Persistent renal and extrarenal immune changes after severe ischemic injury

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Background. Renal ischemia/reperfusion (I/R) injury is associated with delayed graft function and decreased long-term allograft function. However, most experimental studies evaluating renal I/R injury have focused on acute events after ischemia. T cells are potential candidates to link preservation injury, alloimmunity, and fibrosis. We hypothesized that severe renal I/R injury would generate long-term kidney damage and immune changes.

Methods. C57BL/6 mice underwent 60 minutes of warm unilateral I/R injury or sham surgery and were studied for 6 weeks. Serum creatinine, renal histology, and albumin excretion were measured. Phagocyte infiltration, CD4+ infiltration, renal cytokine expression, and splenic lymphocyte intracellular cytokine production were also measured in mice at 6 weeks.

Results. Serum creatinine levels rose following 60 minutes of unilateral I/R injury compared to sham mice. Histologic analysis of ischemic kidneys at 6 weeks revealed a pronounced loss of tubular architecture and infiltration of inflammatory cells. Phagocyte and CD4+ T-cell infiltration were significantly increased in ischemic kidneys. This was accompanied by a significant increase in interleukin (IL)-1β and regulated upon activation, normal T-cell expressed and secreted (RANTES) expression. Despite similar splenic CD4 and CD8 numbers, intracellular cytokine staining of T cells revealed a significant increase in interferon-gamma (IFN-γ) in I/R injury mice compared to sham mice.

Conclusion. Persistent renal and extrarenal immune responses occur after a single episode of severe I/R injury. These immune processes resulting from injury could in turn have long-term consequences on progression of renal disease in transplanted and native kidneys.

Ischemic acute renal failure (ARF) is associated with decreased allograft survival in patients with transplanted kidneys and high mortality in patients with native kidneys [1, 2]. Kidney transplants from living unrelated donors [not well human leukocyte antigen (HLA)-matched] with minimal ischemic injury have improved 3-year allograft survival, compared with grafts from well-matched cadaveric donors with significant ischemia [3]. This implies that renal ischemia/reperfusion (I/R) injury can have important consequences on long-term graft survival.

Although much attention has focused on mechanisms of early injury following an ischemic insult, the mechanisms of long-term injury after severe ischemia are incompletely understood. Most experimental work has focused on the first 5 days following an ischemic insult. Renal ischemic injury has been found to permanently damage peritubular capillaries causing hypoxia, which may be involved in the progression of chronic renal disease after ARF [4, 5]. Tubulointerstitial influx of inflammatory cells is found in many forms of chronic renal diseases, including “nonimmune” diseases such as diabetes and hypertension [6]. Thus, immune changes after “nonimmune” injury may participate in progressive dysfunction.

It has recently been demonstrated that the T cell, specifically the CD4+ T cell is an important modulator of renal I/R injury [7, 8]. Given that a single episode of renal I/R injury is associated with increased chronic rejection (chronic allograft nephropathy) [1], we hypothesized that long-term inflammatory changes, particularly in CD4 T cells, would occur. In this study we investigated late immunologic changes after severe renal I/R injury in the mouse (60 minutes I/R injury—to simulate severe injury which has long term clinical consequences; unilateral to allow mouse survival). We found that at 6 weeks after severe I/R injury there was a loss of tubular architecture and this is accompanied by an increase in phagocyte and CD4+ infiltration, as well as up-regulation of interleukin (IL)-1 and regulated upon activation, normal T-cell expressed and secreted (RANTES). When we investigated extrarenal changes in T cells, we unexpectedly found that spleens from ischemic mice, but not sham-operated mice, had significant increases in T-cell interferon-gamma (IFN-γ) production, despite similar numbers of CD4 and CD8 cells. Thus, a single ischemic episode to the kidney...
resulted in both long-term localized and distant immunologic changes.

METHODS
Renal ischemia model
Male C57BL/6J, 5 to 7 weeks of age from Jackson Laboratories (Bar Harbor, ME, USA) underwent 60 minutes unilateral ischemia or sham surgery and were then monitored for 6 weeks postischemia. Briefly, 25 to 35 g mice were anesthetized with intraperitoneal pentobarbital (75 mg/kg), underwent abdominal incisions, and had their right renal pedicle bluntly dissected. A microvascular (75 mg/kg), underwent abdominal incisions, and had their right renal pedicle bluntly dissected. A microvascular clamp was placed on the right renal pedicle for 60 minutes while the animal was kept at a constant temperature (∼37°C) and well hydrated. After 60 minutes, the clamp was removed, wounds sutured, and the animal was allowed to recover. Animals were monitored and maintained for 6 weeks before sacrifice. Sham animals underwent the same surgical procedure without clamping of the renal artery. Since we were interested in examining long-term responses postischemia, we used a severe model of renal I/R injury. Sixty minutes of ischemia was chosen because it produces severe damage to the kidney and was therefore more likely to initiate an immune response. Mild episodes of ischemia to either transplant or native kidneys are not thought to result in significant long-term changes. The unilateral model was chosen to prevent mortality in mice, since they do not survive 60 minutes of bilateral renal ischemia for more than 2 days (data not shown).

Assessment of renal function
Blood samples were obtained from mice at various time points, and serum creatinine levels were measured as markers of renal function. Serum creatinine was analyzed on blood samples using a 557A Creatinine Kit (Sigma Diagnostics, St. Louis, MO, USA) and analyzed on a Cobas Mira S Plus automated analyzer (Roche Diagnostics Corp., Indianapolis, IN, USA).

Urinary albumin clearance
Albuminuria is a key indicator of progressive renal disease [9] and therefore we collected urine for analysis of albumin excretion in I/R injury compared to sham-operated mice. Urine volumes and albumin were analyzed in mice at 0, 3, and 6 weeks postischemia. Mice were placed in specially designed murine metabolic cages for a 24-hour period. Urine collected during this period was analyzed for albumin using an enzyme-linked immunosorbent assay (ELISA) kit (Bethyl Laboratories, Montgomery, TX, USA) per manufacturer’s instructions. Albumin clearance was determined using albumin concentration and urine creatinine.

Assessment of histologic injury
Kidneys were taken at 6 weeks postischemia and analyzed by hematoxylin and eosin staining. Ischemic kidneys were compared to contralateral and sham-operated kidneys by two blinded reviewers.

Myeloperoxidase (MPO) assay to quantify phagocytes
An MPO assay was performed to quantify neutrophils in postischemic murine kidney tissue, as previously described in depth [10]. Kidney samples were homogenized (1:20 wt/vol) in ice-cold KPO4 buffer. Samples were spun at 17,000g for 30 minutes at 4°C, and pellets were washed and spun an additional two times. Then 0.5% hexadecyltrimethylammonium bromide-10 mmol/L EDTA in KPO4 buffer was added to the remaining pellet (6:1). Suspensions were freeze/thawed three times and then incubated for 20 minutes at 4°C. Following final centrifugation, supernatants were measured for MPO. Changes in absorbance over 3.5 minutes were recorded at 460 nm. One unit of MPO activity was defined as a change of absorbance of 1 per minute. Results were expressed as units MPO per mg of protein, which was determined using the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL, USA).

CD4+ and neutrophil detection by immunohistochemistry
Tissue sections were prepared as described above for routine histology. Sections (4 μm) were prepared on a cryostat and mounted on Fisher Superfrost Plus slides, fixed in ice-cold acetone for 1 to 2 minutes, and allowed to air dry. Sections were then blocked with 1:100 normal rabbit serum in phosphate-buffered saline (PBS) containing Vector Avidin DH (Vector Laboratories, Inc., Burlingame, CA, USA). The following primary antibodies were then added to the sections: GK 1.5 (rat antimouse CD4) (American Type Culture Collection, Manassas, VA, USA) and 7/4 (rat antimouse neutrophil) (Accurate Chemical & Scientific Corp., Westbury, NY, USA). Sections were then incubated for 1 hour at room temperature. An isotype control primary antibody was used as a background staining control. Sections were then rinsed in PBS and treated with 3% hydrogen peroxide in biotin (10 μg/L PBS) to block the biotin-binding sites. After three washes in PBS, the slides were incubated with a biotin-conjugated rabbit antirat IgG secondary antibody (Vector Laboratories Inc.) for 35 minutes at room temperature. Sections were once again washed, and were then incubated for 45 minutes in Vector Elite ABC (Vector Laboratories Inc.). Sections were washed and developed with 3-amino-9-ethyl-carbazole, counterstained with hematoxylin, and mounted using Glycergel (Dako Corp., Carpinteria, CA, USA). After the entire kidney section was viewed, ten high-powered (×40) fields were
counted in the area of the corticomedullary junction, and number of cells per high powered field was quantified in a blinded fashion.

**CD4+ and CD8+ splenic populations by flow cytometry**

Spleens were collected from euthanized mice, homogenized, and treated with ammonium chloride to remove red blood cells. Washed cells were filtered through a 70 μm nylon cell strainer and counted in a hemocytometer. One million cells were treated with Fc Block for 30 minutes then stained with anti-CD8 fluorescein isothiocyanate (FITC) and anti-CD4 phycoerythrin (Pe) (Pharmingen, San Diego, CA, USA) for 1 hour on ice. Cells were then washed twice, then resuspended in 1% formaldehyde. Flow cytometry was performed using an Epics II (Coulter, Hialeah, FL, USA) gating on appropriate unstained and isotype controls.

**Bioplex Protein Array System**

A panel of cytokines was measured in kidney samples taken at 6 weeks postischemia. Ischemic, contralateral kidneys and sham kidneys were measured in duplicate using the Bioplex Protein Array System (Bio-Rad, Hercules, CA, USA), according to the instructions of the manufacturer. This is a novel multiplexed, particle based, flow cytometric assay which utilizes anticytokine monoclonal antibodies linked to microspheres incorporating distinct proportions of two fluorescent dyes [11]. The assay enables quantification of several mediators in a sample volume as small as 15 μL. Our assay was customized to detect and quantify IL-1β, IL-2, IL-4, IL-6, IFN-γ, tumor necrosis factor-α (TNF-α), and RANTES. For each cytokine, eight standards ranging from 2 to 3200 pg/mL and the minimal detectable dose was <10 pg/mL.

**Intracellular cytokine staining**

Spleens were collected from animals at 6 weeks postischemia and a lymphocyte-rich cell suspension was obtained using Lympholyte M cell separation media. Anticytokine antibodies were used in conjunction with antibodies against cell surface antigens for the purpose of studying specific lymphocyte populations. Cells were surfaced stained with CD3. Intracellular staining was performed using a fluorescent-conjugated anti-IFN-γ antibody and anti-IL-4 antibody. The final suspension was analyzed on a FACS flow cytometer. Appropriate unstimulated and isotype controls were also used.

**Statistical analysis**

Data was expressed as mean ± SE. Analysis of variance (ANOVA) and Fisher least-significant difference test were used to compare means of multiple groups statistically. For paired data, Student t test was used. Statistical significance was set at P < 0.05.

**RESULTS**

**Long-term functional alterations**

Serum creatinines were followed in I/R injury versus sham-operated mice over 6 weeks. Early injury to the kidney was characterized by a significant increase in serum creatinine at 24, 48, and 72 hours in I/R injury mice compared to sham-operated mice. The increase in creatinine reflects the net function of both kidneys (Fig. 1). Albumin clearance was measured in I/R injury versus sham-operated mice at 0, 3, and 6 weeks following I/R injury (Fig. 2). At 3 and 6 weeks postischemia there is a significant increase in albumin clearance in I/R injury mice compared to sham-operated mice (P < 0.05).
Fig. 3. Histologic analysis of kidney tissue 6 weeks postischemia using hematoxylin and eosin staining. Gross structural changes are observed between contralateral kidneys (A, left) and ischemic kidneys (A, right). Morphologically ischemic kidneys (B) show a loss of tubular architecture, tubular destruction, and cyst formation. Contralateral (C) and sham-operated kidneys (D) show normal histology (representative of N = 12).

Structural characterization of long-term injury

Six weeks following a severe ischemic insult to the kidney, the ischemic kidney is shrunken in size to approximately half that of the contralateral kidney (Fig. 3A). Histologically, kidney sections of ischemic kidneys displayed a loss of tubular architecture with dilation of tubules and cyst formation (Fig. 3B). Contralateral kidneys and kidneys obtained from sham-operated mice (Fig. 3C and D, respectively) show normal histology.

Long-term phagocyte infiltration

Kidneys were analyzed for phagocyte infiltration 6 weeks postischemia (Fig. 4). An MPO assay which detects neutrophils, but may also detect macrophage infiltration, was used [10]. Long-term I/R injury kidneys had a significant increase in MPO levels compared to both contralateral kidneys and kidneys obtained from sham-operated animals. We also performed immunostaining of neutrophils and a similar increase was found in kidney sections from ischemic mice compared to contralateral and sham kidneys (Fig. 5).

CD4+ T-cell infiltration

T-cell infiltration into the kidney 6 weeks postischemia was analyzed. Figure 6 shows representative
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**Long-term renal cytokine changes**

We analyzed a panel of cytokine expression in kidneys at 6 weeks posts ischemia using a cytokine protein array system. We found a significant difference in IL-1β and RANTES expression in ischemic kidneys versus contralateral and sham kidneys (Fig. 8). However, no differences were found in IL-2, IL-4, IL-6, IFN-γ, and TNF-α.

**Systemic immune changes in lymphoid organs**

We hypothesized that long-term extrarenal changes in T cells could occur after an episode of renal I/R injury, and we therefore examined distant lymphoid tissue. Splenic cells were analyzed for CD4+/CD8+ positive cells. FACS analysis showed similar numbers of CD4+/CD8+ cells in spleens obtained from I/R injury and sham-operated mice (Fig. 9). Given that T-cell IFN-γ production has been associated with early renal I/R injury [8, 12], we hypothesized that a long-term phenotypic shift of T cells to produce IFN-γ could occur. Intracellular cytokine staining was performed and Figure 10 shows that splenic T cells from I/R injury mice have an increased production of IFN-γ at 6 weeks posts ischemia compared to sham animals. We also analyzed splenic IL-4 cytokine production, however, found no differences between ischemic and sham animals (data not shown).

**DISCUSSION**

In this study, we demonstrate that a single episode of severe warm ischemic injury to the kidney is associated with significant long-term kidney inflammation, particularly CD4+ infiltration and, unexpectedly, distant immune cell changes. Although the inflammatory pathways involved in renal I/R injury have been extensively evaluated to focus on short-term outcomes, there has been little attention on long-term inflammatory changes after single kidney ischemic injury. Many studies have implicated severe I/R injury as an antigen-independent risk factor for late renal allograft failure [13–15]. With organ shortages for transplantation, more nonheart-beating cadavers as well as brain-dead cadavers with long ischemic times are being used for organ donation. Despite lifelong immunosuppression of recipients, immunologic responses remain the leading factor in the pathogenesis of chronic rejection. Other nonalloreactive factors such as cold ischemia have been investigated and have also been found to contribute to late damage [16].

We used a model of severe ischemia in order to learn about the long-term effects of ischemia since in the kidney transplant patients long ischemic times lead to worse long-term outcomes [17]. Shorter degrees of ischemia are clinically less established to be associated with significant long-term problems. We found a prominent change in gross and microscopic renal structure in the clamped kidney 6 weeks after ischemia. It is quite likely that the ischemic kidney did not recover from injury. The I/R injury kidney had shrunk to approximately half the size of the contralateral kidney. The I/R injury kidney also developed cystic changes, tubular dropout, and interstitial fibrosis. These are the hallmarks of end-stage kidneys [6]. The presence of albumin in urine is a marker of loss of renal barrier function to plasma proteins, and a marker of progressive kidney disease in both allograft and native kidney. There was a significant rise in urine albumin clearance over time in I/R injury mice compared to sham-operated mice, which suggest glomerular dysfunction and/or abnormalities in tubular uptake of albumin. Given that albumin clearance is significantly increased in ischemic mice by 3 weeks, the injured kidney is the most likely source rather than hyperfiltration injury to the contralateral kidney.

In conjunction with the gross structural and histologic changes, we observed a large influx of leukocytes into I/R injury kidneys. There was an increase in MPO levels, reflecting an increase in neutrophils and macrophages. This was confirmed using specific immunostaining for neutrophils which showed a significant increase in neutrophil infiltration into the ischemic kidney. Based on the functional role for CD4+ T cells in early I/R injury, autoimmunity, and allograft rejection, we specifically examined these cells in I/R injury kidney. Unlike the paucity of CD4+ cells within a few days after I/R injury [8], there...
Fig. 6. CD4+ infiltration as measured using specific CD4 antibodies at 6 weeks in sham kidneys (A), contralateral kidneys (B), and ischemic kidneys (C).

was a marked infiltration of CD4+ T cells throughout the I/R injury kidney, but not in the sham or normal kidney. Inflammatory infiltrates characterize progressive renal disease in both human allografts and native kidneys, and are putative mediators of the progressive fibrosis that ultimately leads to a loss of function [6]. This study is the first demonstration of marked CD4+ infiltration late after I/R injury, and may facilitate rejection as well as progression of renal disease.

In conjunction with an increase in infiltrating CD4 T cells was an increase in IL-1β and RANTES. Previous in vitro studies have shown that proinflammatory cytokines such as IL-1β can induce RANTES production by renal tubular epithelial cells. Activation of complement of endothelial cells also contributes to production of chemokines [18]. Cytokines and chemokines released by damaged or activated renal resident cells can direct T-cell infiltration [16]. A recent study has also demonstrated that T cells once recruited can, in turn, promote further chemokine production through combined cognate and soluble factor-mediated interactions with resident tubular epithelial cells [19]. Recently, T-cell activation has been demonstrated to occur totally independently of antigen and RANTES has been shown to directly activate T cells [20]. RANTES up-regulation has been shown to occur in renal I/R injury, so this is one possible mechanism for T-cell activation in the absence of alloantigen [21].

With the marked CD4+ T-cell infiltration in the I/R injury kidney, we postulated that extrarenal lymphoid organs could also undergo changes in immune cells. We examined CD4 and CD8 T-cell counts in spleens of I/R injury and sham-operated animals and found the numbers to be similar in both groups. Given that qualitative changes in T cells may be occurring and that IFN-γ production by T cells has been implicated to be pivotal in many immune diseases, we explored if a functional change in T cells occurred in spleen. Using intracellular cytokine staining, a significant increase in T-cell IFN-γ was observed in I/R injury mice compared to sham-operated mice. IFN-γ has been previously shown to be up-regulated in kidney following renal I/R injury [21]. It is also well known to enhance expression of major histocompatibility complex (MHC) class I and II antigens which are likely to predispose ischemic damaged kidneys to the development of graft rejection [22, 23]. In a human study, it has been recently shown that IFN-γ may have an influence on acute rejection of kidney transplants, particularly those patients on cyclosporine therapy [24]. IFN-γ was also associated with worse long-term graft function [24]. IFN-γ has also recently been shown to mediate Toll-like receptor 4 mRNA expression following renal I/R injury [25]. We speculate that the increase in T-cell IFN-γ in I/R injury animals could play a role in progressive renal injury and susceptibility to alloimmune responses.
Our study was limited by the need of keeping a “normal” kidney in the I/R injury mouse due to inability to dialyze the mice for kidney failure as is performed in humans. The remnant healthy kidney could play a role in the outcomes of the injured kidney. We also recognized that splenic lymphocytes may not be representative of all lymphocytes. Although they may not represent the systemic circulation as a whole, they do represent a large number of immune cells. Furthermore, we used a model of severe injury in order to accentuate the possible injury as well as immune response. In milder injury, as we and others have previously shown in studies conducted in rats and humans, changes to kidney function and structure are less clinically pertinent. It is also important to note that some of these long-term changes may occur earlier (as in after 2 weeks of injury); however, even if changes occur earlier after injury, it is important to point out that these changes persist and are still present at 6 weeks posts ischemia. We have previously studied shorter time intervals (1 week) in rats, and found that the marked kidney fibrosis, mononuclear cell infiltrate, and cyst formation are not present; however, some CD4 infiltration was seen [26].

Based on these novel findings of long-term intra- and extrarenal changes in T cells in this new experimental model, future studies will examine the mechanisms and consequences of the long-term inflammatory changes posts ischemia. Among the key questions is if inflammatory changes mediated by immune cells directly contribute to a progressive decline in kidney injury or if the inflammatory changes mediated by immune cells are merely a secondary phenomenon and response to injury.
REFERENCES