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An Inflammasome-Independent Role for Epithelial-Expressed Nlrp3 in Renal Ischemia-Reperfusion Injury

Alana A. Shigeoka,* James L. Mueller,†‡§ Amanpreet Kambo,* John C. Mathison,* Andrew J. King,¶ Wesley F. Hall,‖ Jean da Silva Correia,* Richard J. Ulevitch,* Hal M. Hoffman,†‡§ and Dianne B. McKay*  

Cytoplasmic innate immune receptors are important therapeutic targets for diseases associated with overproduction of proinflammatory cytokines. One cytoplasmic receptor complex, the Nlrp3 inflammasome, responds to an extensive array of molecules associated with cellular stress. Under normal conditions, Nlrp3 is autoinrepress, but in the presence of its ligands, it oligomerizes, recruits apoptosis-associated speck-like protein containing a caspase recruitment domain (Asc), and triggers caspase 1 activation and the maturation of proinflammatory cytokines such as IL-1β and IL-18. Because ischemic tissue injury provides a potential source for Nlrp3 ligands, our study compared and contrasted the effects of renal ischemia in wild-type mice and mice deficient in components of the Nlrp3 inflammasome (Nlrp3<sup>−/−</sup> and Asc<sup>−/−</sup> mice). To examine the role of the inflammasome in renal ischemia-reperfusion injury (IRI) we also tested downstream caspases 1, 1β, and 18. Both Nlrp3 and Asc were highly expressed in renal tubular epithelium of humans and mice, and the absence of Nlrp3, but not Asc or the downstream inflammasome targets, dramatically protected from kidney IRI. We conclude that Nlrp3 contributes to renal IRI by a direct effect on renal tubular epithelium and that this effect is independent of inflammasome-induced proinflammatory cytokine production. *The Journal of Immunology, 2010, 185: 6277–6285.  

Tremendous interest has evolved over the past decade in innate immune sensing receptors and their role in disease processes. Of the known innate immune receptors, the best described are the membrane-bound TLRs; many recent discoveries have highlighted their importance in human diseases. Another family of intracellular innate sensors, called the nucleotide-binding oligomerization domain-like receptors (NLRs), has attracted focused interest as potential therapeutic targets for diseases associated with the overproduction of proinflammatory cytokines. The NLRs are critical signaling molecules that contribute to formation of intracellular macromolecular complexes and regulate host immune responses to a variety of stimuli, including pathogens, molecules released from injured tissue, and a variety of nonspecific molecules, including immune adjuvants (1).  

The NLR family includes several different groups of intracellular molecules sharing a N-terminal pyrin domain (PYD), a central domain, which acts as an oligomerization module for the complex formation between NLRs (2). The caspase recruitment domain (CARD) (Asc) contains a central caspase recruitment domain (CARD) (Asc) through PYD–PYD interactions (3). The NLR family includes the caspase 1 inflammasome, a multiprotein molecular scaffold necessary for caspase 1 activation (4). Upon ligand sensing, Nlrp3 recruits the adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD) (Asc) through a PYD–PYD interaction. Asc then recruits inflammatory caspases (e.g., caspase 1) to the assembly complex through CARD–CARD interactions (5). The assembly complex brings into close proximity the inflammatory caspases, leading to their activation (6). Activation of caspase 1 is required for processing and release of active proinflammatory cytokines such as IL-1β and IL-18.  

A large variety of structurally unrelated stimuli activate Nlrp3, such as microbial RNA and the imidazolquinoline compounds R837 and R848, muramyl dipeptide, polynosinic-polycytidylic acid, ATP, potassium efflux, asbestos, silica, aluminum hydroxide, amyloid β peptide, pore-forming toxins, cellular components released into the extracellular milieu by distressed cells (including monosodium urate and calcium pyrophosphate dihydrate crystals found in gout and pseudogout), and cholesterol crystals (7–11). The fact that so many different stimuli trigger activation of Nlrp3, and subsequent inflammasome formation, suggests that Nlrp3 is a common hub for many different cellular triggers that lead to overproduction of proinflammatory cytokines.  

Several human diseases have been linked to Nlrp3. For instance, mutations in the Nlrp3 gene cause familial cold autoinflammatory syndrome, Muckle-Wells syndrome, and neonatal-onset multisystem inflammatory disease, which are collectively known as cryopyrinopathies (3, 12–14). More recently, inflammation associated with gout has been linked to Nlrp3 and overproduction of IL-1β (10). A central role for overproduction of IL-1β in the pathogenesis of Nlrp3-related diseases has been identified by the successful treatment of these diseases with IL-1-targeted therapies. The rIL-
IR antagonist anakinra has shown remarkable therapeutic efficacy for patients with cryopyrinopathies as well as gout (15–20). Another of the disease processes linked to innate immune receptors is ischemic kidney injury. Ischemic kidney injury occurs commonly in hospitalized patients and is a complex pathophysiological process contributed to by both cell death and inflammation. Although recent attention has highlighted the important role for TLRs in ischemic injury of the kidney (21, 22), as well as other organs (23–28), less is known about the role of the NLRs in ischemic organ injury (29).

Because Nlrp3 plays a central role in immune regulation associated with cellular injury, we investigated whether Nlrp3 plays a role in acute kidney injury. To mimic acute kidney injury, we used a well-characterized murine model of ischemia-reperfusion injury (IRI). We examined the dependence of kidney injury and cytokine/chemokine secretion on the Nlrp3 inflammasome using mice with targeted deletions in the inflammasome components, Nlrp3 and Asc. Because inflammasome formation activates caspase 1, we also asked whether targeted deletion of caspase 1 influenced acute kidney injury. The dependence of acute kidney injury on IL-1 was tested using an IL-1R antagonist and also using IL-1R knockout mice. The role of IL-18 was tested using IL-18 knockout mice. Our studies are the first to our knowledge to isolate an inflammasome-independent role of the Nlrp3 protein in renal epithelial cells and to identify a new inflammasome-independent function in vivo for Nlrp3.

Materials and Methods

Mice

Mice were housed in the vivariums at The Scripps Research Institute and University of California at San Diego (La Jolla, CA) and approved for use by the Laboratory Animal Care and Use Committee. Asc+/− and Nlrp3+/− mice were provided by Millenium Pharmaceuticals (San Diego, CA). Animals were handled according to the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care, C57BL/6, IL-1R−/−, and IL-18−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Caspase 1−/− mice were obtained from R. Ulevitch (The Scripps Research Institute). All mice used in these experiments had been bred onto a C57BL/6 background by >10 generations.

Detection of Asc and Nlrp3 in wild-type mice and human cells

Asc and Nlrp3 mRNA were detected in renal tubular epithelium (RTE) or lymph node (LN) cells by semiquantitative PCR. To isolate and detect Asc and Nlrp3 from murine RTE, the same protocol was used as noted below (isolation of human RTE), except that collagenase D was used instead of collagenase A for murine RTE. Asc and Nlrp3 were also detected in pooled LN cells from dissected inguinal and axillary LNs. The first strand of cDNA of each sample was synthesized from 1 μg total RNA using a Quantitect RT kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. For RT-PCR, 1 μg total cellular RNA was reverse transcribed and cDNA amplified. The expression of Asc and Nlrp3 mRNA in murine RTE was detected by semiquantitative PCR with 5′-GAGGCGATCCGCTCTTCTATC-3′ as a forward primer and 5′-GGTCCATACCAAGTGGAGGATG-3′ as a reverse primer for Asc mRNA and with 5′-GAGGCGATCCGCTCTTCTATC-3′ as a forward primer and 5′-GGGACATACCAAGTGGAGGATG-3′ as a reverse primer for Asc mRNA and with 5′-GAGGCGATCCGCTCTTCTATC-3′ as a forward primer. The level of GADPH mRNA in each sample was determined by PCR using 5′-TTCTCCAAAGTGGAGGATG-3′ as a forward primer and 5′-TCTCCCAACCTAGGAAGGGTC-3′ as a reverse primer. Detection of Asc in human RTE the forward primer was 5′-GCGGAGGAGCTCAAGAAGGTT-3′ and reverse primer was 5′-TCTCCCAACCTAGGAAGGGTC-3′. For detection of Nlrp3 in HEK293, THP1 and human RTE cells the forward primer was 5′-AAAGAAGACTCTTTGCTGGTCC-3′ and reverse primer was 5′-ATCTGGATCCTCCAGGAC-3′ and reverse primer 5′-CATGATGCTTCCAGCAGGATG-3′. The PCR products were separated on a 1% agarose gel and digital photographs taken on a transilluminator.

Isolation of human RTE

Human kidney tissue was obtained from discarded nephrectomies for renal cell carcinoma (IRB:08-5054, Scripps Clinic and Green Hospital, La Jolla, CA). Immediately postnephrectomy, the renal pathologist removed a wedge section of normal tissue (distal from the site of tumor). After transport to the laboratory in sterile media, the renal capsule was removed and cortex dissected from the medulla. The cortex was then finely minced, placed in collagenase A (Sigma-Aldrich, St. Louis, MO), and incubated at 37°C for 30 min with frequent shaking. Postincubation, the digested mixture was differentially sieved (200–50 μm) and washed three times with fresh media. The sieved contents were then centrifuged, pelleted, and layered over 30% Percoll (Sigma-Aldrich). After spinning the Percoll mixture at 21,500 × g for 30 min, four distinct bands were apparent as previously described (30, 31). The cells were collected from band 3 and washed three times with HBSS. The cells were then cultured in specialized tubular epithelial cell growth media (DMEM–Ham’s-F-12 [Fisher Scientific, Pittsburgh, PA]; insulin [5 μg/ml]; transferrin [5 μg/ml]; selenium [50 nM]; hydrocortisone [0.05 μM; Sigma-Aldrich]; epidermal growth factor [10 ng/ml; Sigma-Aldrich]; tri-iodothyronine [32 ng/ml]; and penicillin/streptomycin [1 ml]) to promote growth of tubular epithelial cells but not other cell types. Within a few days, a cobblestone morphology characteristic of cultured renal tubular epithelium was evident. Confirmation of renal tubular epithelium was made by positive staining for Lotus tetragonolobus lectin (32) ([Vector Laboratories, Burlingame, CA).]
methodology described above (see 3 then washed with sterile PBS and 5 m glutamine [2 mM], FBS [10%), penicillin [100 U/ml], streptomycin [100 µg/ml], 2-ME [0.05 mM], HEPES [10 mM]). The bone marrow cells were then washed with sterile PBS and 5 × 10⁶ WT or Nlrp3⁻/⁻ bone marrow cells in sterile PBS were injected into the tail vein of recipient mice. The mice were kept in microisolator cages for 6 wk to complete engraftment with donor bone marrow and were given trimethoprim-sulfadiazine-enriched water until induction of IRI. Confirmation of Asc⁻/- or Nlrp3⁻/- engraftment after bone marrow transplantation was performed on bone marrow cells of chimeric WT mice engrafted with either Nlrp3⁻/- bone marrow or WT bone marrow. To detect the Nlrp3 mutation, the WT forward and reverse primers were 5'-GCTGTCGGCAGTCC-TAGC-3' and 5'-CAAGGAGCCTTCTTCGAGG-3’, respectively, and the KO allele forward and reverse primers were 5'-GGCAGGAGATTGCCAGAGG-3' and 5'-TCCACACAAATAGGGA-TG-3’, respectively, and the KO allele forward and reverse primers were 5'-GGGATCCTGGCATGATGAG-3' and 5'-GTCACCACAAATAGGGA-TG-3’, respectively.

Detection of active caspase 1 and mature IL-1β

To detect the Nlrp3 targets, caspase 1 and IL-1β, and to assess whether they were activated in WT and/or Nlrp3⁻/⁻ mice after IRI, whole kidneys were harvested immediately following 25 min bilateral renal artery clamping/24 h reperfusion. The kidneys were then lysed in a buffer containing 60 mM Tris, 10% glycerol, and 2% SDS and protease inhibitors. The lysis supernatants were then resolved by SDS-PAGE, transferred to nitrocellulose membranes, and then probed with anti-caspase 1 Ab, anti-cleaved IL-1β Ab, or anti–hsp-90 loading control (all Abs from Cell Signaling Technology, Danvers, MA). The blots were then developed by the ECL system (Amersham Biosciences, Louisville, CO).

Blockade of IL-1 and IL-18

The effect of IL-1 blockade on renal IRI was tested in two ways. The first employed the IL-1R antagonist anakinra (Kinereit, Agen, Thousand Oaks, CA). Anakinra (25 mg/kg) was injected i.p. into WT mice at two intervals upon induction of anesthesia, and at 6 h postoperatively, following published dosing and interval recommendations (35, 36). To assess the potential dose/interval dependence, anakinra was also given over a range of doses from 1–150 mg/kg and a range of administration times (from 6 h presurgery to 12 h postsurgery). Higher/low and/or more frequent anakinra dosing did not show any different results from used regimen. The second method to test for the effect of IL-1 blockade was by inducing IRI in IL-1R⁻⁻ mice using methodology described above (see Induction of in vivo IRI). IL-18⁻⁻ mice were used to test the effect of IL-18 deficiency in renal IRI.

Bone marrow transplantation

To evaluate the role of inflammation in kidney IR injury, Nlrp3⁻/- versus WT bone marrow was transplanted into WT mice. Male WT mice were lethally irradiated with two doses of 5 Gy, separated by 3 h, using a 137Cs Gam- maccell 40 Extractor irradiator (MDS Nordion, Ottawa, Ontario, Canada). The next day, bone marrow was collected from WT or Nlrp3⁻/- mice by flushing femurs and tibia with sterile 10K media (RPMI 1640 containing L-glutamine, 25 mM, FBS [10%], penicillin [100 U/ml], streptomycin [100 µg/ml], 2-ME [0.05 mM], HEPES [10 mM]). The bone marrow cells were then washed with sterile PBS and 5 × 10⁶ WT or Nlrp3⁻/- bone marrow cells in sterile PBS were injected into the tail vein of recipient irradiated mice. The mice were kept in microisolator cages for 6 wk to complete engraftment with donor bone marrow and were given trimethoprim-sulfadiazine-enriched water until induction of IRI. Confirmation of Asc⁻/- or Nlrp3⁻/- engraftment after bone marrow transplantation was performed on bone marrow cells of chimeric WT mice engrafted with either Nlrp3⁻/- bone marrow or WT bone marrow. To detect the Nlrp3 mutation, the WT forward and reverse primers were 5'-GCTGTCGGCAGTCC-TAGC-3' and 5'-CAAGGAGCCTTCTTCGAGG-3’, respectively, and the KO allele forward and reverse primers were 5'-GGCAGGAGATTGCCAGAGG-3' and 5'-TCCACACAAATAGGGA-TG-3’, respectively, and the KO allele forward and reverse primers were 5'-GGGATCCTGGCATGATGAG-3' and 5'-GTCACCACAAATAGGGA-TG-3’, respectively.

Apoptosis

To evaluate for evidence of apoptosis in renal cortical tissue after renal IRI, tissue cryosections (6 µm) were assayed with the TUNEL reaction using the TUNEL assay as provided in the fluorescein Fragmentation detection kit (Calbiochem, San Diego, CA). Cryosections were fixed in 4% formaldehyde and permeabilized in proteinase K (in 10 mM Tris [pH 8]). The sections were then exposed to the TdT equilibration buffer and the 3'-hydroxyl groups of DNA labeled with the fluorescein-conjugated deoxyribonucleotides. Nuclear staining was identified in cell nuclei with DAPI and DNA breakage imaged by fluorescence microscopy. Eight random nonoverlapping sections were viewed and counted under a grid at ×200 original magnification. All tissue sections were blind labeled and viewed without knowledge of experimental groups.

Results

Nlrp3 and Asc are expressed in murine and human kidneys

A pathognomonic finding of renal IRI is renal tubular epithelial injury (37, 38). To evaluate the role of Nlrp3 and Asc in kidney IRI, we first asked whether the Nlrp3 and Asc genes were present in renal tubular epithelial cells of murine and human kidneys. As seen in Fig. 1, using semiquantitative PCR, both Nlrp3 and Asc were well expressed in renal tubular epithelial cells in the two species. These genes were also expressed in murine lymphocytes and in the human monocytic cell line THP1, but not in nontransfected HEK cells, as previously described (1, 5, 39, 40).

Contribution of Nlrp3, Asc, and caspase 1 to kidney injury following ischemia and reperfusion

Because Nlrp3 and Asc were highly expressed in mouse and human renal tubular epithelial cells, the cells most susceptible to renal hypoxia, the participation of these inflammasome component proteins in responses to acute kidney injury were tested in a well-characterized model of murine kidney IRI. Using WT and mutant

![Image](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/)

**FIGURE 1.** Nlrp3 and Asc in murine and human cells. The figure shows three panels depicting the Nlrp3 (left panel), Asc (middle panel), or housekeeping GADPH (right panel) genes. Each panel illustrates gene expression detected by semiquantitative PCR in the RTE of Nlrp3⁻/- mice (Nlrp3⁻/-nRTE), wild-type (WtRTE) mice, lymph node cells from WT mice (WTLN), nontransfected HEK 293 cells (HEK), Thp1 cells (Thp1), or human RTE cells extracted from normal human kidneys (hRTE). The results are representative of four mice per group and five different human kidneys extracted for renal tubular cells with identical results.
FIGURE 2. Kidney IRI in WT, NLR, and caspase 1-deficient mice. WT, Asc−/−, caspase 1−/−, or Nlrp3−/− mice were subjected to 25 min ischemia/24 h reperfusion and plasma obtained at 24 h to detect creatinine from injured WT, Asc−/−, caspase 1−/−, and Nlrp3−/− mice (n = 6 mice/group). Preinjury creatinines were obtained 2 wk earlier on each of the experimental mice: WT is shown in the figure (pre); Asc−/− (0.42 ± 0.01); caspase 1−/− (0.47 ± 0.01); and Nlrp3−/− (0.48 ± 0.04). Creatinines were also obtained from sham-operated mice: WT (0.4 ± 0.1); Asc−/− (0.3 ± 0.1); caspase 1−/− (0.4 ± 0.2); and Nlrp3−/− (0.3 ± 0.2). Error bars represent SD of creatinines, and statistical significance was determined with a one-way ANOVA with Bonferroni’s multiple comparison test. The data represent one of five identical experiments with the same results.

FIGURE 3. Histological renal injury after bilateral renal artery clamping. A shows blinded scoring of tubular necrosis from WT (n = 6), caspase 1−/− (n = 6), Asc−/− (n = 6), Nlrp3−/− (n = 6), and sham-operated (n = 3) mice 24 h after bilateral renal artery clamping (25 min ischemia/24 h reperfusion). The top panel shows the blinded necrosis score (see Materials and Methods for details), and the bottom panel shows a representative micrograph of each group of mice (periodic acid-Schiff, ×200 original magnification). Means of necrosis scores were as follows: WT (5.0 ± 1.1); caspase 1−/− (4.7 ± 1.0); Asc−/− (3.7 ± 2.2); and Nlrp3−/− (2.3 ± 0.8). There were no statistical differences between WT versus caspase 1−/− (p > 0.50) or WT versus Asc−/− (p > 0.50), but significant differences in histological injury were observed between WT versus Nlrp3−/− (p < 0.01) mice. B shows blinded scores of neutrophil infiltration on the same sections (see Materials and Methods). Means of neutrophil scores were: WT (2.1 ± 0.8); caspase 1−/− (1.8 ± 1.3); Asc−/− (1.6 ± 1.1); and Nlrp3−/− (1.4 ± 0.7). No statistical differences in neutrophil infiltration were seen between the groups: WT versus caspase 1−/− (p > 0.05) versus Asc−/− (p > 0.05), or versus Nlrp3−/− (p > 0.05). Statistical significance was determined by a one-way ANOVA with Bonferroni’s multiple comparison test.

Fig. 2 shows plasma creatinine levels 2 wk preinjury for WT mice and 24 h postinjury for each indicated group of mice. Preinjury and sham creatinines are listed in the figure legend for Asc−/−, caspase 1−/−, and Nlrp3−/− mice; no differences were observed between any of the groups. As seen in Fig. 2, there were no significant differences in postinjury creatinines between WT and Asc−/− mice (p < 0.05) or between WT versus caspase 1−/− mice (p < 0.05). However, an extremely significant reduction in serum creatinine was noted in IR-treated Nlrp3−/− mice (p < 0.001), suggesting greater renal protection was afforded by a deficiency of Nlrp3 than by a deficiency in the protein Asc, which collaborates with Nlrp3 to form the inflammasome. Confirming that Nlrp3 can also signal independently of the inflammasome, there was no protection from renal IRI by deletion of caspase 1, the downstream signaling target of this inflammasome.

Acute tubular necrosis in each group of mice was evaluated by a pathologist blinded to the experimental groups (Fig. 3A). Using a modified standard scoring system (21), necrosis was scored 24 h after the initial ischemic insult (Fig. 3A). The means of each group are shown in the figure legend. As seen in the top panel of Fig. 3A, there was no difference in necrosis between WT, caspase 1−/− (p > 0.05), and Asc−/− mice (p > 0.05), but a significant reduction in tubular necrosis was seen between WT mice and mice deficient in Nlrp3 (p < 0.01). A representative photomicrograph is shown for each of the groups below Fig. 3A. Neutrophil infiltration was also assessed in each group, in a blinded manner, by the pathologist (Fig. 3B). There were no significant differences in neutrophil infiltration noted between any of the groups.

IL-18 and IL-1β secretion in WT and Nlrp3−/− mice after kidney IRI

Protection from kidney injury following IRI was most prominent in mice lacking the innate sensor molecule Nlrp3. As such, postinjury
IL-18 and IL-1β secretion was compared and contrasted between WT and Nlrp3<sup>−/−</sup> mice (Fig. 4). Fig. 4A shows there was significantly less IL-18 and IL-1β produced after kidney IRI in the Nlrp3<sup>−/−</sup> than WT mice. In the bottom panels of Fig. 4A, LPS treatment indicated that, as expected, mice deficient in Nlrp3 were unable to secrete the inflammasome-dependent cytokines IL-18 or IL-1β. Fig. 4B shows that mature IL-1β and activated caspase 1 was detected in WT kidneys but not in Nlrp3<sup>−/−</sup> kidneys subjected to renal IRI. The fact that Nlrp3<sup>−/−</sup> mice were not able to activate caspase 1 or produce mature IL-1β following IRI confirms that Nlrp3 is necessary for inflammasome-mediated proinflammatory cytokine production in vivo. But, as seen below in Fig. 5, the renal protection conferred by the deficiency in Nlrp3 is independent of its role in inflammasome-induced proinflammatory cytokine secretion.

**IL-18 and IL-1R blockade and kidney IRI**

Because Nlrp3-deficient mice did not produce IL-18 or IL-1β, the experiments shown in Fig. 5 were performed to determine whether protection afforded by the absence of Nlrp3 was mediated through these cytokines. In Fig. 5A, WT mice were pretreated with an IL-1R antagonist prior to renal artery clamping. IL-1–targeted therapy is highly useful for treatment of several disorders associated with mutations in Nlrp3 (10, 15–20). Shown in Fig. 5A, there were no significant differences in creatinine levels after renal IRI in unmanipulated WT mice compared with WT mice treated with the IL-1Ra anakinra (<i>p = 0.13</i>). A wide range of anakinra doses were compared and contrasted (see Materials and Methods), and there was no renal protection afforded by any of the tested doses of anakinra (data not shown). In Fig. 5B, a slight, but nonsignificant, decrease in renal injury in IL-1R<sup>−/−</sup> mice was noted compared with unmanipulated WT mice (<i>p &gt; 0.05</i>), but no differences were seen in histology score or neutrophil infiltrates (data not shown). In Fig. 5C, there were no differences in creatinines of WT mice versus IL-18<sup>−/−</sup> mice (<i>p &gt; 0.05</i>) treated with bilateral renal artery clamping, consistent with prior reports of the ineffectiveness of IL-18 deficiency on renal IRI (42).

**Inflammation in WT, Asc<sup>−/−</sup>, and Nlrp3<sup>−/−</sup> mice after renal IRI**

To determine whether the absence of Nlrp3 protected kidneys from IRI by abrogating hematopoietic cell-mediated inflammation,
WT mice were transplanted with bone marrow from WT, Asc−/−, or Nlrp3−/− mice, and renal IRI was induced (Fig. 6). As shown in Fig. 6A, there were no differences in creatinine levels after IRI between the WT mice transplanted with WT versus Asc−/− (p > 0.05) or Nlrp3−/− (p > 0.05) bone marrow. Confirming that there were no differences in tubular necrosis or inflammation between the two groups, blinded necrosis scores (Fig. 6B) and neutrophil counts (Fig. 6C) showed no differences between the WT and chimeric mice (Fig. 6B). Fig. 6D confirms that the WT bone marrow was indeed replaced by the mutant marrow in the chimeric mice. Irradiated mutant mice were noted to have severe weight loss, and therefore, reverse bone marrow transplants were not performed until further studies could determine the cause of the irradiation-induced weight loss in the mutant mice.

**Apoptosis and renal IRI**

An early histological finding of kidney IRI is apoptosis of renal tubular cells; necrosis follows longer ischemic intervals (43). To determine whether Nlrp3 contributed to renal tubular apoptosis in our IRI model, kidney sections were examined for evidence of apoptosis in situ by the TUNEL assay. We have previously shown that maximal apoptosis occurs at 4 h postinjury in this model (29) and so focused on this interval, comparing results in WT versus Asc−/− or Nlrp3−/− kidneys. As shown in Fig. 7, there were significant differences in apoptosis between WT and Nlrp3−/− mice (p < 0.001), whereas there were no differences between IRI-treated WT and Asc−/− kidneys (p > 0.05). These data suggest that in addition to the protection from necrosis, the absence of Nlrp3 also protected the kidney from renal tubular apoptosis in our model.

**Discussion**

Renal IRI is a common clinical problem complicated by substantial morbidity and mortality; thus, tremendous interest has evolved in putative triggers of the renal injury. The pathognomonic features of renal IRI include hypoxia-induced tubular epithelial damage followed by postreperfusion inflammation. Multiple cell types and cell signals contribute to the syndrome, but tubular epithelial apoptosis and necrosis are predominant findings, particularly in the ischemic phases of injury.

Many recent studies have pointed to innate pattern recognition receptors as important triggers of tissue injury induced by ischemia (21, 29). Because apoptosis and inflammation are essential components of kidney IRI, our study focused on a recently identified intracellular innate immune receptor, Nlrp3, known to play a role in apoptosis and inflammatory responses to cellular injury.

Within the cytoplasm, Nlrp3 forms a complex with Asc and procaspase 1 called the Nlrp3 inflammasome. Recent studies have suggested that inflammasome components may signal death pathways independent from the inflammasome (44). We investigated the dependence of Nlrp3 on the intact inflammasome in vivo by testing the inflammasome components, Asc and Nlrp3, as well as the downstream targets caspase 1, IL-1β, and IL-18 in our well-characterized ischemic kidney injury model.

Examining murine and human renal tubular epithelial cells, we found that Nlrp3 and Asc were expressed in both species and, importantly, within tissue highly susceptible to renal IRI/renal tubular epithelium. These data are consistent with reports of innate immune receptor expression in many different cell types and in diverse species (45–52), suggesting these phylogenetically conserved proteins are involved in the response to endogenous stress.

To determine whether Nlrp3 plays an important role in tissue responses to renal ischemia, we compared and contrasted injury responses in WT and inflammasome-deficient mice using a well-characterized model of renal IRI. Interestingly, despite the presence of both Nlrp3 and Asc in the cells most susceptible to IRI, only the Nlrp3 deficiency offered protection from tissue injury. Nlrp3 is well known to respond to a wide variety of structurally different ligands, positioning it as a key sensor for cellular injury (53). Our data showing that Nlrp3−/− mice, but not Asc−/− or caspase 1−/− mice, were protected suggest that Nlrp3 might be uncoupled from Asc, and from the inflammasome, in renal tubular epithelium. Consistent with our data, Nlrp3−/− mice have been found to be protected from renal IRI in another model, but these investigators also found that Asc−/− mice were protected (54). The mice used in the study were backcrossed onto C57BL/6 mice for only four generations, and therefore, it is possible that the founder strain might account for the variance in findings. We have carefully confirmed that our mice are on a C57BL/6 background by absence of rejection of skin grafts from the mutant mice on the WT mice.

Because the absence of Nlrp3, but not Asc or caspase 1, protected from renal injury, our data strongly suggest an inflammasome-independent role for Nlrp3. The lack of protection from injury we observed in caspase 1-knockout mice is supported by studies of others using a similar model of renal ischemia (55). Activation of the Nlrp3 inflammasome is known to cause caspase 1 activation and cleavage of pro-IL-1β and pro-IL-18 into their active, mature forms (4, 6). Consistent with this, we found that a deficiency in Nlrp3 blocked IL-1β production, as well as IL-18 production. But, blockade of IL-1 and IL-18 (with either anakinra or with IL-1R−/− or IL-18−/− mice) had no significant effect
on renal injury responses, as previously reported (56). Others have reported that blockade of IL-18 provides either minimal (57) or no (42) protection from renal IRI. Therefore, Nlrp3 does not likely contribute to renal injury primarily through inflammasome-mediated IL-1β or IL-18 production. Our data suggest that Nlrp3, rather than Asc, drives responses to tissue injury in this model and suggests activation of Nlrp3 can induce inflammasome-independent responses in renal epithelial cells.

The pathogenesis of kidney IR is complex, and to date, many causative mechanisms have been proposed. These mechanisms generally involve direct induction of cell death pathways in tubular epithelia (58), glomerular injury, renal endothelial injury and expression of adhesion molecules (59), and leukocyte recruitment and activation (60). Nlrp3 has been detected in the cytosol of various immune cells, such as T cells, macrophages, and dendritic cells (49), and we thought it possible that Nlrp3 might contribute to renal injury through inflammation initiated by hematopoietic-derived immune cells. However, WT mice engrafted with Nlrp3−/− bone marrow were not protected from injury. Although Nlrp3−/− mice do not produce IL-1b or IL-18 (neutrophil chemotaxins),

FIGURE 6. Bone marrow chimeric mice subjected to IRI. A shows WT mice that were irradiated and injected with WT (n = 6), Asc−/− (n = 6), or Nlrp3−/− (n = 6) bone marrow 6 wk earlier. Postengraftment, the three groups of mice were treated with 25 min bilateral renal artery clamping followed by 24 h reperfusion. Precreatinine values were obtained 2 wk preinjury (pre). Sham creatinines were obtained from mice that underwent laparotomy without undergoing bilateral renal artery clamping (n = 3). Error bars represent SDs. B shows the necrosis score of the injured animals. The means of scores were: WT (4.3 ± 0.8), Asc−/− (4.0 ± 1.1), Nlrp3−/− (4.3 ± 0.8); and statistical differences: WT versus Asc−/− (p > 0.05) versus Nlrp3−/− (p > 0.05). C shows the scores of neutrophil infiltration: WT (2.8 ± 0.4), Asc−/− (2.5 ± 0.5), Nlrp3−/− (2.3 ± 0.8); and the statistical differences were: WT versus Asc−/− (p > 0.05) and WT versus Nlrp3−/− (p > 0.05). Statistical significance was obtained by a one-way ANOVA with Bonferroni’s multiple comparison test. D shows that chimeric bone marrow was engrafted in the indicated experimental groups. The control panels show the Nlrp3 knockout allele (indicating the Nlrp3 gene) was present in the Nlrp3−/− mice, but not in the WT or the Asc−/− mice (controls). The WT mice transplanted with the Nlrp3−/− bone marrow show that the Nlrp3 knockout allele was highly expressed (bone marrow chimeras, Nlrp3−/− → WT). Likewise, the Asc knockout allele was expressed in Asc−/− controls but not in the WT controls and also highly expressed in the mice transplanted with Asc−/− bone marrow (bone marrow chimeras, Asc−/− → WT).

FIGURE 7. Apoptosis of RTE cells after renal IRI. WT versus Nlrp3−/−, Asc−/−, or sham mice were treated with 25 min of bilateral renal artery clamping followed by 4 h of reperfusion. The mice were sacrificed and their kidneys removed and placed into formalin for paraffin embedding and TUNEL assay. A shows representative images of TUNEL and DAPI (Nuclei) staining 4 h postreperfusion. Original magnification ×200. B shows results of blinded counting of TUNEL-positive cells, 4 h postreperfusion, in nine random, nonoverlapping sections (n = 3 mice/group). Data are expressed as means ± SD. Statistical significance was determined by a one-way ANOVA with Bonferroni’s multiple comparison test.
neutrophils are still recruited to the injured tissue. Therefore, future studies will need to be conducted to completely exclude any role for Nlrp3 in activation of specific innate and adaptive inflammatory mediators to ischemic renal tissue in our model. A major role, though, for Nlrp3-mediated inflammation does not appear to be relevant from these data.

The most susceptible region of the kidney to ischemia appears to be the renal tubular epithelium located in the outer stripe of the medulla (37, 38). It is thought that ischemia-related reduction in microvascular blood flow to this region of the kidney coupled with a limited capacity of the proximal tubule cells to undergo anaerobic metabolism makes this region particularly susceptible. In fact, the most prominent histologic findings are the loss of the periodic acid-Schiff–positive brush border of the proximal tubular epithelium and scattered single-cell necrosis or desquamation of proximal tubular cells with intact basement membranes (61). Interestingly, those proximal tubular epithelial cells that do not undergo apoptosis/necrosis (e.g., the surviving proximal tubular epithelial cells) divide and repopulate the tubular epithelium, thereby making kidney IRI a reversible disorder, unless all of the epithelium is destroyed by the ischemia (62). Significantly less tubular necrosis was observed in Nlrp3−/− mice than in WT, Asc−/−, or caspase 1−/− mice following renal IRI, consistent with the known role for Nlrp3 as a mediator of necrosis in monocytes and macrophages (63). Interestingly, significantly less renal tubular apoptosis was also seen in the Nlrp3-deficient mice subjected to renal IRI compared with renal IRI treated WT or Asc-deficient mice. These data suggest that Nlrp3 might contribute to renal injury following ischemia/reperfusion by inducing apoptosis within a few hours of the ischemic insult. Future studies are ongoing in our laboratory to identify Nlrp3-dependent signaling events that contribute to renal tubular necrosis and apoptosis following IRI.

Our data point to a role for Nlrp3 in the kidney that is independent of the inflammasome. Future studies will need to focus on additional direct injury mechanisms that might be playing a role in Nlrp3-mediated protection. Our study is the first to our knowledge to show that in renal epithelium Nlrp3 can signal injury responses independent of the inflammasome and independent of proinflammatory cytokines/chemokines.

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Disclosures

The authors have no financial conflicts of interest.

References


Corrections


In regards to Ref. 54 (Iyer, S. S., W. P. Pulskens, J. J. Sadler, L. M. Butter, G. J. Teske, T. K. Ulland, S. C. Eisenbarth, S. Florquin, R. A. Flavell, J. C. Leemans, and F. S. Sutterwala. 2009. Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome. Proc. Natl. Acad. Sci. USA 106: 20388–20393), an error occurred in the Discussion section on p. 6282 of the article, which states that “The mice used in the study were backcrossed onto C57BL/6 mice for only four generations...”. This was a mistake; the mice used in Ref. 54 were backcrossed for nine generations. We apologize for this error and regret any inconvenience.

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