The Journal of Immunology

Phenotypic and Functional Characterization of Kidney-Infiltrating Lymphocytes in Renal Ischemia Reperfusion Injury

Dolores B. Ascon, Sergio Lopez-Briones, Manchang Liu, Miguel Ascon, Vladimir Savransky, Robert B. Colvin, Mark J. Soloski and Hamid Rabb

J Immunol 2006; 177:3380-3387; ;
doi: 10.4049/jimmunol.177.5.3380
http://www.jimmunol.org/content/177/5/3380

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Phenotypic and Functional Characterization of Kidney-Infiltrating Lymphocytes in Renal Ischemia Reperfusion Injury[1]

Dolores B. Ascon,* Sergio Lopez-Briones,* Manchang Liu,* Miguel Ascon,* Vladimir Savransky,* Robert B. Colvin,† Mark J. Soloski,* and Hamid Rabb[2]*

T and B lymphocytes have been implicated in the pathogenesis of renal ischemia reperfusion injury (IRI). The trafficking of lymphocytes into kidneys during IRI has been postulated to underlie this effect, but has not been rigorously studied. We therefore characterized the lymphocyte populations infiltrating into mouse kidneys 3 and 24 h after renal IRI. Immunohistochemistry and flow cytometry staining of kidney lymphocytes showed increased trafficking of $CD3^+$ T cells and $CD19^+$ B cells in both sham-operated and IRI mice 3 h after renal IRI. In the IRI mice, increased infiltration of $NK1.1^+$ and $CD4^+NK1.1^+$ cells compared with normal and sham-operated mice was observed 3 and 24 h after renal IRI, respectively. After 24 h of renal IRI, the decreased percentages of $CD3^+$, $CD19^+$, and $NK1.1^+$ populations in the IRI mice compared with control groups were observed. Increased TNF-α and IFN-γ production of kidney infiltration $CD3^+$ T cells in IRI mice but not sham-operated mice was found. Unexpectedly, isolation and transfer of kidney-infiltrating lymphocytes 24 h after renal IRI into T cell-deficient mice reduced their functional and histological injury after renal IRI, suggesting that kidney-infiltrating lymphocytes could have a protective function. These quantitative, qualitative, and functional changes in kidney lymphocytes provide mechanistic insight into how lymphocytes modulate IRI, as well as demonstrating that abdominal surgery alone leads to lymphocyte changes in kidney. ***The Journal of Immunology,*** **2006, 177: 3380–3387.**

Renal ischemia reperfusion injury (IRI)[3] is a major cause of acute renal failure associated with a high rate of mortality in patients with native kidneys and early kidney dysfunction with enhanced rejection in patients with transplanted kidneys (1). Several inflammatory mediators have been identified in the pathogenesis of renal IRI, including infiltration of neutrophils and macrophages, activation of complement fraction C5a and membrane attack complex, and up-regulation of cytokines, chemokines, and leukocyte adhesion molecules ICAM-1 and VCAM-1 (2–6). Recent studies have identified T lymphocytes as important mediators in renal IRI (7–9) as well as IRI of the liver (10, 11) and the lung (12). Adoptive transfer of wild-type $CD4^+$ T cells into athymic mice (*nu/nu),* which were partially protected from IRI, restored renal injury phenotype. However, transfer of $CD4^+$ T cells deficient in either the molecule CD28 or the ability to produce IFN-γ were unable to restore the ischemic injury in *nu/nu* mice (8). Subsequent studies investigated the $CD4^+$ T cell subsets Th1 or Th2 and their contribution in renal IRI using STAT4 and STAT6 knockout mice, finding that STAT6 deficiency conferred enhanced kidney injury and mild improvement of renal function in the STAT4- deficient group (13) similar to findings in liver IRI (14). B cell-deficient mice were also protected from ischemic injury, supporting the role of the adaptive immune system in the pathogenesis of renal IRI (15). Intriguingly, no protection from renal IRI was observed in the RAG-1-deficient mouse which lacks both T and B lymphocytes (16, 17). Enhanced NK cell activity in these mice was implicated. Taken together, these studies illustrate the complexity of the mechanisms involving T lymphocytes in renal IRI.

Despite several studies demonstrating a role for lymphocytes in renal IRI, there have been no rigorous studies to date to determine whether lymphocytes orchestrate kidney injury from the affected organ or from a distant site possibly releasing inflammatory cytokines. The limited studies on infiltrating lymphocytes in postischemic mouse kidneys are due, in part, because of difficulties in isolating the smaller number of lymphocytes involved in acute renal failure compared with the massive infiltration of $CD8^+$ T cells in kidney allograft rejection (18). Infiltration of CD4 and CD8 T cells has been reported in rat kidneys 3 days after ischemia (19). In contrast, very early infiltration of $CD3^{int}$ T cells (intermediate cells with phenotype $CD3^{int}IL\text{-}2R\beta^+$) was observed in mouse kidneys that underwent ischemic injury (20). There is no information to date about the role of NK cells and NKT cells in renal IRI, which has been recently found to be important mediators in liver IRI (21).

To better understand the role of lymphocytes in the pathophysiology of renal IRI, we used immunohistochemistry and found lymphocyte trafficking into kidneys of sham-operated and IRI mice. We then optimized a technique to effectively elute lymphocytes from mouse kidneys and characterized these lymphocyte populations. Flow cytometry analysis of kidney mononuclear cells (KMNC) identified the following lymphocyte phenotypes

*Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21205; and †Department of Pathology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02115

Received for publication October 25, 2005. Accepted for publication June 20, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This study was supported by grants from the National Institutes of Health minority supplement for the R0–1 DK54770 and a Roche Organ Transplantation Research Foundation award.

[2] Address correspondence and reprint requests to Dr. Hamid Rabb, Johns Hopkins University School of Medicine, Ross Research Building, Room 970, 720 Rutland Avenue, Baltimore, MD 21205. E-mail address: hrabb1@jhmi.edu

[3] Abbreviations used in this paper: IRI, ischemia reperfusion injury; KMNC, kidney mononuclear cell; HPF, high-power field.

0022-1767/06/$02.00

$CD3^+$, $CD4^+$, $CD4^+NK1.1^+$, $CD8^+$, $CD19^+$, and $NK1.1^+$ cells in normal mice (no surgery), sham-operated (laparotomy), and IRI mice (laparotomy and 30-min ischemia). We found that early trafficking of $CD3^+$ T cells and activation of $CD4^+$ and $CD8^+$ T cells into kidneys of sham-operated and IRI mice were largely due to laparotomy and surgical trauma alone. However, the trafficking of the populations NK and NKT cells into postischemic kidneys was closely correlated with increased serum creatinine and kidney injury and not sham surgery. In addition, infiltrating $CD3^+$ T cells increased proinflammatory cytokine production in IRI mice but not in sham-operated mice, which illustrates the importance of functional changes in infiltrating lymphocytes independent of trafficking alone. Finally, we isolated kidney-infiltrating lymphocytes 24 h after renal IRI and transferred the cells into T cell-deficient mice, and found that adoptive transfer of these lymphocytes unexpectedly improved structural and functional outcome after IRI.

Materials and Methods

Mice

Male C57BL/6J wild-type and T cell-deficient *nu/nu* mice (B6.Cg-*Foxn1*^{nu}), 5–10 wk of age, were purchased from The Jackson Laboratory and Taconic Farms, respectively. The mice were maintained under specific pathogen-free conditions. All experiments were performed in accordance with the guide for the Animal Care and Use Committee guidelines.

Mouse renal IRI model

An established model of renal IRI in mice was used (7). Briefly, male mice were anesthetized with an i.p. injection of sodium pentobarbital (75 mg/kg). Following abdominal incisions, renal pedicles were bluntly dissected and a microvascular clamp (Roboz Surgical Instrument) was placed on each renal pedicle for 30 min. During the procedure, animals were kept well hydrated with warm saline and at a constant temperature (37°C). After a 30-min ischemia, the clamps were removed, and the wounds were sutured. Then the animals were allowed to recover, with free access to food and water. Sham-operated mice underwent the same surgical procedure without clamping of the renal pedicle.

Assessment of renal function

Blood samples were obtained at 0, 3, 24, 48, and 72 h after renal IRI. Serum creatinine levels (milligrams per deciliter) were measured to monitor renal function using a commercial creatinine kit (Randox) and an autoanalyzer (Roche).

Histology

At 0, 3, 24, and 72 h of renal IRI, kidneys were dissected from mice and tissue slices were fixed in 10% formalin. For histological examination, formalin tissues were embedded in paraffin and 4-μm sections were stained with H&E.

Immunohistochemistry

Immunohistochemistry staining for T cells was performed on formalin-fixed kidney tissue. Kidney sections (4 μm) were immersed in 3% hydrogen peroxide methanol for 5 min to block endogenous peroxidase. For Ag retrieval, slides were pressure cooked in Ag-decloaker solution (Biocare Medical) for 3 min. After treatment with normal goat serum (1/100) and two drops of avidin D (100 mg/ml PBS), polyclonal rabbit anti-human/mouse CD3 Ab was added to the sections at a 1/200 dilution (Calbiochem). Sections were then incubated overnight at 4°C. An isotype primary Ab was used as a background staining control. Sections were then rinsed in PBS and treated with biotin (10 μg/L PBS) to block the biotin binding sites. After three washes in PBS, the slides were incubated with a biotin-conjugated goat anti-rabbit IgG secondary Ab (Vector Laboratories) for 35 min at room temperature. Sections were once again washed, and were then incubated for 45 min with streptavidin peroxidase (Biogenex). Kidney sections were exposed with romulin AEC chromogen (Biocare Medical) to visualize the immunocomplex and counterstained with hematoxylin. All kidney sections were examined by a pathologist and a nephrologist in a blinded fashion. Ten high-powered fields were counted in the area of the corticomedullary junction, and the total number of cells was quantified (cells per 10 HPF).

Isolation of lymphocytes from mouse kidneys

At the time of sacrifice, mice were first exsanguinated and then both kidneys were collected. Lymphocytes were isolated from two decapsulated kidneys by modifying a protocol previously used to isolate intestinal intraepithelial lymphocytes (22). Kidney tissue was disrupted mechanically in 10 ml of RPMI 1640 medium supplemented with 5% of newborn calf serum using a Stomacher 80 Biomaster (Sewart). To remove debris, samples were passed through a glass wool column prewashed with 50 ml of RPMI 1640 medium with 5 mM HEPES at 37°C. The resulted cell suspension was centrifuged at 300 × *g* for 10 min to pellet the cells. The pellet was then suspended in 36% Percoll (Amersham Pharmacia), gently overlaid onto 72% Percoll, and centrifuged at 1000 × *g* for 30 min at room temperature. Cells were isolated from the Percoll interface and washed twice in medium at 300 × *g* for 10 min at 4°C. Samples were resuspended

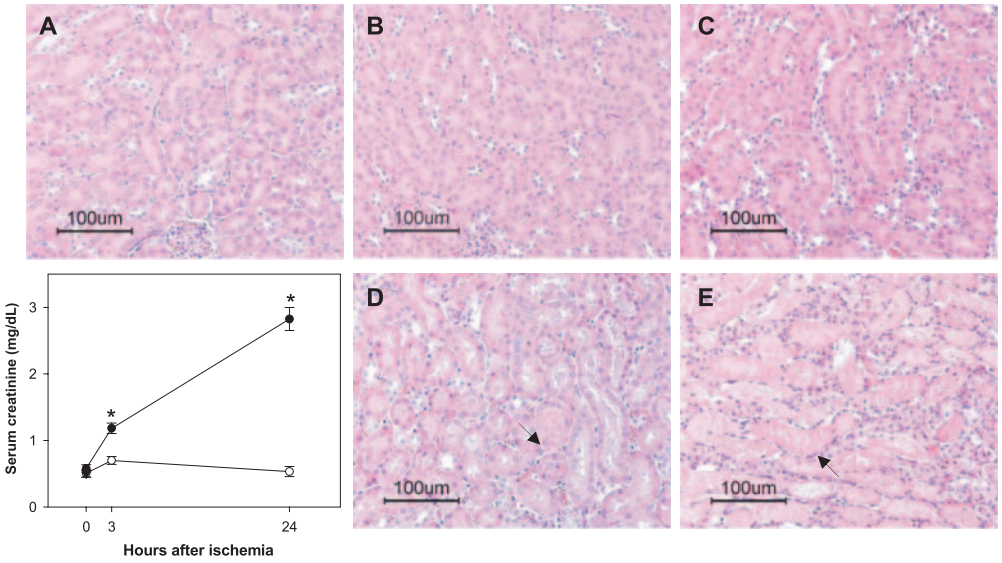

FIGURE 1. Kidney dysfunction in mice following a 30-min bilateral ischemia. Rise in serum creatinine of IRI mice (●) compared with normal (▲) and sham-operated (○) mice 3 and 24 h after renal IRI. Serum creatinine is expressed as mean ± SE of three independent experiments (n = 8/group; $p < 0.05$). Histological kidney injury assessment in tissue sections stained with H&E. *A*, Normal mouse kidney (no IRI). *B* and *C*, Sham-operated mice kidneys showing normal histology 3 and 24 h after surgery, respectively. *D*, IRI mouse kidney showing same proteinaceous casts in tubules 3 h after renal IRI. *E*, IRI mouse kidney showing severe damage 24 h after renal IRI. Original magnification, ×20.

in 250 μl of RPMI 1670 medium and the number of KMNC was assessed using trypan blue exclusion on a hemocytometer, and results were expressed as number of KMNC per two kidneys. The absolute number of infiltrating $CD3^+$ T cells and $CD19^+$ B cells into postischemic kidneys was calculated by multiplying the total number of KMNC by the percentage of positive cells determined by flow cytometry.

Antibodies

The fluorochrome-conjugated mAbs to mouse Ags used for flow cytometry analysis were: anti-CD16/CD32 (2.4G2), anti-CD3ε allophycocyanin (145-2C11), anti-CD4 PerCP (RM4-5), anti-CD8b FITC (53-5.8), anti-CD19 PE (1D3), anti-CD69 PE (H1.2F3), anti-CD25 PE (3C7), and anti-NK1.1 PE (PK136) (BD Pharmingen).

Flow cytometry analysis

KMNC ($\times 10^5$ cells) were preincubated with anti-CD16/CD32 Fc receptor for 10 min to minimize nonspecific Ab binding. Cells were then incubated with various combinations of mAbs for 25 min at 4°C, washed twice with FACS buffer, and fixed with 1% paraformaldehyde. Three-color immunofluorescence staining was analyzed using a FACSCalibur instrument (BD Biosciences). The lymphocytes were gated using forward and side scatter to exclude debris and dead cells, then 10,000 events were acquired in each assay for analysis. The data were analyzed using CellQuest software (BD Biosciences).

Intracellular cytokines staining by flow cytometry

Freshly isolated KMNC were stimulated with 5 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich) in the presence of monensin. The samples were incubated for 4 h at 37°C in a 5% CO_2 humidified atmosphere incubator. Surface staining of stimulated cells was performed with mAb anti-CD3 for 25 min at 4°C. Then, cells were permeabilized with perm/wash solution for 20 min and stained with PE-conjugated mAb anti-TNF-α and anti-IFN-γ or the appropriate isotype-matched control Abs.

Adoptive transfer of KMNC from ischemic wild-type mice to T cell-deficient mice

To study the functional role of lymphocytes infiltrating into postischemic kidneys, we isolated KMNC from wild-type mice 24 h after renal IRI. Cells (1.1×10^6) were transferred by i.v. injection into T cell-deficient mice. The lymphocytes transferred to *nu/nu* mice were $CD3^+$ T cells (46%), $CD4^+$ T cell (22%), $CD8^+$ T cells (12%), and $CD19^+$ B cells (17%). After 24 h, renal ischemia was induced both in the *nu/nu* mice that received the KMNC and in the control *nu/nu* mice group only receiving saline. Assessment of postischemic renal function was monitored at 0, 24, 48, and 72 h after renal IRI. Tissue histological examination was performed 72 h after renal IRI.

Statistical analysis

Data are expressed as mean ± SE. Statistical comparisons between groups were performed by one-way ANOVA test. For paired data, Student's *t* test was used. Statistical significance was determined as a $p < 0.05$. Analysis was accomplished using Sigma Stat 3.0.

Results

Assessment of renal function and histology changes after renal IRI

Kidney function of mice that underwent a 30-min ischemic injury was evaluated by following serum creatinine levels 3 and 24 h after renal IRI. After 3 h of renal IRI, a significant increase in serum creatinine of IRI mice ($n = 8$, 1.18 ± 0.08 mg/dl) when compared with normal ($n = 8$, 0.50 ± 0.05 mg/dl; $p = 0.002$) and sham-operated ($n = 8$, 0.70 ± 0.06 mg/dl; $p < 0.001$) mice was observed. After 24 h of renal IRI, serum creatinine significantly increased in the IRI mice ($n = 8$, 2.83 ± 0.17 mg/dl) as compared with control groups ($p < 0.001$). In the sham-operated mice, serum creatinine was slightly increased compared with normal mice 3 h after surgery (Fig. 1). The kidney structural injury in the cortex and the medulla of IRI mice is shown in Fig. 1. Compared with kidneys of normal mice (Fig. 1*A*) and kidneys of sham-operated mice 3 (Fig. 1*B*) and 24 h (Fig. 1*C*) after surgery, IRI mice show slightly tubular epithelial necrosis 3 h after renal IRI (Fig. 1*D*) and significant tubular injury with loss of tubular structure 24 h after renal IRI (Fig. 1*E*).

Early trafficking of $CD3^+$ T lymphocytes into postischemic kidney determined by immunohistochemistry

Using immunohistochemistry staining, we found $CD3^+$ T lymphocytes in normal mouse kidneys (18 ± 12 cells per 10 HPF) (Fig. 2*A*). In sham-operated mice, the number of $CD3^+$ T cells was slightly increased 3 h after surgery (21 ± 7 cells per 10 HPF; Fig. 2*B*) followed by a decrease 24 h after surgery (7 ± 8 cells per 10

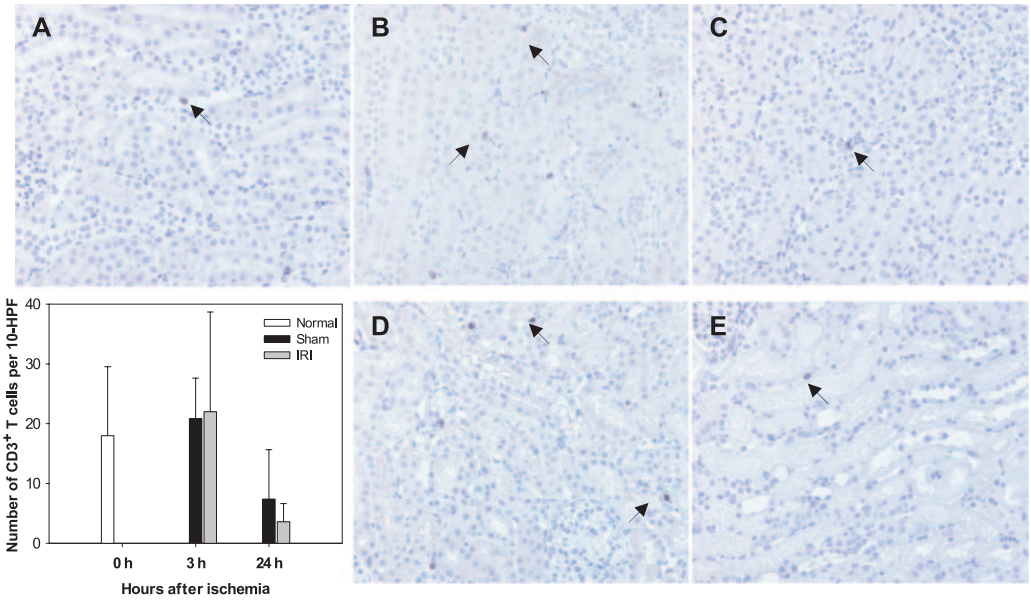

FIGURE 2. Number of $CD3^+$ T lymphocytes in mouse kidneys determined by immunohistochemistry. *A*, Normal mouse kidney (no IRI) showing few $CD3^+$ T cells. *B* and *C*, Kidneys from sham-operated mice showing a slight increase of $CD3^+$ T cells 3 h after renal IRI and few cells 24 h after renal IRI, respectively. *D* and *E*, IRI mice kidneys showing a slight increase of $CD3^+$ T cells 3 h after renal IRI and few T cells 24 h after renal IRI, respectively. $n = 5$/group. Original magnification, ×20.

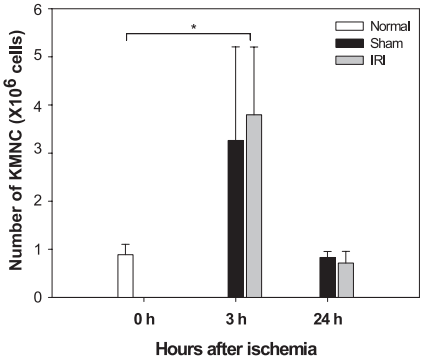

FIGURE 3. Kidney mononuclear cells in sham-operated and IRI mice 3 and 24 h after renal IRI. KMNC were obtained from two kidneys as detailed in *Materials and Methods*. The total cell number was calculated from viable cell counts and expressed as the number of cells per two kidneys (cells). The results are expressed as mean ± SE of three independent experiments ($p = 0.027$).

HPF; Fig. 2*C*) when compared with normal mice. Similarly, in the IRI mice we observed a slight increase of $CD3^+$ T cells 3 h after renal IRI (22 ± 17 cells per 10 HPF; Fig. 2*D*) in the peritubular capillaries, particularly in the medullary outer stripe in kidneys, followed by a decrease 24 h after renal IRI (4 ± 3 cells per 10 HPF; Fig. 2*E*). These results demonstrate that the "sham ischemia," which includes anesthesia and laparotomy, but minimal handling of the kidney, itself induces infiltration of T lymphocytes into mouse kidneys.

Increased infiltration of mononuclear cells into postischemic kidneys

To better characterize the lymphocytes infiltrating into postischemic kidneys, we optimized a technique to efficiently elute mouse KMNC using a mechanical disruption technique followed by a Percoll gradient separation. The total number of KMNC recovered from both kidneys of normal mice was $8.87 \times 10^5 \pm 2.16 \times 10^5$ cells ($n = 8$). Although 3 h after renal IRI serum creatinine of IRI mice was greater than that of control groups, we observed a similar increased number of KMNC in both sham-operated ($3.26 \times 10^6 \pm 1.95 \times 10^6$ cells) and IRI ($3.80 \times 10^6 \pm 1.41 \times 10^6$ cells; $p = 0.027$) mice when compared with normal mice (Fig. 3). Surprisingly, 24 h after renal IRI, while IRI mice exhibit a significant rise of serum creatinine and severe kidney structure damage, the number of KMNC in these mice ($7.15 \times 10^5 \pm 2.42 \times 10^5$ cells) was lower than that of sham-operated ($8.30 \times 10^5 \pm 1.26 \times 10^5$ cells) and normal mice; however, the differences were not significant. These results demonstrated that laparotomy with anesthesia alone increases early infiltration of KMNC into kidneys of sham-operated and IRI mice.

Phenotype of mouse kidney lymphocytes

To characterize the intrarenal lymphocytes, mice were exsanguinated before the kidneys were collected to minimize contamination with lymphocytes from peripheral blood. Three-color flow cytometry analysis of freshly isolated KMNC from mouse kidneys revealed the presence of numerous lymphocyte subsets which include: $CD3^+$, $CD4^+$, and $CD8^+$ T cells, $CD4^+NK1.1^+$, $NK1.1^+$ (phenotype $CD3^-NK1.1^+$) cells, and $CD19^+$ B cells (Figs. 4-6). The predominant population in mouse kidneys was $CD3^+$ T cells. These data show the diversity of lymphocyte populations in mouse kidneys.

T and B lymphocytes infiltrating into mouse kidneys

After 3 h of renal IRI, we observed an increased number of $CD3^+$ T cells in sham-operated ($1.71 \times 10^6 \pm 1.02 \times 10^6$ cells) and IRI ($1.84 \times 10^6 \pm 0.74 \times 10^6$ cells; $p = 0.022$) mice compared with normal mice ($0.35 \times 10^6 \pm 0.13 \times 10^6$ cells). However, 24 h after renal IRI, while the number of $CD3^+$ T cells in sham-operated mice ($0.42 \times 10^6 \pm 0.06 \times 10^5$ cells) reached normal levels, a decreased number of these cells was observed in IRI mice ($0.25 \times 10^6 \pm 0.13 \times 10^6$ cells; $p = 0.040$; Fig. 4*A*).

In view of recent studies showing a protection in B cell-deficient mice from renal IRI (15), we evaluated the infiltration of B lymphocytes into postischemic kidneys. After 3 h of renal IRI, there was a similar increased number of $CD19^+$ B cells in sham-operated ($5.88 \times 10^5 \pm 3.35 \times 10^5$ cells) and IRI ($5.99 \times 10^5 \pm 2.07 \times 10^5$ cells) mice when compared with normal mice ($2.99 \times 10^5 \pm 0.98 \times 10^5$ cells). However, 24 h after renal IRI, the number of $CD19^+$ B lymphocytes decreased significantly in the IRI mice ($0.73 \times 10^5 \pm 0.32 \times 10^5$ cells) compared with normal ($p = 0.041$) and sham-operated ($2.52 \times 10^5 \pm 0.53 \times 10^5$ cells; $p < 0.019$) mice (Fig. 4*B*).

$CD4^+$ T lymphocytes infiltrating into postischemic kidneys

Since previous studies have demonstrated that the CD4 T cells play an important role in the pathogenesis of renal IRI (8), we examined the trafficking of $CD4^+$ and $CD8^+$ T cell subsets into kidneys after ischemic injury. After 3 h of renal IRI, the percentages of $CD4^+$ and $CD4^+NK1.1^+$ cells increased similarly in both sham-operated and IRI mice as compared with normal mice (Fig. 5, *A* and *B*). However, 24 h after renal IRI, while the percentage of $CD4^+$ T cells in the IRI mice was similar to that of control groups, the percentage of $CD4^+NK1.1^+$ cells increased (3.2% ± 0.59%) when compared with normal (1.2% ± 0.24%; $p = 0.010$) and sham-operated (1.6% ± 0.17%) mice (Fig. 5*C*). The percentage of $CD8^+$ T cells was similar in all groups 3 and 24 h after renal IRI and no expression of NK1.1 Ag was observed on these cells (Fig. 5). These results demonstrate that laparotomy itself induces infiltration of $CD4^+$ and $CD4^+NK1.1^+$ cells early after performed

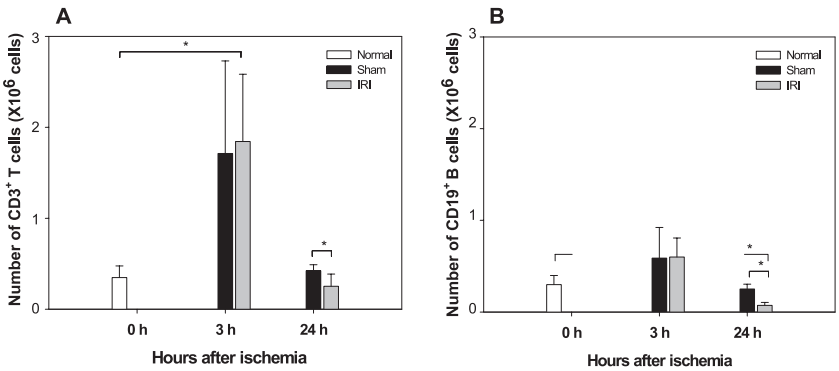

FIGURE 4. Absolute number of $CD3^+$ T cells and $CD19^+$ B cells trafficking into mouse kidneys. *A*, Number of $CD3^+$ T cells in sham-operated and IRI mice 3 ($p = 0.026$) and 24 h ($p = 0.026$) after renal IRI. *B*, After 3 h of renal IRI, the number of $CD19^+$ B cells increased similarly in both sham-operated and IRI mice. After 24 h of renal IRI, the percentage of $CD19^+$ B cells in the IRI mice decreased significantly when compared with normal ($p = 0.003$) and sham-operated ($p < 0.001$) mice. Values shown in the bar graphs represent the percentage mean ± SE of three independent experiments ($n = 8$/group).

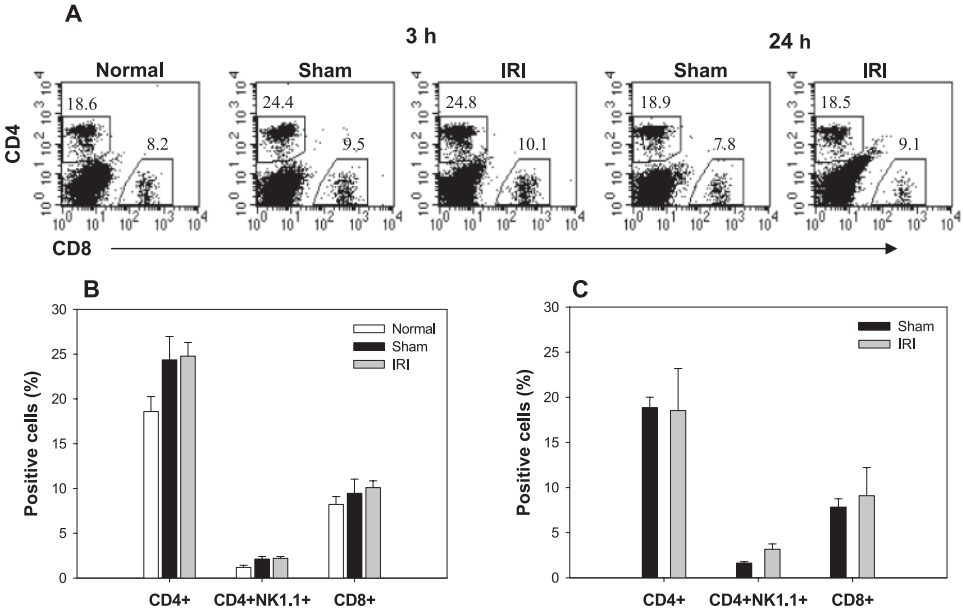

FIGURE 5. Percentages of $CD4^+$, $CD4^+NK1.1^+$, and $CD8^+$ T cells 3 and 24 h after renal IRI by flow cytometry analysis. *A*, The percentages of $CD4^+$ and $CD8^+$ T cells were obtained from the gated lymphocyte area of 10,000 events collected. *B*, After 3 h of renal IRI, similar increased percentages of $CD4^+$ and $CD4^+NK1.1^+$ T cells are shown in sham-operated and IRI mice as compared with normal mice. *C*, After 24 h of renal IRI, the percentage of $CD4^+NK1.1^+$ cells increased in IRI mice. Results are representative of three independent experiments (n = 8/group).

surgical trauma. However, the increased percentage of the $CD4^+NK1.1^+$ cells in the IRI group 24 h after renal IRI could be related to renal ischemic injury because at this time point serum creatinine was increasing and visible kidney structure damage was observed.

NK cells infiltrating into postischemic kidneys

We also determined the trafficking of NK cells (phenotype $CD3^-NK1.1^+$) because these cells have been found infiltrating ischemic allografts (23). Interestingly, after 3 h of renal IRI, the $NK1.1^+$ cells increased significantly in the IRI mice (16% ± 1%) as compared with normal (7% ± 1%; $p < 0.001$) and sham-operated (11% ± 1%; $p < 0.014$) mice. However, 24 h after renal IRI, the $NK1.1^+$ cells decreased significantly in the IRI mice (3.8% ± 0.75%) as compared with normal ($p = 0.010$) and sham-operated (9% ± 0.7%; $p < 0.001$) mice (Fig. 6). These results suggest that NK cells could be involved in the pathogenesis of renal IRI and further studies should be conducted to determine their role in the pathogenesis of renal IRI.

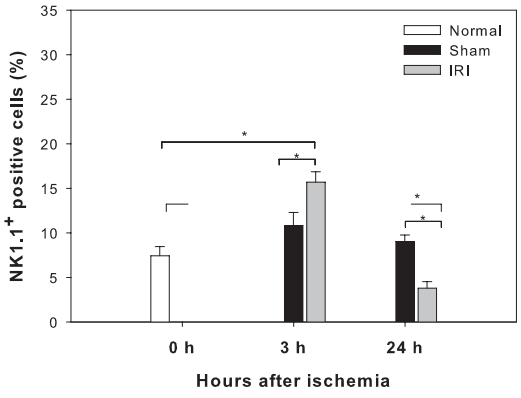

FIGURE 6. Trafficking of $NK1.1^+$ cells into postischemic kidneys. After 3 h of renal IRI, a significant increase of $NK1.1^+$ cells in the IRI mice was observed compared with normal ($p < 0.001$) and sham-operated ($p < 0.014$) mice, followed by a significant decrease 24 h after renal IRI. Data are expressed as the mean percentage of positive cells ± SE of three independent experiments (n = 8/group).

Expression of CD69 on $CD4^+$ and $CD8^+$ T lymphocytes

In diseases involving trafficking of T lymphocytes into affected organs, up-regulation of CD69 Ag was observed (24, 25). We therefore investigated the activation state of the intrarenal $CD4^+$ and $CD8^+$ T cell subsets analyzing the expression of activation markers CD69 and CD25. After 3 h of renal IRI, we observed increased expression of CD69 on $CD4^+$ T cells in sham-operated (14.7% ± 2.35%; $p = 0.024$) and IRI (14.2 ± 1.85%; $p = 0.014$) compared with normal mice (7.1 ± 1.34%) (Fig. 7). CD69 expression on $CD8^+$ T cells tended to increase at 3 h, but was not statistically significant. After 24 h of renal IRI, the expression of CD69 on $CD4^+$ and $CD8^+$ T cells declined to lower levels than normal mice. Moreover, no increased expression of CD25 Ag on $CD4^+$ and $CD8^+$ T cells in any of the studied groups was found (data not shown). These results demonstrated that $CD4^+$ and $CD8^+$ T lymphocytes infiltrating kidneys of sham-operated and IRI mice display some features of activated T lymphocytes.

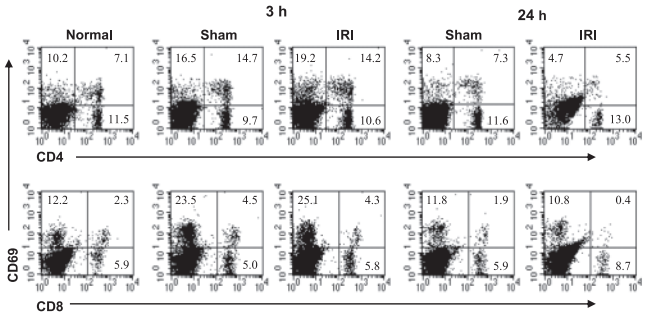

FIGURE 7. Expression of the early activation marker CD69 on $CD4^+$ and $CD8^+$ T lymphocytes in normal, sham-operated, and IRI mice. Freshly isolated KMNC were three-color stained with mAb anti-$CD8^+$ FITC, anti-$CD4^+$ PerCP, and anti-$CD69^+$ PE and analyzed by flow cytometry. Cells were gated in the lymphocyte area and 10,000 events were collected. Significantly increased expression of CD69 on $CD4^+$ T cells of sham-operated and IRI mice as compared with normal mice 3 h after renal IRI was observed. After 24 h of renal IRI, expression of CD69 decreased in both groups. Dot plots are representative examples and show the mean percentage of positive cells ± SE of three independent experiments (n = 8/group).

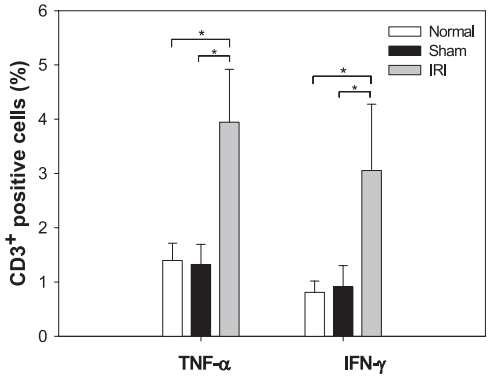

FIGURE 8. Intracellular cytokine staining in kidney $CD3^+$ T cells 24 h after renal IRI. KMNC were isolated from normal, sham-operated, and IRI mice kidneys and stained with mAb anti-CD3 FITC and anti-TNF-α PE or IFN-γ PE and analyzed by flow cytometry. Data are shown as the percentage of $CD3^+$ T cells, TNF-α, or IFN-γ double-positive cells of gated lymphocyte area. Compared with normal and sham-operated mice, IRI mice showed a significant increased percentage of intracellular TNF-α ($p < 0.04$) and IFN-γ ($p < 0.02$) producing $CD3^+$ T cells ($n = 5$/group).

Production of TNF-α and IFN-γ by CD3⁺ T lymphocytes

Several studies have reported the up-regulation of cytokines TNF-α and IFN-γ in renal IRI (5, 26). However, it is not known which cells produced these proinflammatory cytokines. Flow cytometry analysis of freshly isolated KMNC was examined for intracellular cytokine production by $CD3^+$ T lymphocytes. After 24 h of renal IRI, flow cytometry analysis of KMNC of IRI mice revealed increased production of TNF-α and IFN-γ by $CD3^+$ T lymphocytes when compared with normal and sham-operated mice (Fig. 8).

Adoptive transfer of KMNC confers protection from renal IRI

It has been demonstrated that T cell-deficient *nu/nu* mice have less kidney dysfunction after ischemia than wild-type control mice. Adoptive transfer of normal splenocytes to these *nu/nu* mice significantly restores the kidney injury response to IRI (8). To determine the role of infiltrating lymphocytes in mouse kidney, we adoptively transferred KMNC from wild-type mice 24 h after renal IRI into T cell-deficient mice. Renal ischemia was induced both in the *nu/nu* mice that received the KMNC and in the control mice group only receiving saline. We expected worsening of kidney injury after transfer of KMNC to *nu/nu* mice. Unexpectedly, serum creatinines after IRI in *nu/nu* mice that received the KMNC were lower compared with *nu/nu* mice that underwent ischemia but did not received KMNC 24 h ($n = 4$, 0.53 ± 0.23 mg/dl vs 1.53 ± 0.23 mg/dl, $p < 0.03$), 48 h ($n = 4$, $0.35 \pm 0.0.23$ mg/dl vs 1.53 ± 0.52 mg/dl), and 72 h ($n = 4$, 0.1 ± 0.04 mg/dl vs 1.50 ± 0.58 mg/dl) after renal IRI (Fig. 9). Histological examination of kidney tissue of *nu/nu* mice was consistent with the functional data in that mice that did not receive KMNC exhibited more tubular injury than mice undergoing IRI after transfer of KMNC.

Discussion

Recent studies have identified T and B lymphocytes as important mediators of renal IRI (7–9, 15). However, the mechanisms by which these cells mediate kidney injury are unknown. Since little is known about the lymphocyte populations infiltrating kidneys, we optimized a technique to efficiently extract mononuclear cells from mouse kidneys to analyze the surface phenotype of cells trafficking into postischemic kidneys. Our results showed early trafficking of $CD3^+$ T cells and $CD19^+$ B cells into kidneys of both sham-operated and IRI mice 3 h after renal IRI. Thus, laparotomy per se induces very early infiltration of T and B lymphocytes into mouse kidneys, consistent with previous data showing that laparotomy causes a systemic inflammation response syndrome that can involve distant organs (27). These data are also consistent with previous results showing similar infiltration of $CD4^+$ T cells into kidneys of B cell-deficient mice, which were protected from renal IRI, and wild-type mice showing severe kidney damage (15). Although our data suggest that trafficking of T and B lymphocytes into kidneys 3 and 24 h after renal IRI are not correlated with kidney damage, this does not exclude the possibility that accumulation of inflammatory cells into postischemic kidneys is an additional factor that contributes to worsen the already initiated tissue damage (3). Recently, it has been found that ischemic mice treated with FTY720, which prevents renal T cell infiltration, have less kidney dysfunction compared with mice that were not treated (28). Adoptive transfer of KMNC at 24 h after IRI into recipient T cell-deficient mice unexpectedly led to relative protection from renal IRI in the recipient mice, suggesting that at 24 h after IRI, KMNC served a protective role from further ischemic insult and potentially a healing role.

The significant increase of $NK1.1^+$ cells in the IRI group 3 h after renal IRI that correlated with increased serum creatinine suggests a role of these cells in renal IRI. Increased infiltration of NK cells was observed in ischemic allografts with severe tubulointerstitial injury (23). We also detected the presence of a small population of $CD4^+NK1.1^+$ cells in mouse kidneys. Interestingly, in the IRI mice, $CD4^+NK1.1^+$ cells were increased as compared with controls groups 24 h after renal IRI. These new findings are supported by a report in liver ischemia suggesting that NKT cells

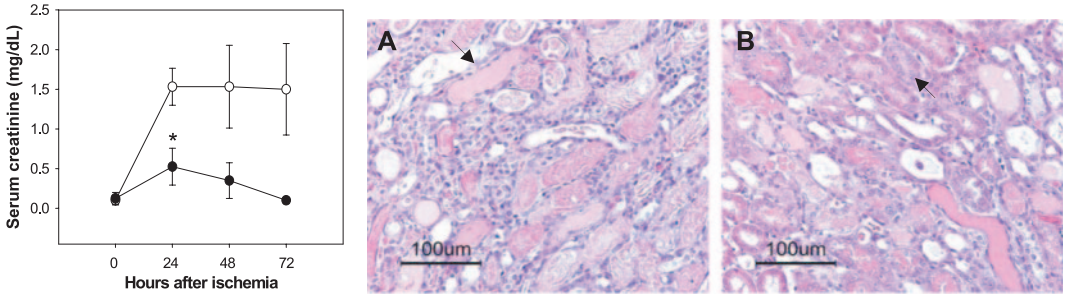

FIGURE 9. KMNC from ischemic mice confers functional and structural protection to T cell-deficient-mice. Reconstituted *nu/nu* mice with KMNC (●) showed reduced serum creatinine at 24 ($p = 0.030$), 48, and 72 h after ischemia compared with *nu/nu* mice that did no receive cells (○). T cell-deficient mice received 1.1×10^6 cells by i.v. injection from littermate mice 24 h before ischemic injury. *A*, After 72 h of renal IRI, histological assessment of tubular injury of *nu/nu* mice that did not receive cells shows moderate tubular damage. *B*, Kidney mononuclear cell-reconstituted *nu/nu* mice show a significant reduction in renal structural injury. Original magnification, ×20.

mediate liver injury after ischemia (21). NKT cells are a subset of lymphocytes with surface markers characteristic of both NK cells (NK1.1) and conventional T cells. Upon activation, NKT cells rapidly produce various immunoregulatory cytokines that trigger the activation and differentiation of a variety of other leukocytes. There is now emerging evidence that NKT cells are playing an important role in the regulation of autoimmunity (29). Furthermore, it has been also found in ischemic kidney that an increased population of $CD3^{int}$ T cells indicates that nonconventional T cells could be also mediating renal IRI (20). These results raise the possibility that NK and NKT cells might be implicated in kidney damage and further studies should be conducted to determine the role of these lymphocytes in renal IRI. The significant decrease of $NK1.1^+$ cells and $CD19^+$ B cells observed in the IRI mice has not been previously reported. The implications of the decrease of these lymphocytes after renal ischemic injury remain to be determined.

Because similar infiltration of $CD4^+$ T cells into kidneys of sham-operated and IRI mice occurred, we hypothesized that functional changes could be more important and examined the activation state of infiltrating T cells into postischemic kidneys. The expression levels of the activation markers CD69 and CD25 on $CD4^+$ and $CD8^+$ T cells were examined. The early activation marker CD69 Ag is a membrane protein rapidly induced on the surface of activated T lymphocytes, which is detectable within 2–4 h following the initial stimulation event, and is generally sustained for 18–24 h (30, 31). In rheumatoid arthritis where T lymphocytes mediated inflammation, T cells infiltrating synovial fluid showed increased expression of CD69 (24). Patients with systemic lupus erythematosus and patients undergoing allograft rejection also show an increased expression of CD69 on T cells (32, 33). We hypothesized that T cells might be activated after renal IRI; however, we found a similarly increased expression of CD69 on the $CD4^+$ and $CD8^+$ T cells in both sham-operated and IRI mice 3 h after renal IRI. No increased expression of the CD25 Ag was observed on T cells of IRI mice or the control groups. These results suggested that laparotomy itself induced activation of infiltrating $CD4^+$ T cells in mouse kidneys.

In renal IRI the up-regulation of cytokines and chemokines including IL-1, IL-2, IL-6, IL-8, IL-10, IFN-γ, TNF-α, KC, MIP-2, and GM-CSF (3, 5, 26, 34) has been identified in kidney tissue. However, it is unknown which type of cytokines are produced by intrarenal T lymphocytes. We found an increased intracellular cytokine production of TNF-α and IFN-γ by $CD3^+$ T cells infiltrating kidneys of IRI mice. Given that TNF-α and IFN-γ have been implicated in the postischemic inflammation as well as directly mediating the outcome from IRI, the observation that IRI leads to production of these mediators from T lymphocytes suggest that lymphocytes infiltrating into the postischemic kidneys could have a major downstream effect on later inflammation and organ dysfunction. Thus, not only the trafficking of T cells postischemia is a potential mechanism, but what those infiltrating cells are doing at the site of injury could be crucial for pathogenesis.

To begin to elucidate the functional role of kidney-infiltrating leukocytes after IRI, we isolated and transferred KMNC 24 h after ischemia to athymic *nu/nu* mice, followed by IRI in the *nu/nu* mice. We expected a heightened injury response in the *nu/nu* mice after KMNC transfer, but unexpectedly found that they had reduced functional and structural injury after IRI. This suggests that KMNC at 24 h after renal IRI could be serving a protective function to decrease the injury response and perhaps could be involved in healing as well. These data are consistent with previous data in kidney IRI (13) and liver IRI (14) that lymphocytes could play a deleterious or protective role in IRI. Furthermore, recent data support a protective role in splenic lymphocytes when harvested 5 days after kidney IRI to confer an "ischemic preconditioning"-like protection when transferred to wild-type mice when they undergo IRI (35). The current data do not identify which particular cell type could be responsible for this effect or whether either earlier or later time point KMNC have a different effect.

In summary, we successfully isolated high yields of viable mononuclear cells from mouse kidneys for phenotypic and functional analysis of lymphocytes. Despite minimal effects of IRI beyond sham surgery or $CD3^+$ T cells trafficking, important increases in proinflammatory cytokine production were found in $CD3^+$ T cells from ischemic kidney. Distinct increases in NK and NKT cells were also seen with IRI mice. Isolation of kidney-infiltrating lymphocytes at 24 h after ischemia, followed by transfer to T cell-deficient mice, demonstrated that these cells could decrease tissue injury after ischemia and could have a potentially protective or healing effect. This work sets the stage for further studies to more closely evaluate the phenotype of kidney-infiltrating cells in IRI, for evaluation of the role of NK and NKT cells in the injury process, and for closer dissection of the possible beneficial role of infiltrating leukocytes in kidney IRI.

Acknowledgments

We thank Dr. Greg Hadley for suggestions with the lymphocyte isolation assay and Shannon Bevans for technical assistance.

Disclosures

The authors have no financial conflict of interest.

References

1. Thadhani, R., M. Pascual, and J. V. Bonventre. 1996. Acute renal failure. *N. Engl. J. Med.* 334: 1448–1460.
2. Bonventre, J. V., and A. Zuk. 2004. Ischemic acute renal failure: an inflammatory disease? *Kidney Int.* 66: 480–485.
3. 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.
4. Day, Y. J., L. Huang, H. Ye, J. Linden, and M. D. Okusa. 2005. Renal ischemia-reperfusion injury and adenosine 2A receptor-mediated tissue protection: role of macrophages. *Am. J. Physiol.* 288: F722–F731.
5. Goes, N., J. Urmson, V. Ramassar, and P. F. Halloran. 1995. Ischemic acute tubular necrosis induces an extensive local cytokine response: evidence for induction of interferon-γ, transforming growth factor-β1, granulocyte-macrophage colony-stimulating factor, interleukin-2, and interleukin-10. *Transplantation* 59: 565–572.
6. Rabb, H., C. C. Mendiola, S. R. Saba, J. R. Dietz, C. W. Smith, J. V. Bonventre, and G. Ramirez. 1995. Antibodies to ICAM-1 protect kidneys in severe ischemic reperfusion injury. *Biochem. Biophys. Res. Commun.* 211: 67–73.
7. 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.
8. Burne, M. J., F. Daniels, A. El Ghandour, S. Mauiyyedi, 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.
9. 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.
10. Zwacka, R. M., Y. Zhang, J. Halldorson, H. Schlossberg, L. Dudus, and J. F. Engelhardt. 1997. $CD4^+$ T-lymphocytes mediate ischemia/reperfusion-induced inflammatory responses in mouse liver. *J. Clin. Invest.* 108: 1283–1290.
11. Kupiec-Weglinski, J. W., and R. W. Busuttil. 2005. Ischemia and reperfusion injury in liver transplantation. *Transplant. Proc.* 37: 1653–1656.
12. Van Putte, B. P., J. Kesecioglu, J. M. Hendriks, V. P. Persy, E. van Marck, P. E. Van Schil, and M. E. De Broe. 2005. Cellular infiltrates and injury evaluation in a rat model of warm pulmonary ischemia-reperfusion. *Crit. Care* 9: R1–R8.
13. Yokota, N., M. Burne-Taney, L. Racusen, and H. Rabb. 2003. Contrasting roles for STAT4 and STAT6 signal transduction pathways in murine renal ischemia-reperfusion injury. *Am. J. Physiol.* 285: F319–F325.
14. Shen, X. D., B. Ke, Y. Zhai, F. Gao, D. Anselmo, C. R. Lassman, R. W. Busuttil, and J. W. Kupiec-Weglinski. 2003. Stat4 and Stat6 signaling in hepatic ischemia/reperfusion injury in mice: HO-1 dependence of Stat4 disruption-mediated cytoprotection. *Hepatology* 37: 296–303.
15. 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.

16. 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.
17. 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.
18. Briscoe, D. M., and H. M. Sayegh. 2002. A rendezvous before rejection: where do T cells meet transplant antigens? *Nat. Med.* 8: 220–222.
19. 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.
20. Miyazawa, S., H. Watanabe, C. Miyaji, O. Hotta, and T. Abo. 2002. Leukocyte accumulation and changes in extra-renal organs during renal ischemia reperfusion in mice. *J. Lab. Clin. Med.* 139: 269–278.
21. Shimamura, K., H. Kawamura, T. Nagura, T. Kato, T. Naito, H. Kameyama, K. Hatakeyama, and T. Abo. 2005. Association of NKT cells and granulocytes with liver injury after reperfusion of the portal vein. *Cell. Immunol.* 234: 31–38.
22. Davies, A., S. Lopez-Briones, H. Ong, C. O'Neil-Marshall, F. A. Lemonnier, K. Nagaraju, E. S. Metcalf, and M. J. Soloski. 2004. Infection-induced expansion of a MHC Class I^b-dependent intestinal intraepithelial $\gamma\delta$ T cell subset. *J. Immunol.* 172: 6828–6837.
23. Coulson, M. T., P. Jablonski, B. O. Howden, N. M. Thomson, and A. N. Stein. 2005. Beyond operational tolerance: effect of ischemic injury on development of chronic damage in renal grafts. *Transplantation* 80: 353–361.
24. Afeltra, A., M. Galeazzi, G. D. Sebastiani, G. M. Ferri, D. Caccavo, M. A. Addessi, R. Marcolongo, and L. Bonomo. 1997. Coexpression of CD69 and HLADR activation markers on synovial fluid T lymphocytes of patients affected by rheumatoid arthritis: a three-colour cytometric analysis. *Int. J. Exp. Pathol.* 78: 331–336.
25. Santamaria, M., M. Marubayashi, J. M. Arizon, A. Montero, M. Concha, F. Valles, A. Lopez, F. Lopez, and J. Pena. 1992. The activation antigen CD69 is selectively expressed on $CD8^+$ endomyocardium infiltrating T lymphocytes in human rejecting heart allografts. *Hum. Immunol.* 33: 1–4.
26. Lemay, S., H. Rabb, G. Postler, and A. K. Singh. 2000. Prominent and sustained up-regulation of gp130-signaling cytokines and the chemokine MIP-2 in murine renal ischemia-reperfusion injury. *Transplantation* 69: 959–963.
27. Jacobi, C. A., J. Ordemann, H. U. Zieren, H. D. Volk, A. Bauhofer, E. Halle, and J. M. Muller. 1998. Increased systemic inflammation after laparotomy vs laparoscopy in an animal model of peritonitis. *Arch. Surg.* 133: 258–262.
28. Suleiman, M., P. M. Cury, J. O. Pestana, E. A. Burdmann, and V. Bueno. 2005. FTY720 prevents renal T-cell infiltration after ischemia/reperfusion injury. *Transplant. Proc.* 37: 373–374.
29. Mars, L. T., J. Novak, R. S. Liblau, and A. Lehuen. 2004. Therapeutic manipulation of iNKT cells in autoimmunity: modes of action and potential risks. *Trends Immunol.* 25: 471–476.
30. Ziegler, S. F., F. Ramsdell, and M. R. Alderson. 1994. The activation antigen CD69. *Stem Cells* 12: 456–465.
31. Risso, A., D. Smilovich, M. C. Capra, I. Baldissarro, G. Yan, A. Bargellesi, and M. E. Cosulich. 1991. CD69 in resting and activated T lymphocytes: its association with a GTP binding protein and biochemical requirements for its expression. *J. Immunol.* 146: 4105–4114.
32. Crispin, J. C., A. Martinez, P. De Pablo, C. Velasquillo, and J. Alcocer-Varela. 1998. Participation of the CD69 antigen in the T-cell activation process of patients with systemic lupus erythematosus. *Scand. J. Immunol.* 48: 196–200.
33. Posselt, A. M., F. Vincenti, M. Bedolli, M. Lantz, J. P. Roberts, and R. Hirose. 2003. CD69 expression on peripheral CD8 T cells correlates with acute rejection in renal transplant recipients. *Transplantation* 76: 190–195.
34. Daemen, M. A., C. van't Veer, T. G. Wolfs, and W. A. Buurman. 1999. Ischemia/reperfusion-induced IFN-γ up-regulation: involvement of IL-12 and IL-18. *J. Immunol.* 162: 5506–5510.
35. Burne-Taney, M. J., M. Liu, W. M. Baldwin, L. Racusen, and H. Rabb. 2006. Decreased capacity of immune cells to cause tissue injury mediates kidney ischemic preconditioning. *J. Immunol.* 176: 7015–7020.