Pathophysiological role of T lymphocytes in renal ischemia-reperfusion injury in mice

HAMID RABB,1 FRANK DANIELS,1 MICHAEL O’DONNELL,1 MAHMUD HAQ,2 SABIIHA R. SABA,3 WILLIAM KEANE,1 AND WINSON W. TANG4

1Department of Internal Medicine, Hennepin County Medical Center, University of Minnesota Medical School, Minneapolis, Minnesota 55415; Departments of 2Surgery and 3Pathology, University of South Florida and Veterans Administration Hospital, Tampa, Florida 33612; and 4Amgen, Thousand Oaks, California 91320

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Rabb, Hamid, Frank Daniels, Michael O’Donnell, Mahmud Haq, Sabiha R. Saba, William Keane, and Winson W. Tang. Pathophysiological role of T lymphocytes in renal ischemia-reperfusion injury in mice. Am J Physiol Renal Physiol 279: F525–F531, 2000.—Mononuclear cell infiltrates are found in human renal ischemia-reperfusion injury (IRI), and peritubular T lymphocytes have been identified in experimental IRI. However, the role of T cells in the pathogenesis of renal IRI is unknown. We hypothesized that T cells are one of the important mediators of renal IRI. To test this hypothesis, we used an established mouse model of renal IRI, and evaluated mice with genetically engineered deficiency of both CD4+ and CD8+ T cells. At 48 h postischemia, CD4/CD8-knockout (KO) mice had marked improvement in renal function compared with control C57BL/6 mice (serum creatinine: 0.7 ± 0.4 vs. 2.5 ± 0.3 mg/dl, respectively; P < 0.05). Tubular necrosis score in CD4/CD8 KO mice, compared with control mice, was significantly less at 48 h (0.4 ± 0.1 vs. 2.4 ± 0.2, respectively; P < 0.05). Because adhesion between T cells and renal tubular epithelial cells (RTECs) may underlie the pathophysiological role of T cells in renal IRI, we also measured T cell adhesion to primary murine RTECs in vitro. Exposure of RTECs to 2 h of hypoxia followed by 1 h of reoxygenation increased T cell adhesion more than twofold. Phorbol ester treatment, which activates integrins, increased T cell adhesion threefold. These data suggest that T lymphocytes can mediate experimental renal IRI. Moreover, adhesion of infiltrating T cells to renal tubular cells may provide a potential mechanism underlying postischemic tubular dysfunction.

ISCHEMIC ACUTE RENAL FAILURE is associated with decreased allograft survival in patients with transplanted kidneys and high mortality and morbidity in patients with native kidneys (10, 32). However, the specific mechanisms that mediate renal ischemia-reperfusion injury (IRI) are incompletely understood (27, 30). A number of studies have examined the role of leukocytes and their surface adhesion molecules in the pathogenesis of postischemic renal injury (20). Most of these studies have focused on the role of neutrophils in IRI. The specific role of neutrophils in the pathogenesis of renal IRI, however, is still unclear, despite studies either supporting or disputing their role.

The role of other inflammatory cells, particularly the lymphocyte, in the development of renal IRI has been largely overlooked in experimental studies. A role for lymphocytes is not immediately intuitive based on classic immunologic paradigms. There are, however, at least five lines of evidence suggesting that lymphocytes play an important role in renal IRI: 1) lymphocytes are found in postischemic human kidney (25); 2) lymphocyte-related cytokines are upregulated in postischemic kidney (5, 14, 29); and 3) leukocyte adhesion molecules CD11/CD18 and intercellular adhesion molecule-1 (ICAM-1) mediate experimental renal IRI (11, 12, 17, 18). These pathways were originally studied because of their role in adhesion of neutrophils to endothelium, but they also mediate lymphocyte adhesion, migration, and intracellular function (26); 4) T lymphocytes mediate murine liver IRI (35); and 5) blocking the CD28-B7 costimulatory pathway, important for lymphocyte activation, significantly abrogates postischemic renal dysfunction in rats (28).

We therefore hypothesized that T cells could be one of the important mediators of renal IRI. To test this hypothesis, we used mice genetically engineered to be deficient in both CD4 and CD8 lymphocytes and evaluated their response to renal IRI using an established mouse model (8, 21). We found that CD4/CD8-deficient mice had a marked functional protection from renal IRI compared with wild-type, strain-matched controls. This was accomplished by reduced tubular necrosis and...
attenuated neutrophil infiltration. To begin to explore mechanisms underlying the role of T cells in renal IRI, we then hypothesized that T cell-renal tubular epithelial cell (RTEC) adhesion is important in the pathogenesis of renal IRI. We tested this hypothesis by investigating T cell-RTEC adhesion in an in vitro murine T cell-RTEC coculture system. Phorbol ester treatment was used as a positive control agonist due to its established effect on integrins (26), which play a role in the pathophysiology of renal IRI. A marked increase in T cell adherence to RTECs occurred after stimulation with phorbol ester. Moreover, T cell adhesion to RTECs was markedly increased when RTECs were exposed to hypoxia-reoxygenation, a maneuver designed to mimic IRI in vitro. We conclude from these experiments that T cells play an important role in the pathogenesis of renal IRI, perhaps in part through direct interactions with RTECs in the postischemic kidney. The study of T cells in acute renal failure is a new approach in this area of investigation and opens the potential of harnessing developments in T cell biology for novel IRI therapeutics.

METHODS

Renal ischemia-reperfusion model. Animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals (Dept. of Health and Human Services, Publ. No. 80–23, Revised 1978). An established model of renal IRI in mice was used (8, 21). Briefly, 25- to 35-g male mice were anesthetized with 35–50 mg/kg pentobarbital and underwent bilateral flank incisions and dissection of the renal pedicles. A microvascular clamp (Roboz, Rockville, MD) was placed on each renal pedicle for 30 min while the animal was kept at constant temperature (continuously monitored) and well hydrated. After 30-min ischemia, the clamps were removed, the wounds were sutured, and mice were allowed to recover. At designated time points postischemia, the mice were euthanized, and blood and kidney samples obtained.

T cell-deficient mice. To test the hypothesis that T cells are important in renal IRI, we studied mice that were deficient in both CD4 and CD8 cells (23). CD4/CD8-deficient mice were individually generated using homologous recombination in pluripotent embryonic stem cells. These mice were crossbred, and double heterozygous mice were bred to produce mice heterozygous for both T receptor deficiency. The double heterozygous mice were mated and offspring were screened by flow cytometry to confirm the total absence of the respective population of the T cells. These CD4/CD8-deficient mice have been well characterized in other studies and have been shown to develop less renal injury in a model of crescentic glomerulonephritis (33). Although these mice lack CD4 and CD8 cells, they do have an increase in α/β-cells and have some cytotoxic response to alloantigen (Table 1) (33). Wild-type C57BL/6 mice were used as controls as they are the genetic background from which the T cell-deficient mice were derived.

Assessment of renal function. Blood samples were obtained from all mice, and serum creatinine levels were measured as markers of renal function using a 550 Express autoanalyzer (Ciba Corning, Oberlin, OH).

Evaluation of renal pathology. Coronal kidney samples were cut and embedded in paraffin, and 4-mm sections were prepared. The sections were then stained with hematoxylin and eosin, reviewed in a blinded fashion by a renal pathologist and nephrologist, and scored with a previously described semiquantitative scale designed to evaluate the degree of tubular necrosis (17, 21). Higher scores represent more severe damage, with maximum score being 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). Neutrophil infiltration into the interstitium was quantified by using Leder stain in 10 high-power fields of the corticomedullary junction.

Preparation of mouse RTECs. Kidneys were removed from adult C57BL/6 mice, and cortex tissue was excised, minced, and digested for 30 min at 37°C in 0.5 mg/ml collagenase (Sigma) (24). Washed cell suspensions were plated in tissue culture flasks. Culture medium consisted of RPMI 1640 containing 1% fetal bovine serum, 50 U/ml penicillin, 50 µg/ml streptomycin, 2 g/l sodium bicarbonate, 4 µg/ml thymine, 36 ng/ml hydrocortisone, 10 ng/ml epidermal growth factor (EGF), 25 µg/ml insulin, 25 µg/ml transferrin, and 25 ng/ml sodium selenite. After 24 h, flasks were washed several times to remove unattached cells, and the culture medium was replaced. RTECs were removed from the flasks at day 5 by brief incubation in trypsin/EDTA. The cells were washed several times, plated in 24-well plates, and grown to confluence for in vitro T cell adherence assays.

Preparation of mouse T lymphocytes. Spleens were removed from adult C57BL/6 mice and mashed though 60 mesh screens (3). Collected cells were layered on lymphocyte separation media (LSM, Organon Teknika-Cappel, Durham, NC), and red blood cells were removed by centrifugation for 30 min at 400 g. An enriched T lymphocyte population was prepared by separation on a nylon wool column after a 1-h incubation at 37°C. Eluted cells were counted, adjusted to 1 × 10⁶ cells/ml, and incubated for 30 min with 5 mM carboxyfluorescin diacetate succinimidyl ester (Molecular Probes, Eugene, OR). Washed T cells were resuspended in RPMI containing 1% fetal bovine serum.

Adherence assay. The T cell-RTEC adhesion assay was modified from a fluorescence-based RTEC adhesion assay previously described in depth (19). T cells (2 × 10⁵) and adherent RTECs were coincubated at 37°C for 2 h. Unattached T cells were removed by four gentle washes with PBS. Cells remaining in the wells were solubilized by the addition of solution containing 0.1% SDS and 0.1 N sodium hydroxide. Fluorescence was measured in a fluorescence spectrophotometer (Perkin-Elmer) at 485-nm excitation and 515 nm emission.

In vitro simulation of IRI conditions. Activation of leukocyte adhesion by integrins occurs in IRI (20). Phorbol ester treatment is a potent inducer of integrin activation (26). Therefore, we added phorbol 12-myristate 13-acetate (50 ng/ml) to the T cell-RTEC preparation as a positive control to assess effects on adherence. Next, to mimic a “physiological” stimulus for leukocyte adhesion in IRI, we constructed a hypoxic chamber where hypoxia-reoxygenation was used as the stimulus for T cell-RTEC adhesion. This experimental
CD4/CD8-deficient mice have improved renal function after renal ischemia reperfusion. We used a model of IRI which is sublethal in nature and is designed to model the severe but nonlethal kidney injury, which is likely to be the nature of the insult in acute tubular necrosis. The CD4/CD8-knockout (KO) mice had a pronounced and statistically significant decrease in serum creatinine at 48 h postischemia (Fig. 1). Earlier, at 24 h postischemia, CD4/CD8 KO mice had a reduced rise in serum creatinine compared with C57BL/6 control mice, but this did not reach statistical significance (Fig. 1).

CD4/CD8-deficient mice have reduced tubular necrosis after renal ischemia-reperfusion injury. The tubular necrosis score, like the serum creatinine level, tended to be less in the CD4/CD8-KO mice at 24 h postischemia, compared with that in the control mice, but this did not reach statistical significance (Fig. 2). However, CD4/CD8-deficient mice had a marked and statistically significant reduction in the tubular necrosis score at 48 h postischemia (Fig. 2).

CD4/CD8-deficient mice have reduced neutrophil migration into postischemic kidney. Neutrophils are abundant in postischemic murine kidney, and may be important in the pathogenesis of renal IRI (12, 20). Using Leder stain to highlight neutrophils, as well as localize them to the corticomedullary junction, we found a significant decrease in the number of infiltrating neutrophils in the CD4/CD8-deficient mice at 24 h postischemia, compared with that in controls (Fig. 3). Neutrophil infiltration in the CD4/CD8-deficient mice was also markedly diminished at 48 h postischemia (Fig. 3).

T cells adhere to renal tubular cells, and the adherence is increased by cell activation. We hypothesized that adhesion of infiltrating T cells to RTECs could be an important and previously unrecognized interaction underlying the role of T cells in renal IRI. We initially studied immortal murine T cell lines and found high baseline adhesion to primary mouse RTECs. We therefore isolated primary mouse T cells from spleen and found only a mild baseline adhesion to murine RTECs. This adhesion was significantly increased when phorbol ester stimulation was administered to the coculture (Figs. 4 and 5). Spectrophotometric quantification revealed that phorbol 12-myristate 13-acetate induced a greater than threefold increase in the T cell-RTEC adhesion (Fig. 5).

Increased T cell adhesion to RTECs during hypoxia-reoxygenation. To better simulate IRI in vitro, we used a hypoxic chamber (Fig. 6). Preliminary studies were performed to determine the degree of RTEC injury.
assessed by lactate dehydrogenase (LDH) release] caused by varying times of hypoxia. We found that 1 h of hypoxia followed by 2 h of reoxygenation resulted in viable RTECs. Under these conditions, there was a twofold increase in T cell-RTEC adhesion (Fig. 7).

DISCUSSION

These data demonstrate that CD4/CD8-deficient mice, compared with wild-type control mice, have improved recovery from renal ischemia reperfusion, with decreased tubular necrosis and reduced infiltrating neutrophils. We also found that T cell adhesion to

Fig. 4. A: sparse baseline adhesion of mouse T cells to cultured mouse renal tubular epithelial cells (RTECs). B: increased adhesion of mouse T cells to the RTECs occurs after phorbol ester (10 ng/ml) stimulation. Background adhesion to both tissue culture plates was comparable, reflecting equivalent washes.

Fig. 5. Quantification of adhesion of murine T cells to primary murine RTECs under control and phorbol ester-stimulated conditions. *P < 0.01.
RTECs occurs in vitro and can be upregulated by phorbol ester, as well as by hypoxia-reoxygenation used to simulate the IRI conditions that occur in vivo. These findings support our hypothesis that the T cell is an important mediator of renal IRI. Moreover, the present results are consistent with increasing evidence from a number of other groups regarding the importance of inflammatory pathways in renal IRI (2, 11, 4, 16).

The mechanisms by which T cells might mediate renal IRI are not known and may include both indirect and direct effects. The present results suggest the intriguing possibility that T cells are, in some way, required for neutrophil infiltration into postischemic tissue. Wild-type control mice had marked neutrophil infiltration into postischemic renal tissue, while neutrophil infiltration in the CD4/CD8-deficient mice was dramatically attenuated. Some investigators have suggested that infiltrating neutrophils are important mediators of postischemic tissue injury (9, 13), although others dispute a significant role of neutrophils in renal IRI (31). If, indeed, neutrophils are primary mediators of renal IRI, then T cells might indirectly participate in renal IRI by stimulating neutrophil infiltration. Alternatively, one could postulate that T cells are direct mediators of renal IRI and that the reduced numbers of neutrophils found in postischemic kidneys of the CD4/CD8-deficient mice were due to less T cell-mediated renal injury. Careful focus on the very early inflammatory events within the first few hours of reperfusion may help determine whether it is the T cell or the neutrophil that is the more proximal mediator.

We observed a more marked difference in tubular injury and serum creatinine in CD4/CD8-deficient mice at 48 h compared with that at 24 h. It is likely that we are actually observing a continuum of response from T cell abrogation at both early and later times on inflammatory response (F. Epstein, personal communication). However, it is also possible that T cell manipulation has a more distinct role in the maintenance and recovery phase of renal IRI compared with the initial injury response.

To begin to study mechanisms by which T cells might directly mediate postischemic tubular cell injury and dysfunction, we investigated adhesive interactions between murine T cells and cultured murine RTECs. The rationale for studying T cell-RTEC interactions was provided by the recognition that the renal tubular epithelial cell, and particularly the proximal tubule cell, is the major target cell of ischemic injury in the kidney (30). Moreover, T cells have been found in the interstitium and peritubular areas of postischemic kidney, as well as in other renal diseases such as acute and chronic allograft rejection (7) and chronic glomerulonephritis (22). Our observation that T cell-RTEC adhesion is significantly enhanced after exposure of RTECs to hypoxia-reoxygenation suggests a possible mechanism by which T cells might mediate postischemic renal tubular dysfunction in vivo. Moreover, the observation that phorbol ester also stimulated T cell-RTEC adhesion suggests that this adhesion is mediated, at least in part, by leukocyte integrins. This is pertinent in light of prior data demonstrating that...
leukocyte integrins are one of the mediators of renal IRI (20).

The present results are also consistent with a number of studies that have demonstrated a role for leukocyte adhesion molecules in renal IRI. Lymphocytes require engagement with adhesion molecules to extravasate into the parenchyma of injured kidney. Up-regulation of ICAM-1 has been found in postischemic human and animal kidneys, including those in mice (12), and blockade of ICAM-1 has attenuated renal IRI in animal models (6, 11, 15, 18). ICAM-1 blockade was investigated in previous studies because of the recognized role of ICAM-1 in mediating neutrophil functions. It should be noted, though, that ICAM-1 is also important for lymphocyte migration and serves as an accessory molecule for lymphocyte activation (26).

Thus the effect of ICAM-1 blockade in previous studies to reduce renal IRI could conceivably have involved an effect to reduce T cell infiltration and activation.

Activation of T cells is a critical step in T cell function and is the target of some current therapeutic interventions. The mechanism by which T cells might become activated to participate in renal IRI is not clear and was not specifically addressed in the present study. Classically, T cell activation has been thought to require “foreign” antigens bound to a self major histocompatibility complex molecule together with costimulatory signals by an antigen-presenting cell. Thus T cell activation in renal IRI would not conventionally be anticipated. Nonetheless, T cell activation and the production of associated cytokines have been described in experimental renal IRI (5, 14, 28, 29). The absence of foreign antigens suggests that alloantigen-independent T cell activation may be involved in renal IRI. Recently, an antigen-independent mechanism of T cell activation that involves the chemokine regulated on activation normal T cells expressed and secreted (RANTES) has been described (1). Of significance, we have recently found upregulation of RANTES in this mouse model of renal IRI (14).

This study was potentially limited by the use of KO mice, which might have alterations independent of what were originally targeted. In addition, use of combined CD4/CD8-KO mice did not allow us to identify which T cell population might be more important for renal IRI. Thus future studies using specific CD4- and CD8-KO mice, as well as T cell depletion induced in wild-type mice by anti-CD4 or anti-CD8 antibodies, are warranted to further investigate the role of specific T cell subsets in renal IRI. In addition, future in vitro studies will be useful to further characterize the adhesive interactions between T cells and RTECs, including the adhesion molecules involved. Together with the present results, such studies will help elucidate the role of T cells in acute kidney injury, as well as potential interactions of T cells with RTECs that may mediate or amplify postischemic renal damage.

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REFERENCES


