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Decreased Capacity of Immune Cells to Cause Tissue Injury Mediates Kidney Ischemic Preconditioning

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Ischemic preconditioning (IP) is a well-established phenomenon, and the underlying mechanisms of IP are thought to involve adaptive changes within the injured tissue. Because one of the main functions of immune cells is to harbor memory, we hypothesized that circulating immune cells could mediate IP by responding to an initial ischemia reperfusion injury (IRI) and then mediate decreased injury after a second IRI event. C57BL/6 mice underwent 30 min of bilateral renal clamping or sham operation. At 5 days after ischemia, purified leukocytes from spleen were adoptively transferred into T cell-deficient (nu/nu) mice. After 1 wk, these mice underwent 30 min of renal IRI. The nu/nu mice receiving leukocytes from ischemic wild-type mice had significantly reduced renal injury compared with nu/nu mice receiving leukocytes from sham-operated, wild-type mice. Infiltration of neutrophil and macrophage in postischemic kidney did not correlate with the protection. No difference in kidney C3d or IgG deposition was detected between groups. Given that inducible NO synthase (iNOS) has been implicated in IP, leukocytes from ischemic or sham-operated, iNOS-deficient mice were transferred into nu/nu mice. Effects similar to those of wild-type transfer of ischemic leukocytes were demonstrated; thus, iNOS was not mediating the IP effect of leukocytes. This is the first evidence that immune cells are primed after renal IRI and thereby lose the capacity to cause kidney injury during a second episode of IRI. This finding may also be relevant for elucidating the mechanisms underlying cross-talk between injured kidney and distant organs. The Journal of Immunology, 2006, 176: 7015–7020.

Immune cells are now established to be important mediators of renal ischemia reperfusion injury (IRI) (7); however, there have been no studies to investigate their involvement in IP in the kidney. Some have suggested that a decrease in leukocyte-endothelial interaction confers protection in the preconditioned kidney (8). This could be due to a number of factors, including a decrease in immune cell infiltration or a decrease in immune-mediated cytokines and chemokines.

For this study we hypothesized that circulating immune cells could mediate IP by responding to an initial ischemic event and then mediating decreased tissue injury after a second ischemic event. To test this hypothesis, we initially established an IP model in mice. After confirming the IP effect, we transferred immune cells from ischemic and sham-operated mice into T cell-deficient (nu/nu) mice that then underwent an episode of ischemic injury. We found that T cell-deficient mice that received leukocytes from mice that had undergone an ischemic period 5 days before death had a reduced amount of injury compared with T cell-deficient mice that received leukocytes from sham-operated mice. Given the putative role for iNOS in IP, we tested whether leukocyte iNOS played a role in this immune preconditioning by transferring iNOS-deficient mouse leukocytes after renal IRI into T cell-deficient mice. We found that iNOS deficiency did not alter the protective effect of immune-mediated IP. This is the first demonstration that immune cells are mediators of an IP response.

Materials and Methods

Mice

T cell-deficient mice (B6.Cg-Foxn1nu/J), iNOS-deficient mice (B6.129P2-Nos2<sup>−/−</sup>em1J), as well as C57BL/6 wild-type (WT) littermates were purchased from The Jackson Laboratory. Mice were housed under pathogen-free conditions according to National Institutes of Health guidelines. To confirm knockout and adoptive transfer status, spleens were collected from mice at death and analyzed for T cell populations using flow cytometric

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FIGURE 1. Experimental groups and design.

Group 1: IRI
Group 2: Sham surgery

Day 0
Donors
IRI or sham surgery
Day 5
Donors sacrificed and leukocytes transferred to recipients
Day 12
Recipients undergo renal IRI
Day 15
Recipients sacrificed

analysis. All animal studies were reviewed and approved by the Johns Hopkins University animal care and use committee.

Renal ischemia reperfusion model

An established model of renal IRI was used (9). Animals were anesthetized with 75 mg/kg i.p. sodium pentobarbital. Abdominal incisions were made, and the renal pedicles were bluntly dissected. A microvascular clamp was placed on both renal pedicles for 30 min. During the procedure, animals were kept well hydrated with saline and kept at a constant temperature (~37°C). After the clamps were removed, the wounds were sutured, and the animals were allowed to recover.

Adoptive transfer experiments

At 5 days after ischemia, spleens were collected from donor IRI-operated or sham-operated mice. Splenic cells were collected by centrifugation, and RBC were removed by lysis in NH₄Cl for 5 min. Cells were counted, and ~15 x 10⁶ enriched cells were injected i.v. into nu/nu mice. IRI was induced in the recipient mice 1 wk after transfer (Fig. 1).

Assessment of posts ischemic renal function

Blood samples were obtained from the tail vein at 0, 24, 48, and 72 h after ischemia. Serum creatinine (Scr; milligrams per deciliter) was used as a measure of renal function and was assessed with a creatinine 557A kit (Sigma-Aldrich) using an autoanalyzer (Roche).

Tissue histological analysis

Animals were killed at 72 h after ischemia, and kidneys were harvested. Tissue slices were fixed with 10% formalin and then stained with H&E after paraaffin embedding. Tissue sections were scored in a blinded fashion, using a previously described semiquantitative scale designed to evaluate the degree of tubular necrosis (10, 11). Higher scores represent more severe damage; the maximum score is 4: 0, normal kidney; 1, minimal necrosis (<5% involvement); 2, mild necrosis (5–25% involvement); 3, moderate necrosis (25–75% involvement); and 4, severe necrosis (>75% involvement).

Myeloperoxidase assay

Myeloperoxidase (MPO) activity, an indicator of neutrophil and macrophage accumulation in tissues, was measured as described by Lai (12). Kidney tissue was homogenized in a solution containing 0.5% (w/v) hexadecyltrimethylammonium bromide dissolved in 50 mM potassium phosphate buffer (pH 6.0) and centrifuged for 30 min at 20,000 x g at 4°C. Samples were incubated at 60°C for 2 h in a water bath, then centrifuged at 4,000 x g for 12 min. The supernatant (40 µl) was incubated with 160 µl of a reaction mixture containing 1.6 mM tetramethylbenzidine and 3 mM H₂O₂ diluted in 80 mM phosphate buffer (pH 5.4) in a 96-well microwell. The rate of change in absorbance at 630 nm was measured spectrophotometrically, and MPO activity was expressed as absorbance per minute per milligram of wet tissue.

Renal CD3 and IgG immunofluorescence staining

To exclude the possible role of contamination by complement or Ig with adoptive transfer from the donor mice, immunofluorescence staining for CD3 or IgG was performed on both groups of recipients’ kidneys. Briefly, kidneys were sliced, embedded in OCT compound (Tissue-Tek; Sakura Finetek), and frozen with liquid nitrogen. OCT-embedded frozen kidneys were sectioned at 6-µm thickness, fixed in ice-cold acetone for 20 min, and allowed to air dry for 1 h. Dried sections were immersed in PBS for 5 min, then in serum-free protein block (DakoCytonation) for 20 min at room temperature. A diluted (1/20) FITC-conjugated rabbit anti-human/mouse CD3 (DakoUSA) or rabbit anti-human/mouse IgG (1/250) was applied to the sections for 1 h at room temperature. Sections were finally rinsed with PBS and coverslipped using Vectashield with 4’,6-diamido-2-phenylindole hydrochloride (Vector Laboratories) mounting medium.

Hemoglobin assay in transferred cell suspensions

To exclude the likelihood of contamination of hemoglobin (which can alter tissue injury responses) when purifying the donor splenocytes, a hemoglobin assay was performed on the leukocyte suspensions with a QuantiChrom hemoglobin assay kit (DIHB-250; BioAssay Systems) according to the manufacturer’s instructions.

Flow cytometry

Splenocytes were isolated and stained by two-color flow cytometry (Beckman Coulter) using PE- or FITC-conjugated mAbs CD3, CD4, CD8, and CD19 (BD Pharmingen). A hemocytometer was used to count total cell numbers.

Statistical analysis

Data are expressed as the mean ± SE. Comparison of group means was performed using a one-way ANOVA. A value of p < 0.05 was considered significant.

Results

IP experimental design

We first sought to establish that we could induce preconditioning in our model of renal IRI. We subjected two groups of mice to either 30-min bilateral renal clamping or sham surgery and followed their SCr for 3 days after ischemia to confirm a rise in creatinine in the ischemic group. On day 6 after ischemia, both groups of mice received 30 min of bilateral renal clamping, and once again their creatinine was followed for 3 days. Fig. 2 shows the SCr levels of both groups. The mice that received an ischemic insult on day 0 had a significant rise in SCr compared with mice that underwent sham or IRI surgery on day 0. On day 6, both groups of mice (IRI and sham) underwent renal IRI and were followed for 72 h after ischemia. After a second ischemic insult, IRI mice had protection from renal injury with significantly reduced SCr. (●, I/R primed-mice; ○, sham surgery-primed mice. *, p < 0.05, n = 4).
that underwent sham surgery. On day 6 both groups of mice underwent renal IRI. The second ischemic event in the IRI group did not produce a rise in creatinine, demonstrating ischemic preconditioning. The mice that underwent an initial sham surgery followed by renal IRI on day 6 showed a significant increase in SCr.

Transfer of ischemic leukocytes protects the kidney from injury
To test the hypothesis that immune cells directly mediated IP, we took two groups of mice and subjected one group to 30 min renal IRI and one group to sham surgery. Five days after ischemia, splenic cells were harvested, and leukocytes were isolated. We then transferred the same number of cells from both groups i.v. to nu/nu mice. After 1 wk, all recipient mice underwent 30-min renal IRI. The positive control for the experiment was 30 min IRI in WT mice, which produces a rise in SCr by 24 h after ischemia. The negative control was nu/nu mice as previously described (9), which do not show as much increase in SCr after renal IRI. We demonstrated in this study that nu/nu mice that receive leukocytes from mice that have undergone an ischemic insult are significantly protected from renal IRI (no rise in SCr) compared with nu/nu mice that receive leukocytes from sham-operated mice, which have a significant rise in SCr 24 h after ischemia (Fig. 3). FACS analysis was used to confirm that both groups of mice received similar numbers of immune cells. Splenocytes from mice from each group were analyzed 72 h after ischemia. These results are presented in Fig. 4 and show that similar amounts of immune cells were transferred into each group of mice.

Along with a functional protection from IRI, we found that mice that receive IRI leukocytes before IRI also show marked structural protection. Fig. 5 represents sections of kidneys 72 h after ischemia. WT mice show a typical pattern of tissue injury after ischemia with dilatation of tubules and patchy necrosis. The nu/nu mice, however, show a marked protection from structural injury 72 h after ischemia, as previously demonstrated (9). When IRI leukocytes were transferred before ischemia, nu/nu mice retained their structural protection of injury. However, when sham leukocytes were transferred before ischemia, this protection was abolished, and nu/nu mice showed structural injury more closely resembling that of WT mice. Tubular injury scores were determined and are presented in Fig. 6. The nu/nu mice that received IRI

**FIGURE 3.** T cell-deficient (nu/nu) mice that received IRI leukocytes had significantly reduced renal injury compared with nu/nu mice that received sham leukocytes. *, p < 0.05 vs. nu/nu + IRI leukocytes; WT and nu/nu, positive and negative control.

**FIGURE 4.** Normal WT levels of CD4 and CD8 are shown in A, and pretransfer levels for nu/nu mice are represented in B (n = 4). Similar numbers of CD4 and CD8 T cell reconstitution occurred in nu/nu mice adoptively transferred with IRI (C) or sham-primed leukocytes (D) 72 h after ischemia.

**FIGURE 5.** Representative kidney histology 72 h after ischemia. T cell-deficient (nu/nu) mice receiving IRI-primed leukocytes before ischemia had decreased histological damage, compared with those receiving sham surgery-primed leukocytes before undergoing renal IRI. (wild type and nu/nu, positive and negative control)

**FIGURE 6.** Tubular injury scores demonstrate that nu/nu mice that received IRI leukocytes before ischemia have significantly reduced structural injury, compared with nu/nu mice that received sham leukocytes before IRI at 72 h after ischemia. The nu/nu mice had markedly reduced structural injury after ischemia, compared with WT littermate controls, as previously demonstrated (9). *, p < 0.05 vs nu/nu + sham (n = 8).
leukocytes before ischemia were significantly protected from renal injury compared with \textit{nu/nu} mice that received sham leukocytes. 

Lack of hemoglobin in transferred cell suspensions

To exclude the possibility of significant contamination of hemoglobin when purifying the donor splenocytes, a hemoglobin assay was performed on the transferring splenocyte suspensions, and minimal hemoglobin was detected in WT sham, WT ischemic, iNOS sham, or iNOS ischemic cell suspension (0.002–0.007 g/dl in cell suspension compared with 0.22g/dl in serum as a positive control).

Neutrophil and macrophage infiltration in preconditioned kidneys

We evaluated the levels of infiltrating neutrophils and macrophages after ischemia as a potential mechanism of immune-mediated IP. Postischemic kidneys were analyzed at 72 h for MPO activity as an indicator of neutrophil and macrophage infiltration. Fig. 7 shows MPO activity for all four groups at 72 h after ischemia. We found no differences in neutrophil and macrophage infiltration between groups.

Complement and Ig deposition in the kidney

To exclude the possibility that the transferred B cells produced Abs to the kidney or the likelihood of contamination of complement with adoptive transfer from donor mice, immunofluorescence staining for C3d or IgG was performed on both groups of recipients’ kidneys, and the results were reviewed blindly by a pathologist (W.M.B.). C3d stained as a discontinuous linear pattern in the tubular basement membrane of \textit{nu/nu} mice receiving leukocytes from donors undergoing sham treatment or renal ischemia.
examined neutrophil and macrophage infiltration into the kidney. To determine a possible mechanism of action, we have shown previously that these mice are protected from renal injury (9). They do, however, have a normal population of other leukocytes. Deficient mice are athymic and thus produce no mature T cells. The mechanism proposed was that iNOS works by the generation of NO, which, in turn, attenuates posts ischemic interactions between leukocytes and endothelium. We therefore investigated whether iNOS was working directly with leukocytes to mediate preconditioning. We transferred ischemia or sham-primed leukocytes from iNOS-deficient mice to nu/nu mice 1 wk before renal IRI. Fig. 9 displays the course of injury in nu/nu mice after transfer of ischemia- or sham surgery-primed leukocytes from iNOS-deficient mice. We found that, similar to IRI in WT mice, iNOS-deficient ischemic leukocytes transferred into nu/nu mice before renal IRI resulted in subsequently reduced renal IRI compared with the nu/nu mice receiving sham surgery-primed leukocytes. These data demonstrate that iNOS deficiency does not abrogate the immune cell’s role in IP.

Role of iNOS in immune cell-mediated preconditioning

The role of iNOS in preconditioning has been described recently (1). The mechanism proposed was that iNOS works by the generation of NO, which, in turn, attenuates posts ischemic interactions between leukocytes and endothelium. We therefore investigated whether iNOS was working directly with leukocytes to mediate preconditioning. We transferred ischemia or sham-primed leukocytes from iNOS-deficient mice to nu/nu mice 1 wk before renal IRI. Fig. 9 displays the course of injury in nu/nu mice after transfer of ischemia- or sham surgery-primed leukocytes from iNOS-deficient mice. We found that, similar to IRI in WT mice, iNOS-deficient ischemic leukocytes transferred into nu/nu mice before renal IRI resulted in subsequently reduced renal IRI compared with the nu/nu mice receiving sham surgery-primed leukocytes. These data demonstrate that iNOS deficiency does not abrogate the immune cell’s role in IP.

Discussion

The mechanisms underlying IP-mediated renal protection have not fully been elucidated. Most studies have focused on the roles of heat shock proteins (13, 14), endothelin (15), NO (5, 16), and adenosine (3, 17). Because the inflammatory response mediates the injury response to renal IRI (18), we hypothesized that leukocytes also played a role in IP. We found that T cell-deficient mice (nu/nu) that received immune cells from ischemic mice were protected from a rise in serum creatinine and tubular injury compared with nu/nu mice that received immune cells from sham-operated mice. Exposure of immune cells to the milieu of renal IRI thus alters them so as to render them less able to injure the kidney when they are subsequently exposed to a second episode of ischemia. The effects were independent of phagocytosis trafficking into postischemic kidney as well as of iNOS.

We did not specifically study which population of leukocytes was responsible for protection from injury; however, we limited the complexities between donor and recipient by using T cell-deficient mice as recipients of the leukocyte population. These T cell-deficient mice are athymic and thus produce no mature T cells. They do, however, have a normal population of other leukocytes. We have shown previously that these mice are protected from renal injury (9). To determine a possible mechanism of action, we examined neutrophil and macrophage infiltration into the kidney after ischemia. Kidney samples from each group were analyzed; however, we found no difference in infiltration after ischemia.

Because iNOS is an important contributor to the prolonged protective effects of ischemic preconditioning in the mouse kidney (1), we investigated whether leukocyte iNOS could explain our finding that immune cells are important in IP. We demonstrated in this study that when leukocytes from iNOS-deficient ischemic mice were transferred into T cell-deficient mice, a response similar to that of WT ischemic leukocytes was seen compared with those leukocytes from sham surgery, iNOS-deficient or WT mice. Therefore, it appears that iNOS is not involved with an immune cell-mediated IP.

To exclude other, more conventional mechanisms of ischemic preconditioning, we stained the kidneys from nu/nu mice receiving either ischemic or sham surgery leukocytes with IgG or/and C3d to determine whether the protected kidneys contain Abs and/or complement. Little IgG Ab or complement was detected in either group of recipient kidneys. Because hemoglobin from lysed erythrocytes could affect IP, we measured the hemoglobin levels in these transferred cell preparations and did not detect significant hemoglobin contaminations. These data demonstrate that circulating Ab or complement is less likely to mediate the ischemia-primed leukocyte protective effect.

Several recent studies have focused on the use of IP in a remote organ to protect another organ against later, extended ischemia. Attenuation of lung IRI damage in a porcine model of lung IRI could be achieved by brief, repeated phases of sublethal ischemia to the hind limb skeletal muscle (19). The results showed that functional characteristics of lung IRI were completely reversed; however, the brief ischemia to the hind limb was not sufficient to completely ameliorate the systemic inflammation. These findings are in agreement with our findings showing that neutrophil and macrophage infiltrations were not different in the protected and unprotected groups. This hind limb ischemia study did not elucidate the mechanism of this systemic preconditioning; however, in our study we found that circulating immune cells are able to mediate, in part, the kidney IP response.

Another study that supported the concept of immune cell-mediated IP demonstrated that adoptive transfer of splenocytes from E-selectin-tolerized rats to normal rats resulted in protection from injury in a rat model of ischemic brain injury (20). Our current study found that a similar response occurs with the transfer of ischemia-exposed leukocytes.

In conclusion, this is the first evidence that an immune cell distant from the kidney can directly mediate IP in the kidney. This finding may also be relevant for elucidating the mechanisms underlying cross-talk between injured kidney and distant organs. Identifying the specific leukocyte population as well as the pathways that mediate IP could lead to novel therapies for IRI.

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Disclosures

The authors have no financial conflict of interest.

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