRI. (A and B) Mice treated with IL-18BP (■) demonstrated significantly lower serum creatinine (A) and less tubular damage, assessed by semiquantitative scoring of hematoxylin- and eosin-stained tissue sections (B) compared with controls (□) at 24 and 72 h after reperfusion. (C and D) Immunohistochemistry staining for neutrophils (stain) and macrophages (stain) demonstrated that IL-18BP pretreatment significantly reduced cell accumulation for both neutrophils (stain) and macrophages (stain). Data are means ± SD; n = 9 per group.
IL-18BP has been used as a therapeutic agent in a number of studies. An IL-18BP-Fc construct has been shown successfully to neutralize IL-18 and prevent Fas/FasL-mediated liver disease. IL-18BP seems to be a potent protective mediator to counter skin inflammation during contact hypersensitivity. Collagen-induced arthritis in mice is inhibited by treatment with murine IL-18BP. We demonstrated the benefit of IL-18BP in protecting the kidney from injury in the adriamycin nephropathy model of FSGS. Administration of IL-18BP after disease induction resulted in a significant reduction in the clinical manifestations of disease, including proteinuria, kidney dysfunction, glomerulosclerosis, and cellular interstitial infiltration (unpublished data 2007). One advantage of using IL-18BP therapy is that it avoids the problems that usually complicate long-term antibody-based treatments, such as immunogenicity. In this study, neutralization of IL-18 with IL-18BP provided protection against kidney IRI, achieving a comparable degree of suppression of downstream effector molecules and cytokines to that seen in the IL-18−/− mice. Thus, strategies targeting IL-18 by using IL-18BP may have therapeutic possibilities in a clinical setting.

This study confirms the pathogenic role of IL-18 in kidney IRI, which may be mediated by downstream effector molecules of IL-18 (TNF-α and iNOS) and macrophage infiltration directed by MCP-1. BM-derived cells, particularly macrophages, play the major role in IL-18−/−-induced injury. IL-18BP pretreatment affords significant protection against kidney IRI and has clear therapeutic potential.

**CONCISE METHODS**

**Animals**

IL-18−/− mice on a C57BL/6 background were generously provided by Animal Services at the Australian National University and were backcrossed more than nine times onto C57BL/6 background. C57BL/6 mice used as WT controls were obtained from the Animal Resource Centre (Perth, Western Australia, Australia). The mice were maintained in a specific pathogen–free facility in the University of Sydney and received food and acidified water *ad libitum*. Male mice housing at least 1 wk after their arrival and weighing 25 to 30 g were used in all experiments. Experiments were conducted by following established guidelines for animal care and were approved by the animal ethics committee of the University of Sydney.

**Induction of Kidney IRI**

Induction of kidney IRI was as described previously. Briefly, using a midline abdominal incision, both renal pedicles were clamped for 21 min with microaneurysm clamps. During the period of ischemia, body temperature was maintained by placing the mice on a 37°C heat pad. After removal of the clamps, the kidneys were inspected for 1 min for restoration of blood flow, returning to their original color. And the abdomen was closed. Sham-operated mice received identical surgical procedures except that microaneurysm clamps were not applied. For maintaining fluid balance, all mice were supplemented with 1 ml of saline administered subcutaneously. Mice were killed 24 and 72 h after reperfusion (*n* = 6 to 10 per group).
IL-18BP Pretreatment
WT mice received 300 μg of IL-18BP intraperitoneally (provided by SeroNo Int., Geneva, Switzerland) 40 min before the induction of ischemia and an additional 300 μg of IL-18BP at the time of reperfusion. Control mice received PBS instead of IL-18BP.

Blood and Tissue Samples
Blood and kidney tissues were harvested at time of killing. Tissue slices were divided to be fixed with 10% neutral-buffered formalin for paraffin embedding, fixed with periodate-lysine-paraformaldehyde fixative to be frozen in OCT compound (Sakura Finetek, Torrance, CA), or snap-frozen in liquid nitrogen for subsequent mRNA extraction.

Generation of BM Chimeric Mice
BM chimeric mice were generated by adoptive transfer of donor BM cells into irradiated recipient animals as described previously.47 Chimeric mice were produced in the following recipient/donor combinations: WT/WTBM, IL-18+/−/IL-18+/− BM, WT/IL-18−/− BM, and IL-18−/−/WTBM. The recipient mice were lethally irradiated using a 137Cs irradiator (Gammacell 40 Exactor; Nordion Int., Kanata, Ontario, Canada), with a single dose of 9.5 Gy from a cesium source. Recipient irradiated mice were administered an injection of 1 × 10⁷ BM cells via the tail vein 6 h after irradiation. Mice were housed for 8 to 10 wk to allow full cellular reconstitution before being subjected to 21 min of kidney ischemia. Full chimerism of each mouse was confirmed by genotyping of DNA from whole blood using REDExtract-N-AmpTM Blood PCR kits (Sigma-Aldrich, St. Louis, MO).

Assessment of Renal Function
Serum creatinine was measured using the modified Jaffe rate reaction by the Biochemistry Department of the Royal Prince Alfred Hospital (Sydney, Australia).

Histology and Immunohistochemistry
Kidneys embedded in paraffin were sectioned at 3 μm and stained with hematoxylin and eosin. The percentage of tubules that exhibited cellular necrosis, loss of brush border, and tubule dilation were assessed with hematoxylin and eosin. The percentage of tubules that exhibited IL-18

Primary Culture of Mouse RTEC
Primary mouse RTEC were generated following the method as described previously.47 Briefly, kidneys were flushed with saline in vivo to remove blood cells, then removed. The kidney cortices from WT mice were cut into pieces of approximately 1 mm³ and then digested in HBSS containing 3 mg/ml collagenase at 37°C for 25 min and washed in DMEM/F12 (Invitrogen, Grand Island, NY). The kidney digest was washed through a series of sieves (mesh diameters of 250, 150, 75, and 40 μm). The cortical tubular cells were spun down at 300 × g for 5 min and further washed. The cell pellet was resuspended in defined K1 medium.48 The cell suspension was then plated on cell culture Petri dishes and incubated at 37°C for 2 to 3 h to facilitate adherence of contaminating glomeruli. The nonadherent tubules were then collected and cultured on collagen-coated Petri dishes (BD Biosciences, Bedford, MA) in K1 medium until epithelial colonies were established. Expression of the epithelial cell marker cytokeratin was verified by immunofluorescent staining with an anti-cytokeratin antibody (Sigma-Aldrich). Cells were 96 to 100% cytokeratin positive as previously reported.47 Experiments were commenced after the cells had reached 80 to 90% confluence, which was usually between 5 and 7 d after the isolation procedure.

Isolation of Resident Peritoneal Macrophages
After injection of 10 ml of cold PBS into the peritoneal cavity of WT mice, resident peritoneal macrophages were harvested then plate- adhered in serum-free medium for 4 h, and nonadherent cells were removed by washing. The adherent peritoneal macrophages were cultured in complete DMEM with 10% FBS. Expression of the macrophage marker was verified by staining with PE-conjugated antimouse F4/80 antibody (ABD Serotec, Oxford, UK) and analyzed on a calibur flow cytometer using CellQuest software (Becton Dickinson, Mountain View, CA). The purity of these cells was defined by positive staining for the macrophage marker (F4/80) and was 97.3%.

Induction of RTEC and Macrophage Ischemia In Vitro
A mouse renal tubular epithelial cell line (MCT) was a gift from Prof. E Neilson.49 A macrophage cell line (RAW264.7) was obtained from the American Type Culture Collection (Manassas, VA). MCT cells, primary RTEC, RAW264.7 cells, and peritoneal macrophages were rendered transiently ischemic by immersing the cellular monolayer in mineral oil as described previously.47 This immersion induced ischemia by restricting cellular exposure to oxygen and nutrients as well as by limiting metabolite washout. Primary RTEC were placed in serum-free K1 medium and other cells in serum-free DMEM (Invitrogen) for 24 h, washed twice with PBS, and immersed in mineral oil (Sigma-Aldrich) for 40 min at 37°C for cell lines and for 60 min at 37°C for primary RTEC and peritoneal macrophages. After extensive washing with PBS, cells were incubated in K1 medium or complete DMEM with 10% FBS. The cells were collected at 1 h after medium replace-
ment for IL-18 expression. Control group cells were exposed to serum-free K1 or DMEM alone as the nonischemic control. In another set of experiments, the supernatant from ischemic MCT or the nonischemic control was collected at 16 h after medium replacement for macrophage stimulation.

**Macrophage Stimulation with the Supernatant from Ischemic MCT**

Macrophages (RAW264.7) were placed in serum-free DMEM (Invitrogen) for 24 h and then cultured with the supernatant collected at 16 h from ischemic MCT cells or the nonischemic control. Macrophages were collected at 4 h after co-culture with the supernatant for IL-18 mRNA expression.

**Immunofluorescent Double Staining**

For macrophage and IL-18 immunofluorescent double staining, adherent peritoneal macrophages on culture chamber slides were blocked with 1% BSA in PBS for 20 min and incubated with rat anti-mouse F4/80 antibody (ABD Serotec) and goat polyclonal antibodies to mouse IL-18 (SC-6177; Santa Cruz Biotechnologies, Santa Cruz, CA) for 60 min followed by anti-rat IgG conjugated with AlexaFluor 595 and anti-goat IgG conjugated with AlexaFluor 488 (Molecular Probes, Eugene, OR). The slides were mounted with DAPI medium.

**RNA Extraction and cDNA Synthesis**

Total RNA was extracted from kidney tissue and cells using TRIzol (Invitrogen). cDNA was synthesized using oligo d(T)16 (Applied Biosystems, Foster City, CA) and the SuperScript III Reverse Transcriptase kit (Invitrogen).

**Real-Time PCR**

cDNA was amplified in 1× Universal Master Mix (Applied Biosystems) with gene-specific primers and probe on the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Specific Taqman primers and probes for IL-18 (Mm00434225_m1), TNF-α (Mm00443258_m1), and iNOS (Mm00440485_m1) were obtained from Applied Biosystems. Specific Taqman primers and probes for MCP-1 and MIP-2 were previously described.47 The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control to normalize the mRNA content of each sample as described previously.47 All results are expressed as ratio to GAPDH.

**Statistical Analysis**

All data are expressed as means ± SEM. Statistical differences between two groups were analyzed by unpaired, two-tailed t tests, and multiple groups were compared using one-way ANOVA with post hoc Bonferroni correction (Graph Pad Prism 4.0 software; GraphPad, San Diego, CA). P < 0.05 was considered significant.

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