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

Melissa J. Burne-Taney, Manchang Liu, William M. Baldwin, Lorraine Racusen and Hamid Rabb

*J Immunol* 2006; 176:7015-7020; doi: 10.4049/jimmunol.176.11.7015
http://www.jimmunol.org/content/176/11/7015

References

This article cites 20 articles, 7 of which you can access for free at:
http://www.jimmunol.org/content/176/11/7015.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Decreased Capacity of Immune Cells to Cause Tissue Injury Mediates Kidney Ischemic Preconditioning

Melissa J. Burne-Taney,* Manchang Liu,* William M. Baldwin, † Lorraine Racusen, † and Hamid Rabb2*†

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-Nos2tm1Lau/J), 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

Received for publication April 8, 2005. Accepted for publication March 3, 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.

*This work was supported by National Institutes of Health Grant R01 DK54770 (to H.R.), a National Kidney Foundation/Bristol Myers Squibb Young Investigator Award (to M.J.B.-T.), and by NHLBI P50 HL073944 (to H.R. and M.L.).

† Address correspondence and reprint requests to Dr. Hamid Rabb, Johns Hopkins University School of Medicine, Ross 965, 720 Rutland Avenue, Baltimore, MD 21205. E-mail address: hrabb1@jhmi.edu

1 Abbreviations used in this paper: IP, ischemic preconditioning; iNOS, inducible NO synthase; IRI, ischemia reperfusion injury; MPO, myeloperoxidase; SCr, serum creatinine; WT, wild type.
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 × 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 paraffin 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 Laight (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 × g at 4°C. 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 microplate. 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 C3d 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 C3d 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 blocker (DakoCytomation) for 20 min at room temperature. A diluted (1/20) FITC-conjugated rabbit anti-human/mouse C3d (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 Vectorshield 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 leucocyte suspensions with a Quantichrome 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

![Figure 1](image-url): Experimental groups and design.

![Figure 2](image-url): Confirmation of IP after a second ischemic event. Mice 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...
leukocytes before ischemia were significantly protected from renal injury compared with 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 0.22 g/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 nu/nu mice receiving leukocytes from donors undergoing sham treatment or renal ischemia.

FIGURE 7. MPO activity at 72 h after ischemia revealed similar amounts of kidney-infiltrating phagocytes in WT, nu/nu, as well as nu/nu mice that received sham or IRI leukocytes before renal IRI (n = 4).

FIGURE 8. Immunofluorescence staining for C3d (A and B) and IgG (C and D) on kidneys from nu/nu mice receiving leukocytes primed by either sham surgery (A and C) or renal ischemia (B and D). C3d stained nonspecifically with a discontinuous linear pattern in the tubular basement membrane, and IgG staining was minimal in both groups of mice. There was no difference in the low levels of C3d staining between groups.
examined neutrophil and macrophage infiltration into the kidney during renal injury (9). To determine a possible mechanism of action, we transferred ischemia or sham surgery-primed leukocytes from iNOS-deficient IRI mice transferred into nu/nu mice before renal IRI and 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 the effects 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.

### Acknowledgments

We acknowledge the expert technical assistance of Karen Talbot-Fox with immunofluorescence staining.

### Disclosures

The authors have no financial conflict of interest.

### References


