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NK Cells Induce Apoptosis in Tubular Epithelial Cells and Contribute to Renal Ischemia-Reperfusion Injury¹

Zhu-Xu Zhang,^{2*†§¶} Shuang Wang,^{*¶} Xuyan Huang,^{*¶} Wei-Ping Min,^{*†§¶} Hongtao Sun,^{*¶} Weihua Liu,^{*¶} Bertha Garcia,^{*§¶} and Anthony M. Jevnikar^{2*†§¶}

Renal ischemia-reperfusion injury (IRI) can result in acute renal failure with mortality rates of 50% in severe cases. NK cells are important participants in early-stage innate immune responses. However, their role in renal tubular epithelial cell (TEC) injury in IRI is currently unknown. Our data indicate that NK cells can kill syngeneic TEC in vitro. Apoptotic death of TEC in vitro is associated with TEC expression of the NK cell ligand Rae-1, as well as NKG2D on NK cells. In vivo following IRI, there was increased expression of Rae-1 on TEC. FACS analyses of kidney cell preparations indicated a quantitative increase in NKG2D-bearing NK cells within the kidney following IRI. NK cell depletion in wild-type C57BL/6 mice was protective, while adoptive transfer of NK cells worsened injury in NK, T, and B cell-null Rag2^{-/-}γc^{-/-} mice with IRI. NK cell-mediated kidney injury was perforin (PFN)-dependent as PFN^{-/-} NK cells had minimal capacity to kill TEC in vitro compared with NK cells from wild-type, FasL-deficient (*gld*), or IFN-γ^{-/-} mice. Taken together, these results demonstrate for the first time that NK cells can directly kill TEC and that NK cells contribute substantially to kidney IRI. NK cell killing may represent an important underrecognized mechanism of kidney injury in diverse forms of inflammation, including transplantation. *The Journal of Immunology*, 2008, 181: 7489–7498.

The kidney is vulnerable to diverse forms of injury that can lead to dysfunction and, in the most severe cases, acute renal failure. Renal ischemia-reperfusion injury (IRI),³ which follows reduced renal blood flow, is a major cause of acute renal failure and if severe enough to lead to acute renal failure, it can result in a very high mortality rate of 50% (1). In the case of renal transplantation, injury that occurs during the early stage of transplant surgery may lead to delayed graft function and both early allograft loss and chronic allograft dysfunction after kidney transplantation (2). At present, there are no specific treatments available to prevent IRI, as many of the mechanisms have not been clearly identified. A complex network between kidney cells and ischemia/inflammatory factors can lead to completely different outcomes, namely injury or recovery.

IRI is considered an Ag-independent inflammatory process that involves multiple factors leading to cellular dysfunction. In the kidney, endothelial cells as well as tubular epithelial cells (TEC) are susceptible to injury (3–5). We have demonstrated the contri-

bution of TEC in kidney injury and dysfunction following exposure to inflammation and exposure to proinflammatory cytokines (6–8). Following ischemia, oxidative stress induces cytokine and chemokine up-regulation, which can enhance the recruitment of inflammatory cells capable of mediating injury directly and indirectly through the expression of cytokines such as IFN-γ and TNF-α (9, 10). Cell adhesion molecules are responsible for recruitment of leukocytes into the injury site, as demonstrated in both in vitro and in vivo studies (10, 11). Previous studies have demonstrated that kidney-infiltrating macrophages and neutrophils are significantly increased after ischemic injury, leading to tubular dysfunction (12–15). Recent studies suggest that in addition to innate immune responses, diverse cell types of the adaptive immune system also participate in kidney IRI. In particular, CD4⁺ T cells appear to be crucial for renal IRI (16–21). Interestingly, CD4⁺ T cells infiltrate into the kidney within 1 h following warm (22, 23) as well as cold ischemia (24), with great relevance to transplantation and the impact of cold solution hypothermic preservation. These early infiltrating CD4⁺ T cells are independent of neutrophil infiltration (16, 25). Recent studies also indicate that early T cell infiltration at 4 h can resolve by 24 h, the so-called “hit-and-run” phenomenon (19, 26, 27). In addition to T cells being significantly increased in IRI kidneys, CD19⁺ B cells, CD4⁺NK⁺ cells, and CD3⁻NK1.1⁺ cells also infiltrate into the kidney shortly after injury (19), and resident dendritic cells appear to be a prominent source of TNF-α in early IRI (28). Furthermore, NKT cells promote neutrophil infiltration and IFN-γ production after IRI (29). A recent study has shown that B cell deficiency can protect kidneys from IRI (30). Clearly, further studies are required to define the interaction between innate and adaptive immunity in renal IRI and the capacity of mononuclear cells to mediate kidney tissue damage, either directly or indirectly.

NK cells, which play a significant role in the innate immune response, can lyse target cells immediately without Ag presensitization. Interestingly, T and B cell-deficient Rag1^{-/-} mice can develop IRI, suggesting a role for NK cells or other innate immune

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³ Abbreviations used in this paper: IRI, ischemia-reperfusion injury; PFN, perforin; 7-AAD, 7-aminoactinomycin D; TEC, tubular epithelial cell.

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responses in this type of injury (31, 32). Furthermore, Rae-1, a cell surface receptor recognized by NKG2D on NK cells, is increased following kidney IRI, supporting a potential link between NK cell activity and kidney injury (33). The capacity of NK cells to induce injury of parenchymal cells of the kidney of which TEC predominate has not been determined. The cytotoxic function of NK cells appears to be regulated by their Ly-49 (34, 35) and NKG2 surface receptors (36–38). These molecules can interact with class I MHC or class I MHC-like ligands on target cells, which in turn control the reactivity of NK cells, becoming either inhibitory or stimulatory (39). An inhibitory effect through Ly-49 engagement prevents NK cells from attacking “self-MHC” class I-expressing cells. In contrast, a stimulatory effect through NKG2D engagement promotes cytotoxicity of NK cells and permits attack of cells that express “non-self” MHC molecules as well as autologous cells that have deficient/reduced self-MHC class I molecules. In the present study we have demonstrated for the first time that TEC express ligands specific for NK cell engagement that permit NK cell-induced apoptosis. Since depletion of NK cells attenuates renal IRI, whereas adoptive transfer of NK cells augments IRI, our data suggest that NK cell-TEC interactions may also occur *in vivo* and the data also support a major role for NK cell-TEC interactions in promoting kidney dysfunction following IRI. Targeting NK cell-TEC interactions may lead to novel and clinically useful approaches to limit IRI in diverse forms of renal inflammatory injury, including renal transplantation.

Materials and Methods

Animals

C57BL/6 (B6, H-2^b), B6Snn.C3-*FasL gld (gld)*, IFN- γ ^{-/-}, and perforin (PFN)^{-/-} mice were purchased from The Jackson Laboratory. Rag2^{-/-} and Rag2^{-/-} γ_c ^{-/-} mice were purchased from Taconic Farms. The animals were maintained in the animal facility at the University of Western Ontario using approved protocols and procedures.

TEC culture

The TEC line B6 NG1.1 was developed as previously described (6). Primary TEC were obtained from kidneys after being digested with collagenase for 15 min. All TEC were grown in complete K1 culture medium (DMEM/Hams F12, 50:50; Invitrogen) supplemented with 5% bovine calf serum, hormone mix (5 μ g/ml insulin, 1.25 ng/ml prostaglandin E₁, 34 pg/ml triiodothyronine, 5 μ g/ml transferrin, 1.73 ng/ml sodium selenite, and 18 ng/ml hydrocortisone), and 25 ng/ml epidermal growth factor. Cells were trypsinized before each passage. Phenotypes of TEC were confirmed by staining with anti-CD13, CD26, and E-cadherin.

Antibodies

Cells were characterized with fluorescent-conjugated mAbs that specifically recognize CD3, CD4, CD8, NK1.1, DX5 (CD49b), NKG2D, H60 and MULT-1 (eBioscience). Expression of Rae-1 was determined by anti-Rae-1 (clone c-20) and rabbit anti-goat IgG (Santa Cruz Biotechnology).

Data were acquired and analyzed on a Cytomics FC500 flow cytometer (Beckman Coulter). NK cell depletion Ab, anti-asialo GM1, was purchased from Cedarlane Laboratories. The isotype control IgG was obtained from Sigma-Aldrich. Depletion of NK cells was optimized, and only 40–50 μ l Ab (not less) and two injections before IRI give a clear depletion of NK cells in mice (data not shown).

Isolation of NK cells

NK cells were purified from C57BL/6 (B6) mice spleen using anti-CD49b MACS beads selection after depleting CD3⁺ T cells (Miltenyi Biotec). They were then activated for 4–8 days in the presence of IL-2 (1000 IU/ml) in RPMI 1640 supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μ g/ml), glutamine (2 mM), sodium pyruvate (1 mM), HEPES (10 mM), and 2-ME (0.5 mM). The activated NK cells were used as effector cells. Purity of B6 NK cells was confirmed by flow cytometry and >92% cells were CD3⁻CD49b⁺.

To isolate infiltrating NK cells, kidneys were collected after 0 (sham), 4, and 24 h after IRI (renal artery clamping, 45 min at 32°C) and then digested

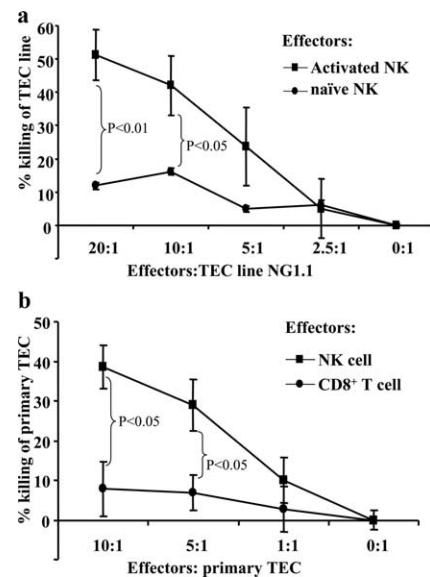


FIGURE 1. Activated NK cells kill TEC. Naive NK cells were purified from B6 mice spleen using CD49b MACS beads selection (Miltenyi Biotec) after depleting CD3⁺ T cells. The purified NK cells were activated by IL-2 (1000 IU/ml) for 3–7 days before being used as effector. TEC line B6 NG1.1 or B6 primary TEC (10⁴) were labeled with 10 μ Ci/ml of [³H]thymidine and used as target (triplicate). After NK cells and target cells were cocultured for 5 h, the cells were harvested and counted in a beta scintillation counter. Specific cell lysis was calculated as described in *Materials and Methods*. Results are from three separate experiments. *a*, Activated NK cells kill TEC lines. *b*, B6 NK cells kill B6 primary TEC.

with collagenase to make single-cell suspensions. All kidney cells were then suspended in 36% Percoll before being laid onto 72% Percoll. After 1000 \times g and 30 min of centrifugation, leukocytes were collected at the interface of Percoll and counted before being stained with anti-CD45, anti-CD3, and anti-CD49b (DX5).

Cytotoxicity assay

NK cells were purified by CD49b⁺ selection on MACS beads column (Miltenyi Biotec) and then stimulated by IL-2 (1000 IU/ml) for 4–7 days. Any contaminating T cells were further depleted with anti-CD3 MACS beads (Miltenyi Biotec) after flow cytometry analysis. We measured DNA fragmentation and death of target cells as described in previous studies and in current protocols in immunology (40–42). Briefly, TEC were labeled with 10 μ Ci/ml of [³H]thymidine at 37°C overnight, washed, and then used as targets. After coculture with NK cells at 37°C for 5 h, the cell mixtures were harvested and counted in a beta scintillation counter (PerkinElmer). Specific cell lysis was calculated using the following equation: % Specific killing = $(S - E) / S \times 100$, where *E* (experimental) is cpm of retained DNA in the presence of NK cells, and *S* (spontaneous) is cpm of retained DNA in the absence of NK cells.

Cell death was confirmed with 7-aminoactinomycin D (7-AAD) staining, according to the manufacturer’s protocol (BD Biosciences). TEC were labeled with 0.5 μ M CFSE (Molecular Probes/Invitrogen) for 10 min and washed three times with medium. The CFSE-labeled TEC were mixed with NK cells or cultured alone for various lengths of time. 7-AAD staining and flow cytometry were used to determine the percentage of the apoptotic TEC by gating on CFSE⁺ cells.

Kidney reperfusion injury

Ischemia was induced in B6 mice by clamping the left kidney for 45 min at 32°C on a thermoregulated pad. After the clamps were released, the right kidney was removed. Kidneys were collected at different time points after being perfused with PBS to remove blood. Sham controls were mice that were treated with the same operative procedure as in the injury group but kidneys were not clamped. Serum was collected for creatinine detection by a Jaffe reaction method with an automated CX5 clinic analyzer (Beckman Coulter).

Purified CD49b⁺ NK cells or whole spleen cells from Rag2^{-/-} mice were transferred to Rag2^{-/-} γ_c ^{-/-} mice. Five days afterward, reconstituted

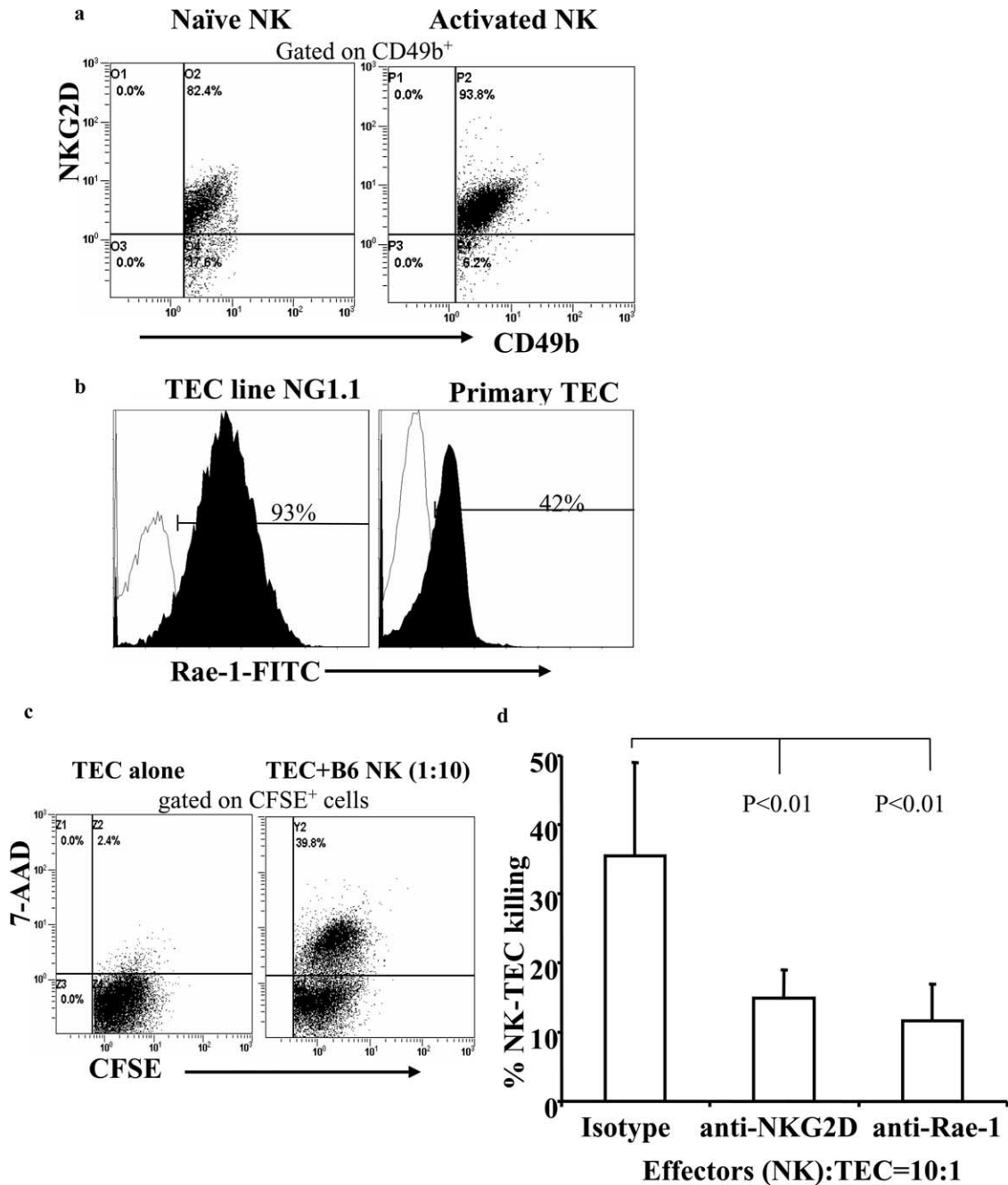


FIGURE 2. Expressions of NKG2D on NK cells and Rae-1 on TEC play important roles in NK cell-mediated TEC killing. NK cells were purified and activated as described in *Materials and Methods*. Expression of NKG2D (CD3⁻CD49b⁺NKG2D⁺) was determined by anti-CD3, anti-NKG2D, and anti-CD49b. TEC were stained by anti-Rae-1 by flow cytometry (BD Biosciences). Rat IgG2b-FITC was used as control. *a*, NK cells express NKG2D before and after activation. *b*, B6 primary TEC were developed according to the protocol and used for staining by anti-Rae-1. TEC line NG1.1 and primary TEC express Rae-1 (filled area, clone c-20, Santa Cruz Biotechnology). *c*, B6 NK cells were activated as described in *Materials and Methods* and used as effectors. CFSE-labeled TEC line NG1.1 was used as target. The mixtures were incubated for 5 h and used for 7-AAD staining to detect apoptotic death of TEC by being gated in CFSE⁺ cells. TEC alone was used as control. *d*, Blockade of NKG2D or Rae-1 inhibits NK cell-mediated TEC killing. Cytotoxicity assay was performed as described in *Materials and Methods*. CFSE-labeled TEC were used as target and stained by 7-AAD after incubation with NK cells. Anti-NKG2D (5 μg/ml, CX5, eBioscience), anti-Rae-1 (5 μg/ml, clone c-20, Santa Cruz Biotechnology), and control rabbit anti-goat IgG (5 μl/ml) were added into wells, respectively. The mixtures were cultured for 5 h before harvest. CFSE⁺7-AAD⁺ cells were calculated. The results were averaged from quadruplicate wells. Similar results were obtained in two separate experiments.

NK cells were confirmed by flow cytometry. Mice then underwent kidney ischemia injury at 35°C for 45 min.

Real-time PCR

Total RNA was extracted from kidneys with a spin column, according to the manufacturer's protocol (Qiagen). cDNA pools were synthesized with the First-Strand Synthesis System according to the manufacturer's

protocol (Stratagene). Primers were designed using Primer Express, primer designing software from Applied Biosystems: B6 subtype Rae-1δ: GGA TAC ACC AAC GGG CTA GA and CCT GGG TCA CCT GAA GTC AT; β-actin: CCA GCC TTC CTT CCT GGG TA and CTA GAA GCA TTT GCG GTG CA. The gene sequences were obtained from the www.ncbi.nlm.nih.gov database. Real-time quantitative PCR was performed on standardized quantities of cDNA using the Brilliant

SYBR Green QPCR Master Mix kit, and amplified DNA products were generated and detected using the Mx4000 system (Stratagene). Each PCR amplification condition was set up in triplicate. β -actin amplification was used as the endogenous control. The normalized delta threshold cycle value and relative expression levels ($2^{-\Delta\Delta Ct}$) were calculated according to the manufacturer's protocol.

Histology and immunohistochemistry

Formalin-fixed kidney sections were stained with H&E and injury was scored in a blinded fashion by pathologists, using an arbitrary scoring system (0, no change; 1+, <25% area change; 2+, 25–50% area change; 3+, 50–75% area change; 4+, >75% area change). Criteria for kidney injury included tubular necrosis, immune cell infiltration, cast, and glomerular necrosis.

Kidney sections were embedded in OCT compound and snap frozen in liquid nitrogen. Cryostat sections were fixed in acetone and stained with Ab using a standard protocol. All slides were stained by a streptavidin-biotin immunoperoxidase and its substrate method as described in the standard protocol.

Statistical analysis

Data were compared using Student's *t* test (Stats program) for unpaired values and one-way ANOVA for multi-group differences. *p*-values of <0.05 were considered to be significantly different.

Results

In vitro-activated NK cells can directly lyse TEC

To determine whether NK cells participate in renal IRI, we first studied if NK cells could directly kill syngeneic TEC. NK cells, purified from B6 mice spleen using anti-CD49b MACS beads selection after depleting CD3⁺ T cells, were activated for 3–5 days in the presence of IL-2 (1000 IU/ml) before being used as effector cells. The B6 TEC line NG1.1 was cocultured with NK cells for 5 h. As shown in Fig. 1*a*, activated NK cells, but not naive NK cells, were capable of killing syngeneic TEC lines in a dose-dependent manner.

To further prove NK cell-mediated TEC killing, primary TEC were cultured from kidneys of B6 mice and similarly used as targets. As shown in Fig. 1*b*, NK cells could also lyse cultured primary TEC in a dose-dependent manner. NK cell-induced killing of primary TEC was substantially greater than B6 CD8⁺ T cells (activated by anti-CD3) (Fig. 1*b*). These data indicate that NK cells are able to directly and efficiently kill syngeneic TEC.

NK cell-mediated TEC killing through NKG2D/Rae-1 interaction

In general, NK cells kill target cells through an Ag-independent mechanism. Ly-49 receptors inhibit the reactivity of NK cells (39). In contrast, a stimulatory effect of NKG2D promotes NK cell attack of cells expressing ligands, such as Rae-1, MULT-1, and H60, on murine cells. Therefore, we studied whether NK targeting molecules are present on NK cells and TEC.

First, we characterized NK cells using flow cytometry. B6 NK cells were purified from B6 spleen cells and activated in the presence of IL-2. As shown in Fig. 2*a*, as expected, NKG2D receptors were detected on NK cells. Hence, the NK cells used in our studies expressed activation surface molecules.

We then tested by flow cytometry for TEC expression of ligands that can interact with NKG2D. The TEC line NG1.1 was found to express high surface levels of Rae-1 (Fig. 2*b*), whereas no significant levels of H60 and MULT were detected (data not shown). To analyze if primary TEC also had similar expression, freshly isolated TEC, obtained from normal B6 mice, were cultured for 6–10 days and expressed MHC class I, CD13, CD26, and E-cadherin (data not shown). Interestingly, primary B6 TEC expressed Rae-1 but little or no surface H60 and MULT (Fig. 2*b* and data not shown).

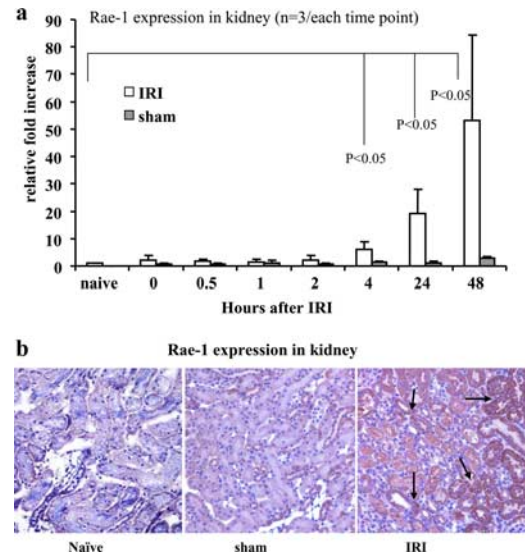


FIGURE 3. Kidneys express Rae-1 after IRI. *a*, Real-time PCR analysis of Rae-1 expression. The kidneys were collected at different reperfusion time points after being perfused with PBS to remove blood from kidneys. Total RNA was extracted (Qiagen) and cDNA pools were synthesized (Stratagene). Real-time quantitative PCR was performed using the Brilliant SYBR Green QPCR Master Mix kits and the Mx3005P system (Stratagene). β -actin amplification was used as the endogenous control. The normalized delta threshold cycle value was calculated according to the manufacturer's protocol. The results represent average data from three mice at each time point. *b*, Expression of Rae-1 in 48-h IRI kidneys was determined by immunohistochemistry (clone c-20, and biotin rabbit anti-goat IgG, Santa Cruz Biotechnology; Olympus DX51 microscope, $\times 400$, SPOT4.5 software). Similar staining was obtained from other mice in the same group. The positive staining areas are depicted by arrows in the images. Sham and naive kidneys were compared.

To test whether NK cell-mediated TEC killing is mediated through NKG2D and Rae-1 interaction, we used anti-NKG2D or anti-Rae-1 Abs to block NK cell-mediated TEC killing. 7-AAD staining was used to detect death of CFSE-labeled TEC (Fig. 2*c*). As shown in Fig. 2*d*, blocking either NKG2D or Rae-1 significantly inhibited NK cell-mediated TEC killing ($p < 0.01$). Hence, NKG2D expressed by NK cells can interact with Rae-1 on TEC to mediate TEC death.

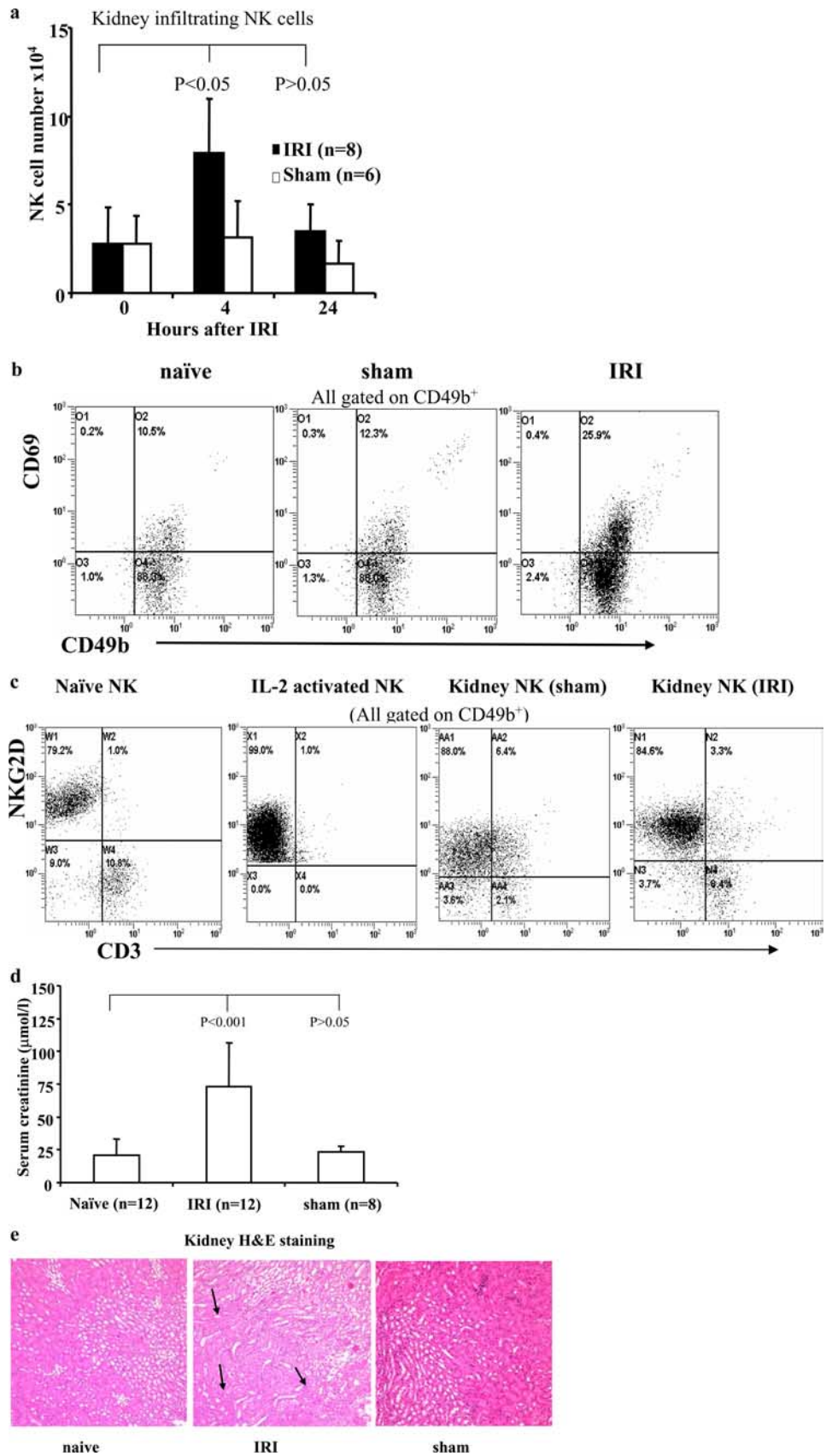
Kidneys express Rae-1 after IRI

Our *in vitro* data demonstrated the capacity of NK cells to injure TEC *in vitro*. A previous study has shown that expression of Rae-1 was increased in kidney after IRI (33). To determine whether NK cell-mediated TEC killing might occur also *in vivo*, we first tested whether kidney parenchymal cells express Rae-1 after ischemic injury. Kidneys from B6 male mice were processed after 45 min of ischemia. The kidneys were collected after different reperfusion times (from 0 to 48 h, $n = 3$ for each time point) and analyzed with real-time PCR. Naive untreated mice were used as a control. Rae-1 mRNA gradually increased after IRI (Fig. 3*a*) and was maximal by 4–48 h. Consistent with PCR results, immunohistochemistry indicated that marked expression of Rae-1 protein was present in tubular cells after 48 h of injury (Fig. 3*b*). Hence, these *in vivo* data support the hypothesis that NK cells interact with TEC through NKG2D/Rae-1 interaction to mediate kidney damage following IRI.

NK cells infiltrate into the kidney after IRI

Previous studies have demonstrated that leukocytes infiltrate into the kidney shortly after IRI, typically within 30 min to 3 h (16–21, 26,

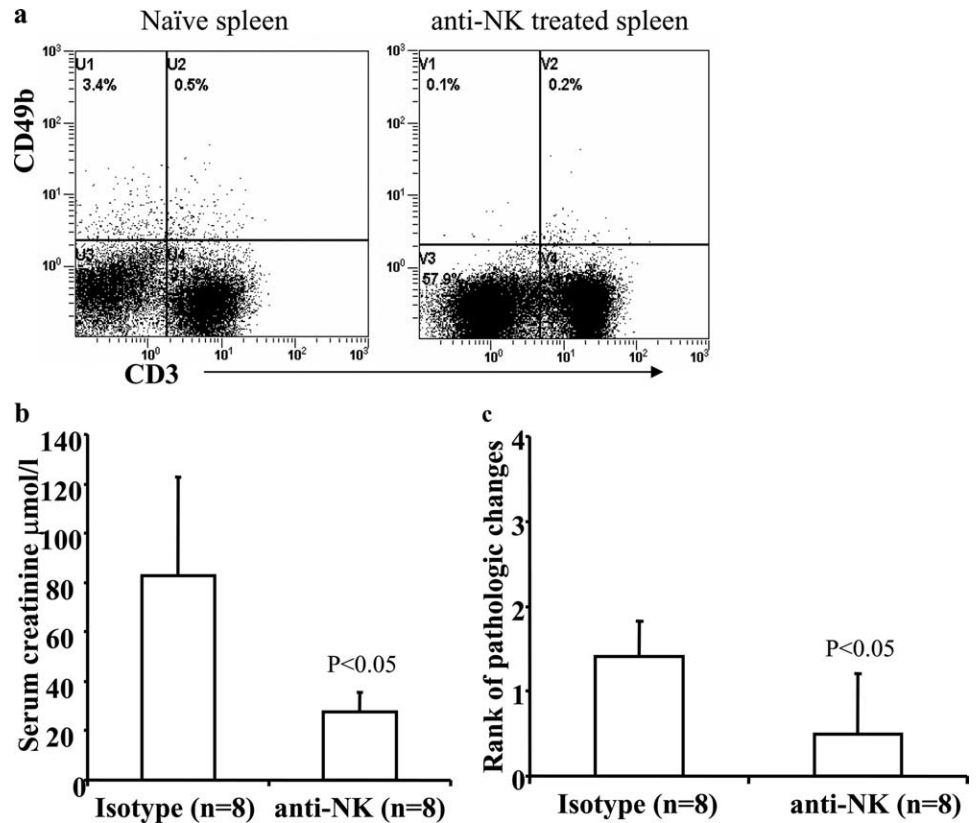
FIGURE 4. NK cells quickly infiltrate into the kidney after IRI. Kidneys were collected after 4 and 24 h of IRI. After being digested by collagenase, the cells were suspended in 36% Percoll (Amersham) and overlaid on 72% Percoll, then centrifuged at $1000 \times g$ for 30 min. Leukocytes were collected at the interface of Percoll and counted before being stained with anti-CD45, anti-CD3, anti-NKG2D, CD69, and anti-CD49b (DX5). *a*, Average number of CD3⁻CD49b⁺ NK cells from each mouse before and after IRI. The results represent pooled data from eight mice. Sham mice were used as control ($n = 6$). *b*, Infiltrating NK cells express activation marker CD69. Infiltrating NK cells were purified after 24 h of IRI and used for staining of lymphocyte activation marker CD69 by being gated on CD49b⁺ cells. Data represent one mouse from each group. *c*, Infiltrating NK cells express NKG2D. Infiltrating NK cells were stained by anti-CD49b (CX5), NKG2D, and CD3. Expression of NKG2D was determined by being gated on CD49b⁺ NK cells. IL-2-activated spleen NK cells and naive NK cells were compared. Data represent one mouse from each group. Similar data were obtained from all mice in the same group. *d*, Serum creatinine level after IRI. Data represent average value from 12 mice. Sham mice were used as control ($n = 8$). *e*, Representative histology of IRI kidney (Olympus DX51 microscope, $\times 200$, SPOT4.5 software). Data represent one mouse from each group. Similar data were obtained from all mice in the same group. Sham and naive kidney were compared. The injury areas are depicted by arrows in the images.



27). A low level of infiltrating NK cells has also been noted in post-ischemic kidneys (19), although infiltrating leukocytes appear to return to low levels by 24 h after injury. As there may be differences in the ischemic models studied, we analyzed NK cell infiltration in B6

male mice that underwent 45 min of ischemia, followed by 4 or 24 h of reperfusion. Whole kidneys were digested with collagenase V and kidney cells were separated from infiltrating leukocytes using gradient Percoll. Isolated total cells were analyzed by flow cytometry.

FIGURE 5. Depletion of NK cells attenuates kidney injury. Anti-asialo GM1 Ab ($3 \times 50 \mu\text{l}$) was i.v. injected into B6 mice on days -4 , -2 , and -1 to deplete NK cells. Control mice received isotype control rabbit IgG. Mice then underwent 45 min of ischemia. *a*, Depletion of NK cells was confirmed by staining spleen cells with anti-CD49 and CD3 before and after depletion. *b*, Sera, collected after 48 h of IRI, were used for creatinine level analysis. Data represent average of eight experiment mice in each group. *c*, Kidneys were collected for pathology scoring analysis as described in *Materials and Methods*. Data represent average of eight mice in each group. Criteria for kidney injury included tubular necrosis, immune cell infiltration, cast, and glomerular necrosis (scored as 0, no change; 1+, <25% area change; 2+, 25–50% area change; 3+, 50–75% area change; 4+, >75% area change). Data were obtained using six mice from each group.



Consistent with previous reports, total leukocytes as measured by CD45 expression quickly increased in the injured kidney, then substantially dropped after 24 h (data not shown). NK cells ($\text{CD3}^+ \text{CD49b}^+$) had a similar pattern and increased quickly after 4 h and could be identified after 24 h (Fig. 4a, $p < 0.05$). Interestingly, the lymphocyte activation marker CD69 was expressed by some infiltrating NK cells 24 h after IRI, supporting a role for activated NK cells in kidney injury (Fig. 4b). Importantly, infiltrating NK cells strongly expressed NKG2D in all mice (Fig. 4c).

To confirm kidney injury in this model, sera were collected for creatinine level analysis as a marker of kidney function. Creatinine levels were significantly increased 24 and 48 h after reperfusion, indicating marked kidney injury (Fig. 4d, 73.2 vs 20.6 $\mu\text{mol/L}$, $p < 0.001$). Consistent with creatinine levels, histology of kidneys demonstrated obvious tubular injury with extensive tubular necrosis/vacuolization (Fig. 4e, arrows), consistent with the increased serum creatinine at the same time point.

Depletion of NK cells inhibits kidney IRI

Our data demonstrate that NK cells infiltrate into the kidney following IRI, TEC express ligands to engage NK cells, and that NK cell-TEC interactions lead to apoptosis of syngeneic TEC in vitro. To test the cytotoxic role of NK cells in IRI in vivo, anti-asialo GM1 Ab was used to deplete NK cells in wild-type B6 mice before ischemia (Fig. 5a). Serum creatinine levels were significantly lower in NK cell-depleted B6 mice compared with isotype control-treated B6 mice after 48 h (Fig. 5b, 82.6 ± 52 vs $25.4 \pm 6.8 \mu\text{mol/L}$, $p < 0.05$). Pathology scoring analysis of injury was performed in a blinded fashion using an arbitrary scoring system. Consistent with the improvement in serum creatinine levels, anti-asialo GM1-treated mice had less kidney tissue damage than did control IgG treated mice (Fig. 5c, 1.4 ± 0.5 vs 0.5 ± 0.8 , $p < 0.05$). Collectively, these data demonstrate that depletion of NK cell greatly attenuates kidney IRI.

Adoptive transfer of NK cells worsens kidney injury in NK, T, and B cell-deficient mice

Previous studies have demonstrated that T cells, and in particular CD4^+ T cells, are necessary for renal IRI (16–21). T cell infiltration is transient with appearance by 4 h and resolution by 24 h (19, 26, 27). Additionally, CD19^+ B cells can participate in kidney IRI (30). Hence, T or B cells have been implicated in having a substantial role in kidney IRI, but these studies do not rule out contribution of NK cells. While we have demonstrated that Ab depletion of NK cells reduces kidney injury in normal B6 mice (Fig. 5), our results do not exclude potential indirect effects of other effector cells being in turn regulated by NK cells. Furthermore, anti-asialo GM1 depletes NK cells, but might also affect some NKT cells, as well as macrophages and monocytes. Hence, to more directly assess the participation of NK cells in mediating kidney tissue damage, we used $\text{Rag2}^{-/-} \gamma_c^{-/-}$ mice that lack NK, T, and B cells in which adoptive transfer of NK cells to reconstitute NK cell activity presents a system in which NK activity may be assessed in the absence of T and B cells (43). CD49b^+ NK cells ($1.5\text{--}2 \times 10^6$) from $\text{Rag2}^{-/-}$ mice were adoptively transfused into $\text{Rag2}^{-/-} \gamma_c^{-/-}$ mice; PBS-injected $\text{Rag2}^{-/-} \gamma_c^{-/-}$ mice were used as controls. All mice ($n = 10/\text{group}$) received 45 min of ischemic injury. Sera were collected after 48 h reperfusion and kidneys were subjected to histopathology analyses. Analysis of spleens following adoptive transfer confirmed the presence of NK cells in $\text{Rag2}^{-/-} \gamma_c^{-/-}$ mice (Fig. 6a). Importantly, reconstituted $\text{Rag2}^{-/-} \gamma_c^{-/-}$ mice that had received NK cells adoptively showed significant higher creatinine levels after IRI compared with PBS-injected NK-deficient mice (Fig. 6b, 63.5 ± 22 vs $34.4 \pm 16.7 \mu\text{mol/L}$, $p < 0.01$). Additionally, $\text{Rag2}^{-/-} \gamma_c^{-/-}$ mice that received NK cell transfers showed obvious large areas of tubular injury, in contrast to mild injury in kidneys from NK, B, and T cell-deficient $\text{Rag2}^{-/-} \gamma_c^{-/-}$ mice (Fig. 6c). Histopathology analyses of kidney injury

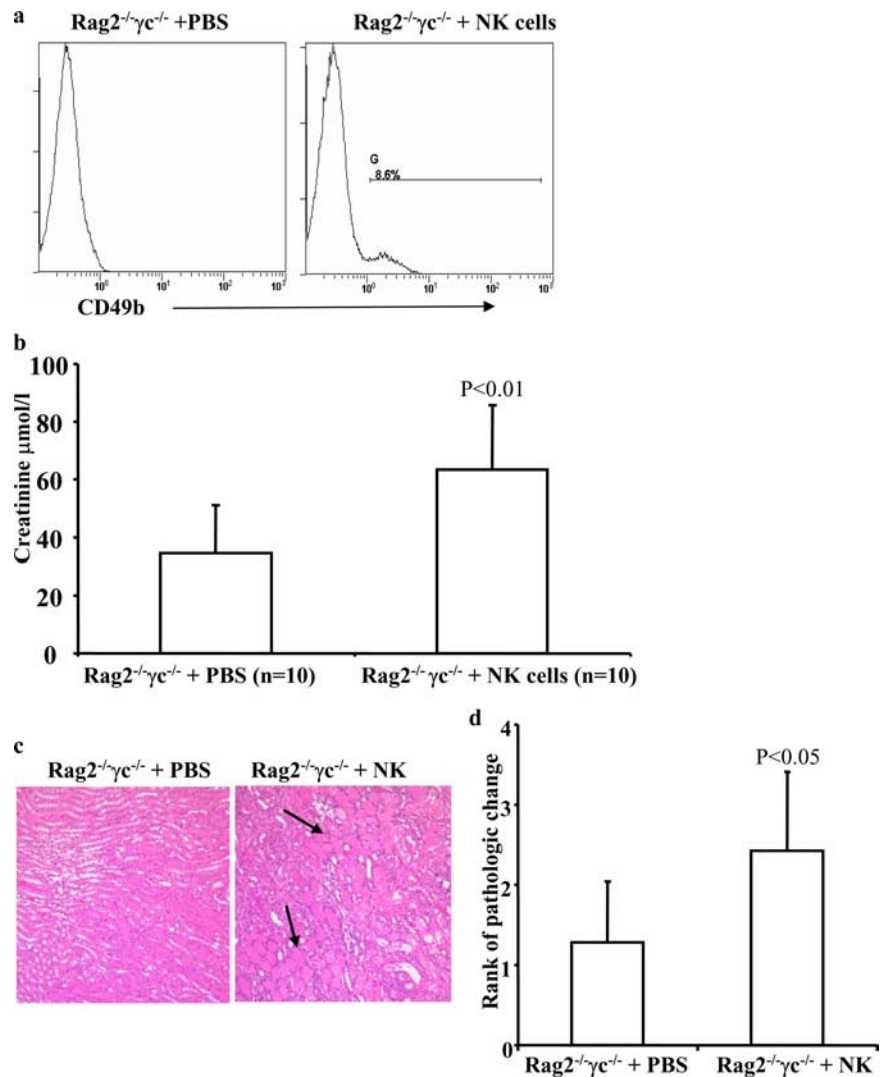


FIGURE 6. Adoptive transfer of NK cells induces strong kidney injury in Rag2^{-/-}γ_c^{-/-} mice. CD49b⁺ NK cells (1.5–2 × 10⁶) from Rag2^{-/-} mice were i.v. transfused into Rag2^{-/-}γ_c^{-/-} mice. After 5 days, treated mice underwent 35°C and 45 min of ischemic injury and 48 h of reperfusion injury. Mice that received PBS were used as control. *a*, The spleens of NK cell-infused Rag2^{-/-}γ_c^{-/-} mice were collected to confirm that NK cells reconstituted, as shown by staining with anti-CD49b-PE. *b*, Serum creatinine level was determined. *c*, Histology results (H&E staining, Olympus DX51 microscope, ×200, SPOT4.5 software) are presented with one mouse from each group. The injury areas are indicated by arrows. *d*, Kidneys were collected for pathology scoring analysis in a double blind fashion. Criteria for kidney injury are described in *Materials and Methods* and in Fig. 5. Data were obtained using eight mice from each group.

performed in a double blind fashion (Fig. 6*d*) showed greater injury in NK cell-transferred mice ($n = 10$ /group, 2.4 ± 1.0 vs 1.3 ± 0.8 , $p < 0.05$). Taken together, these data support a critical role for NK cell mediated-kidney injury in IRI. While this did not appear to be T and B cell-dependent in this model, there may be additive effects of these other effectors along with NK cells in a mixed cell environment.

NK cell-mediated kidney injury is PFN-dependent

NK cells may be cytotoxic by the expression of PFN/granzyme B, FasL, or by proinflammatory cytokines. To determine whether NK cells were biased to a mechanism of cytotoxicity in renal IRI, we purified NK cells from FasL-deficient (*gld*), PFN-deficient (PFN^{-/-}), and IFN-γ^{-/-} mice and transferred these into Rag2^{-/-}γ_c^{-/-} mice. All mice ($n = 6$ /group) received 45 min of ischemic injury. Sera were collected after 48 h reperfusion for creatinine analysis. Interestingly, we found that adoptive transfer of PFN^{-/-} NK cells was associated with minimal kidney injury in Rag2^{-/-}γ_c^{-/-} mice as compared with transfer of wild-type B6 NK cells, *gld* (FasL mutant) NK cells, or IFN-γ^{-/-} NK cells (Fig. 7*a*, $p < 0.05$), suggesting a preferential use of PFN in NK cell-mediated kidney injury after NK cell transfer.

To further support a primary role for perforin in kidney injury, we performed TEC cytotoxicity assays. As with adoptive transfers,

NK cells were purified from B6, PFN^{-/-}, *gld*, and IFN-γ^{-/-} mice activated with IL-2 (1000 IU/ml) and used as effectors in mixed NK cell-TEC cultures. As shown in Fig. 7*b*, PFN^{-/-} NK cell-mediated TEC killing was significantly decreased in vitro, whereas NK cells from B6, *gld*, and IFN-γ^{-/-} mice showed similar TEC killing capacities. Taken together, these data suggest that PFN play a central role in NK cell-mediated kidney injury in vivo and TEC death in vitro.

Discussion

TEC comprise >75% of renal parenchymal cells, and their susceptibility to injury directs the long-term function of allografts, as tubular injury can be a major cause for nephron loss (44). Post-transplant, TEC death by apoptosis occurs with ischemia, reactive oxidative metabolites, NO, proinflammatory cytokines (TNF-α, IL-1), and CTLs (FasL/Fas, PFN/granzyme B). In this study, we have demonstrated for the first time that NK cells can induce kidney TEC death in vitro and that NKG2D and Rae-1 interactions play a critical role in this killing. Furthermore, NK cell infiltration occurs after ischemia, and it is likely that NK cells can also interact with TEC expressing Rae-1 in vivo, as both NK and Rae-1 are increased within the same time period after IRI. In this study, NK cell depletion was protective in IRI, while enhancing NK cell numbers using adoptive transfer enhanced kidney dysfunction and

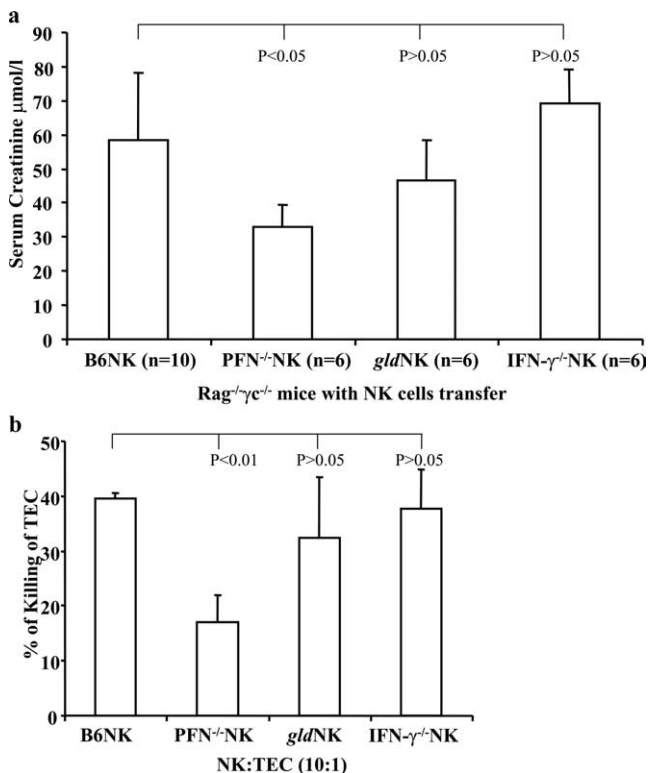


FIGURE 7. NK cell-mediated kidney injury and TEC death are PFN-dependent. *a*, CD49b⁺ NK cells ($1.5\text{--}2 \times 10^6$) from Rag2^{-/-}, PFN^{-/-}, *gld*, and IFN-γ^{-/-} mice were purified as described in *Materials and Methods* and then i.v. transfused into Rag2^{-/-}γ_c^{-/-} mice. After 5 days, treated mice underwent 35°C and 45 min of ischemic injury and 48 h of reperfusion injury. Serum creatinine level was determined. Data were obtained using 10 or 6 mice from each group. *b*, To measure NK cell-mediated TEC killing, NK cells were purified from B6 Rag2^{-/-}, PFN^{-/-}, *gld*, and IFN-γ^{-/-} mice using anti-CD49b MACS bead selection (Miltenyi Biotec) after depleting CD3⁺ T cells. The purified NK cells were activated by IL-2 (1000 IU/ml) for 3–5 days before being used as effectors. TEC line B6 NG1.1 (10⁴) was labeled with CFSE and used as target (triplicate). After NK cells and target cells were cocultured for 5 h, the cells were harvested and stained for 7-ADD expression. Specific cell apoptosis was calculated by measuring CFSE⁺7-AAD⁺ TEC as described in *Materials and Methods*. Experiments were repeated three times and similar results were obtained.

histological injury after IRI. Furthermore, we have found that NK cell-mediated kidney injury and TEC death appear to be PFN biased. As therapeutic strategies become available to alter NK cell-TEC interactions, targeting of NK cells to attenuate IRI may represent an important strategy to limit IRI in renal transplantation and other forms of renal inflammatory injury, along with anti-T and other participatory cell therapeutics.

A number of factors contribute to TEC injury during inflammation and following ischemia. Studies have demonstrated that extracellular and intracellular changes in TEC as well as inflammatory factors contribute to kidney IRI, including Fas-FasL interaction between TEC (6, 45), endogenous TNF-α (7, 28, 46–49), activation of complement C3–C5 (50, 51), and oxidative stress (52, 53). In the past decade, TGF-β has been increasingly implicated as a major cytokine that contributes to tubular injury and dysfunction (54). Furthermore, IRI is considered an Ag-independent inflammatory sequence of events and processes that leads to tubular epithelial and endothelial dysfunction (3–5). Previous studies have demonstrated that kidney-infiltrating macrophages and neutrophils significantly increased after injury, indicating that in-

nate immunity plays an important role in tubular dysfunction (12–15). Recent studies have demonstrated that diverse cell types of the adaptive immune system also participate in kidney IRI and that in addition to classical involvement of neutrophils and macrophages, T cells, especially CD4⁺ T cells, are central effectors in renal IRI (16–21, 24). Interestingly, CD19⁺ B cells, CD4⁺NK⁺ cells, as well as CD3⁻NK1.1⁺ cells also infiltrate into the kidney shortly after injury (19), suggesting that a multicellular inflammatory response is triggered by kidney IRI. While depletion of T cells or B cells can protect kidneys from IRI (18, 25, 30, 32), T and B cell-deficient Rag1^{-/-} mice can develop kidney IRI, reflecting the complex biology of IRI and suggesting that additional cells are involved. NK cells are important participants in early-stage innate immune responses. Unlike T and B cells responses, NK cells can lyse target cells nearly instantly without presensitization. Expression of NKG2D receptors on target cells, however, clearly plays a crucial role in NK cell-mediated killing. In this study, we found that NK cells can clearly kill TEC in vitro. As blockade of either NKG2D or Rae-1 by Abs significantly inhibits NK cell-mediated TEC killing, the cytotoxic effect of NK cells on TEC requires close cellular approximation to permit NKG2D and Rae-1 interaction as well as perforin effect. We have found using quantitative PCR that MULT-1, another NKG2D ligand, is also increased in kidney after IRI (data not shown). However, we were unable to detect MULT-1 expression by flow cytometry or immunohistochemistry, which may be related to a low level of expression or a low affinity of currently available detecting Ab. Hence, we have not excluded a possible contribution of NKG2D and MULT-1 in promoting injury.

Although NK cells infiltrate into kidneys as early as 30 min after IRI (data not shown and Fig. 4a), Rae-1 expression in kidney was detected by PCR by 4 h (Fig. 3). This may suggest that NK cells might use other molecule(s) to interact with TEC rather than NKG2D and Rae-1 in the very early stages of injury. As macrophages, T cells, B cells, NKT cells, dendritic cells and, of course, neutrophils all contribute to IRI, it is clear that the complex biology of IRI and multicellular interactions during development of injury require further investigation to optimize any clinical strategies to attenuate injury.

Our data confirm that NK cells quickly infiltrate into injured kidneys (Fig. 4a) (19). With enhanced expression of Rae-1 in kidneys following IRI (Fig. 3) (33), the scenario would be expected to lead to TEC killing by infiltrating NK cells. Indeed, our in vivo results support a critical role for NK kidney injury using NK depletion and adoptive NK cell transfer experimental approaches. Despite the potential utilization of several cytotoxic mechanisms by NK cells, reduced injury of kidneys was apparent only with PFN^{-/-} NK cells in Rag2^{-/-}γ_c^{-/-} mice with minimal effect of loss of FasL or IFN-γ. Hence, PFN appears to play a crucial role for cytotoxic function of NK cells during development of kidney injury. While the main effector function of NK cells may be cellular cytotoxicity, IFN-γ and TNF-α production by NK cells forms an essential component of their innate immune response (55–57). As we have demonstrated that TEC can undergo self-injury by the coexpression of Fas and FasL (6, 7), which is enhanced by these proinflammatory cytokines, NK cell-mediated renal injury in IRI could involve both direct and indirect mechanisms. Further investigation of the role of NK cells in TEC self-injury is required.

Previous studies have demonstrated that T cell or B cell deficiency can protect kidneys from IRI (18, 25, 30, 32) although the mechanisms by which adoptive transfer of T or B cells can inhibit kidney IRI are not clear (32). T cell infiltration is evident as early as 4 h and appears to resolve by 24 h (19, 26, 27). It is

unknown whether these infiltrating T cells can have a direct cytotoxic effect on kidney cells or whether indirect effects, such as expression of cytokines to enhance TEC self-injury or chemotactic factors for other effectors, lead to kidney injury. Consistent with this, a recent study has shown that IFN- γ -producing CD4⁺ T cells worsen kidney injury, whereas IFN- γ -deficient CD4⁺ T cells protect kidneys from injury (18). It may be that IFN- γ -producing CD4⁺ T cells enhance NK cell function in kidney to worsen injury. While these previous reports reflect the complex biology of IRI and the role of adaptive immunity in this process, it is clear from the present results that non-T cells, including NK cells, also participate in kidney IRI and might be targeted to attenuate injury. In summary, this study demonstrates for the first time that NK cells can directly kill TEC in vitro and are major participants in kidney IRI injury in vivo. Further work is required to define the regulation of NK ligands such as Rae-1 by TEC and to find strategies to block NK cell-TEC interactions to limit renal IRI. Our data highlight the previously unrecognized role of NK cells in TEC killing and IRI and may lead to novel and clinically useful approaches to limit injury in diverse causes of kidney inflammation, including transplantation.

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Disclosures

The authors have no financial conflicts of interest.

References

- Thadhani, R., M. Pascual, and J. V. Bonventre. 1996. Acute renal failure. *N. Engl. J. Med.* 334: 1448–1460.
- Shoskes, D. A., and P. F. Halloran. 1996. Delayed graft function in renal transplantation: etiology, management, and long-term significance. *J. Urol.* 155: 1831–1840.
- Bonventre, J. V. 2003. Dedifferentiation and proliferation of surviving epithelial cells in acute renal failure. *J. Am. Soc. Nephrol.* 14 (Suppl. 1): S55–S61.
- Molitoris, B. A., and T. A. Sutton. 2004. Endothelial injury and dysfunction: role in the extension phase of acute renal failure. *Kidney Int.* 66: 496–499.
- Boros, P., and J. S. Bromberg. 2006. New cellular and molecular immune pathways in ischemia/reperfusion injury. *Am. J. Transplant.* 6: 652–658.
- Du, C., J. Jiang, Q. Guan, Z. Yin, M. Masterson, A. Parbtani, R. Zhong, and A. M. Jevnikar. 2004. Renal tubular epithelial cell self-injury through Fas/Fas ligand interaction promotes renal allograft injury. *Am. J. Transplant.* 4: 1583–1594.
- Du, C., S. Wang, H. Diao, Q. Guan, R. Zhong, and A. M. Jevnikar. 2006. Increasing resistance of tubular epithelial cells to apoptosis by shRNA therapy ameliorates renal ischemia-reperfusion injury. *Am. J. Transplant.* 6: 2256–2267.
- Du, C., Q. Guan, Z. Yin, R. Zhong, and A. M. Jevnikar. 2005. IL-2-mediated apoptosis of kidney tubular epithelial cells is regulated by the caspase-8 inhibitor c-FLIP. *Kidney Int.* 67: 1397–1409.
- Toledo-Pereyra, L. H., F. Lopez-Nebolina, and A. H. Toledo. 2004. Reactive oxygen species and molecular biology of ischemia/reperfusion. *Ann. Transplant.* 9: 81–83.
- Millar, T. M., V. Phan, and L. A. Tibbles. 2007. ROS generation in endothelial hypoxia and reoxygenation stimulates MAP kinase signaling and kinase-dependent neutrophil recruitment. *Free Radic. Biol. Med.* 42: 1165–1177.
- Cassie, S., M. F. Masterson, A. Polukoshko, M. M. Viskovic, and L. A. Tibbles. 2004. Ischemia/reperfusion induces the recruitment of leukocytes from whole blood under flow conditions. *Free Radic. Biol. Med.* 36: 1102–1111.
- Bonventre, J. V., and A. Zuk. 2004. Ischemic acute renal failure: an inflammatory disease? *Kidney Int.* 66: 480–485.
- Ysebaert, D. K., K. E. De Greef, S. R. Vercauteren, M. Ghielli, G. A. Verpooten, E. J. Eyskens, and M. E. De Broe. 2000. Identification and kinetics of leukocytes after severe ischaemia/reperfusion renal injury. *Nephrol. Dial. Transplant.* 15: 1562–1574.
- De Greef, K. E., D. K. Ysebaert, M. Ghielli, S. Vercauteren, E. J. Nouwen, E. J. Eyskens, and M. E. De Broe. 1998. Neutrophils and acute ischemia-reperfusion injury. *J. Nephrol.* 11: 110–122.
- Dragun, D., U. Hoff, J. K. Park, Y. Qun, W. Schneider, F. C. Luft, and H. Haller. 2000. Ischemia-reperfusion injury in renal transplantation is independent of the immunologic background. *Kidney Int.* 58: 2166–2177.
- Burne, M. J., F. Daniels, A. El Ghandour, S. Mauyyedi, R. B. Colvin, M. P. O'Donnell, and H. Rabb. 2001. Identification of the CD4⁺ T cell as a major pathogenic factor in ischemic acute renal failure. *J. Clin. Invest.* 108: 1283–1290.
- De Greef, K. E., D. K. Ysebaert, S. Dauwe, V. Persy, S. R. Vercauteren, D. Mey, and M. E. De Broe. 2001. Anti-B7-1 blocks mononuclear cell adherence in vasa recta after ischemia. *Kidney Int.* 60: 1415–1427.
- Day, Y. J., L. Huang, H. Ye, L. Li, J. Linden, and M. D. Okusa. 2006. Renal ischemia-reperfusion injury and adenosine 2A receptor-mediated tissue protection: the role of CD4⁺ T cells and IFN- γ . *J. Immunol.* 176: 3108–3114.
- Ascon, D. B., S. Lopez-Briones, M. Liu, M. Ascon, V. Savransky, R. B. Colvin, M. J. Soloski, and H. Rabb. 2006. Phenotypic and functional characterization of kidney-infiltrating lymphocytes in renal ischemia reperfusion injury. *J. Immunol.* 177: 3380–3387.
- Ysebaert, D. K., K. E. De Greef, S. R. Vercauteren, A. Verhulst, M. Kockx, G. A. Verpooten, and M. E. De Broe. 2003. Effect of immunosuppression on damage, leukocyte infiltration, and regeneration after severe warm ischemia/reperfusion renal injury. *Kidney Int.* 64: 864–873.
- Rabb, H., F. Daniels, M. O'Donnell, M. Haq, S. R. Saba, W. Keane, and W. W. Tang. 2000. Pathophysiological role of T lymphocytes in renal ischemia-reperfusion injury in mice. *Am. J. Physiol.* 279: F525–F531.
- Takada, M., A. Chandraker, K. C. Nadeau, M. H. Sayegh, and N. L. Tilney. 1997. The role of the B7 costimulatory pathway in experimental cold ischemia/reperfusion injury. *J. Clin. Invest.* 100: 1199–1203.
- Azuma, H., K. Nadeau, M. Takada, H. S. Mackenzie, and N. L. Tilney. 1997. Cellular and molecular predictors of chronic renal dysfunction after initial ischemia/reperfusion injury of a single kidney. *Transplantation* 64: 190–197.
- Takada, M., K. C. Nadeau, G. D. Shaw, K. A. Marquette, and N. L. Tilney. 1997. The cytokine-adhesion molecule cascade in ischemia/reperfusion injury of the rat kidney. Inhibition by a soluble P-selectin ligand. *J. Clin. Invest.* 99: 2682–2690.
- Yokota, N., F. Daniels, J. Crosson, and H. Rabb. 2002. Protective effect of T cell depletion in murine renal ischemia-reperfusion injury. *Transplantation* 74: 759–763.
- Friedewald, J. J., and H. Rabb. 2004. Inflammatory cells in ischemic acute renal failure. *Kidney Int.* 66: 486–491.
- Lai, L. W., K. C. Yong, S. Igarashi, and Y. H. Lien. 2007. A sphingosine-1-phosphate type 1 receptor agonist inhibits the early T-cell transient following renal ischemia-reperfusion injury. *Kidney Int.* 71: 1223–1231.
- Dong, X., S. Swaminathan, L. A. Bachman, A. J. Croatt, K. A. Nath, and M. D. Griffin. 2007. Resident dendritic cells are the predominant TNF-secreting cell in early renal ischemia-reperfusion injury. *Kidney Int.* 71: 619–628.
- Li, L., L. Huang, S. S. Sung, P. I. Lobo, M. G. Brown, R. K. Gregg, V. H. Engelhard, and M. D. Okusa. 2007. NKT cell activation mediates neutrophil IFN- γ production and renal ischemia-reperfusion injury. *J. Immunol.* 178: 5899–5911.
- Burne-Taney, M. J., D. B. Ascon, F. Daniels, L. Racusen, W. Baldwin, and H. Rabb. 2003. B cell deficiency confers protection from renal ischemia reperfusion injury. *J. Immunol.* 171: 3210–3215.
- Park, P., M. Haas, P. N. Cunningham, L. Bao, J. J. Alexander, and R. J. Quigg. 2002. Injury in renal ischemia-reperfusion is independent from immunoglobulins and T lymphocytes. *Am. J. Physiol.* 282: F352–F357.
- Burne-Taney, M. J., N. Yokota-Ikeda, and H. Rabb. 2005. Effects of combined T- and B-cell deficiency on murine ischemia reperfusion injury. *Am. J. Transplant.* 5: 1186–1193.
- Feng, L., F. Cheng, Z. Ye, S. Li, Y. He, X. Yao, Q. Tang, and Y. Li. 2006. The effect of renal ischemia-reperfusion injury on expression of RAE-1 and H60 in mice kidney. *Transplant. Proc.* 38: 2195–2198.
- Hoglund, P., J. Sundback, M. Y. Olsson-Alheim, M. Johansson, M. Salcedo, C. Ohlen, H. G. Ljunggren, C. L. Sentman, and K. Karre. 1997. Host MHC class I gene control of NK-cell specificity in the mouse. *Immunol. Rev.* 155: 11–28.
- Kane, K. P., K. J. Lavender, and B. J. Ma. 2004. Ly-49 receptors and their functions. *Crit. Rev. Immunol.* 24: 321–348.
- Brooks, A. G., P. E. Posch, C. J. Scorzelli, F. Borrego, and J. E. Coligan. 1997. NKG2A complexed with CD94 defines a novel inhibitory natural killer cell receptor. *J. Exp. Med.* 185: 795–800.
- Vance, R. E., J. R. Kraft, J. D. Altman, P. E. Jensen, and D. H. Raulet. 1998. Mouse CD94/NKG2A is a natural killer cell receptor for the nonclassical major histocompatibility complex (MHC) class I molecule Qa-1^p. *J. Exp. Med.* 188: 1841–1848.
- Snyder, M. R., C. M. Weyand, and J. J. Goronzy. 2004. The double life of NK receptors: stimulation or co-stimulation? *Trends Immunol.* 25: 25–32.
- Raziuddin, A., D. L. Longo, M. Bennett, R. Winkler-Pickett, J. R. Ortaldo, and W. J. Murphy. 2002. Increased bone marrow allograft rejection by depletion of NK cells expressing inhibitory Ly49 NK receptors for donor class I antigens. *Blood* 100: 3026–3033.
- Matzinger, P. 1991. The JAM test: a simple assay for DNA fragmentation and cell death. *J. Immunol. Methods* 145: 185–192.
- Hoves, S., S. W. Krause, J. Scholmerich, and M. Fleck. 2003. The JAM-assay: optimized conditions to determine death-receptor-mediated apoptosis. *Methods* 31: 127–134.
- Ayres, F. M., M. Narita, M. Takahashi, L. Alldawi, A. Liu, Y. Osman, T. Abe, T. Yano, M. Sakaue, K. Toba, et al. 2003. A comparative study of the JAM test and ⁵¹Cr-release assay to assess the cytotoxicity of dendritic cells on hematopoietic tumor cells. *Immunol. Invest.* 32: 219–227.
- Prlic, M., B. R. Blazar, M. A. Farrar, and S. C. Jameson. 2003. In vivo survival and homeostatic proliferation of natural killer cells. *J. Exp. Med.* 197: 967–976.
- Pagtalunan, M. E., J. L. Olson, N. L. Tilney, and T. W. Meyer. 1999. Late consequences of acute ischemic injury to a solitary kidney. *J. Am. Soc. Nephrol.* 10: 366–373.
- Nogae, S., M. Miyazaki, N. Kobayashi, T. Saito, K. Abe, H. Saito, P. K. Nakane, Y. Nakanishi, and T. Koji. 1998. Induction of apoptosis in ischemia-reperfusion

- model of mouse kidney: possible involvement of Fas. *J. Am. Soc. Nephrol.* 9: 620–631.
46. Daemen, M. A., M. W. van de Ven, E. Heineman, and W. A. Buurman. 1999. Involvement of endogenous interleukin-10 and tumor necrosis factor- α in renal ischemia-reperfusion injury. *Transplantation* 67: 792–800.
 47. Donahoo, K. K., B. D. Shames, A. H. Harken, and D. R. Meldrum. 1999. Review article: the role of tumor necrosis factor in renal ischemia-reperfusion injury. *J. Urol.* 162: 196–203.
 48. Zhang, X., X. Zheng, H. Sun, B. Feng, G. Chen, C. Vladau, M. Li, D. Chen, M. Suzuki, L. Min, et al. 2006. Prevention of renal ischemic injury by silencing the expression of renal caspase 3 and caspase 8. *Transplantation* 82: 1728–1732.
 49. Zheng, X., X. Zhang, H. Sun, B. Feng, M. Li, G. Chen, C. Vladau, D. Chen, M. Suzuki, L. Min, et al. 2006. Protection of renal ischemia injury using combination gene silencing of complement 3 and caspase 3 genes. *Transplantation* 82: 1781–1786.
 50. de Vries, B., J. Kohl, W. K. Leclercq, T. G. Wolfs, A. A. van Bijnen, P. Heeringa, and W. A. Buurman. 2003. Complement factor C5a mediates renal ischemia-reperfusion injury independent from neutrophils. *J. Immunol.* 170: 3883–3889.
 51. Puri, T. S., and R. J. Quigg. 2007. The many effects of complement C3- and C5-binding proteins in renal injury. *Semin. Nephrol.* 27: 321–337.
 52. Du, C., Q. Guan, H. Diao, Z. Yin, and A. M. Jevnikar. 2006. Nitric oxide induces apoptosis in renal tubular epithelial cells through activation of caspase-8. *Am. J. Physiol.* 290: F1044–F1054.
 53. Glynne, P. A., and T. J. Evans. 1999. Inflammatory cytokines induce apoptotic and necrotic cell shedding from human proximal tubular epithelial cell monolayers. *Kidney Int.* 55: 2573–2597.
 54. Bottinger, E. P. 2007. TGF- β in renal injury and disease. *Semin. Nephrol.* 27: 309–320.
 55. Lodoen, M. B., and L. L. Lanier. 2006. Natural killer cells as an initial defense against pathogens. *Curr. Opin. Immunol.* 18: 391–398.
 56. Zingoni, A., T. Sornasse, B. G. Cocks, Y. Tanaka, A. Santoni, and L. L. Lanier. 2005. NK cell regulation of T cell-mediated responses. *Mol. Immunol.* 42: 451–454.
 57. Vujanovic, N. L. 2001. Role of TNF family ligands in antitumor activity of natural killer cells. *Int. Rev. Immunol.* 20: 415–437.