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High Renal Ischemia Temperature Increases Neutrophil Chemoattractant Production and Tissue Injury During Reperfusion Without an Identifiable Role for CD4 T Cells in the Injury

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Abstract

Various leukocyte populations, including neutrophils and CD4 T cells, have been implicated as mediators of acute renal ischemic injury. The influence of ischemic temperature on molecular and cellular mechanisms mediating this injury was tested in a mouse model. Wild-type C57BL/6, B6.CD4^{-/-}, B6.CD8^{-/-}, and B6.RAG-1^{-/-} mice subjected to bilateral renal pedicle occlusion for 30 minutes at a higher (37°C) but not a lower (32°C) ischemic maintenance temperature had clear evidence of renal dysfunction and histopathology. Ischemia imposed at the higher temperature also increased CXCL1/KC and CXCL2/MIP-2 levels and neutrophils, but not T cells or macrophages, infiltrating into the ischemic kidneys. Depletion of neutrophils but not T cells attenuated the acute ischemic injury. These results indicate the influence of ischemic temperature and time on the production of neutrophil chemoattractants and subsequent neutrophil infiltration to mediate acute ischemic injury and fail to identify a role for adaptive immune components in this injury.

Keywords

Acute ischemic injury; Kidney; Neutrophil; CD4 and CD8 T cells; Chemokines; Mouse model

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Conflict of Interest

None of the authors has any potential financial conflict of interest related to this manuscript.

Introduction

Tissue ischemia and reperfusion injury (IRI) are inherent events in solid organ transplantation. Oxygen deprivation followed by the stress of reperfusion induces injury to the vasculature and parenchymal tissues of transplanted organs. Longer ischemic times produce greater tissue damage upon reperfusion indicating that mechanisms mediating tissue injury in response to reperfusion are either increased or unregulated with increasing ischemic time [1,2]. In support of this, longer ischemic times correlate with delayed graft function, earlier acute rejection episodes and poorer renal allograft outcome [3–6].

Many molecular and cellular mechanisms contribute to the injury provoked by reperfusion of ischemic tissues. IRI is induced in part by the formation of reactive oxygen radicals by the ischemic endothelium during reperfusion [7,8]. These and other molecular mediators induce leukocytic infiltration into ischemic tissues. Neutrophils are typically the first leukocytes to infiltrate inflammatory sites including ischemic tissues within hours of reperfusion [9,10]. This infiltration is directed by activated endothelium expression of selectins as well as the production of a battery of neutrophil chemoattractants that include CXC chemokines and complement activation products [11–15]. In addition to directing tissue inflammation, chemokines activate neutrophils to degranulate releasing proteolytic enzymes and proinflammatory cytokines that are critical mediators of the tissue damage observed following IRI [16,17]. Studies from many laboratories have demonstrated that depletion of neutrophils or neutralization of neutrophil chemoattractants inhibits infiltration and attenuates the tissue damage that normally follows imposition of IRI [12,14,18–21].

Evidence supporting a role of other leukocyte populations in mediating tissue damage during IRI has also been reported. A series of studies have recently implicated CD4 T cells in mediating tissue damage following reperfusion of ischemic kidneys in rodent models [22–24]. These studies have documented the presence of CD4 T cells in ischemic kidneys during reperfusion and reported that IRI is attenuated in T cell deficient mice subjected to renal IRI or in wild-type mice depleted of CD4 T cells prior to imposition of renal IRI. Furthermore, transfer of CD4 T cells to T cell deficient mice restores injury when renal IRI is imposed. In contrast, two laboratories have reported no difference in the extent of injury when renal IRI is imposed on wild type and T cell deficient $\text{TCR}\alpha^{-/-}$ mice [25,26].

Several procedural factors may influence the intensity of ischemic injury both clinically and in animal models. In addition to the duration of tissue ischemia, the temperature during ischemia may impact the level and type of resulting injury. Cooler ischemic maintenance temperatures usually result in lower levels of vascular injury following reperfusion and underlie the practice of chilled organ storage during transport for transplant. The cellular and molecular sequelae of ischemia imposed at different temperatures remain poorly understood. The following studies were initiated to investigate mechanisms of injury at different ischemic maintenance temperatures and to test if differences in CD4 T cell mediated injury during renal IRI in a mouse model were attributable to differences in ischemic temperature. The results indicate no role for CD4 or CD8 T cells in mediating renal IRI at high or low ischemic temperatures. The results also show that ischemia imposed at higher temperatures results in the production of significantly greater levels of neutrophil chemoattractants in the ischemic kidney during reperfusion and this is a major factor influencing the intensity of neutrophil recruitment and activation to mediate tissue injury following reperfusion.

Materials and Methods

Mice

Wild-type C57BL/6, B6.CD4^{-/-}, B6.CD8^{-/-} and Rag1^{-/-} mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Adult males 8–12 weeks old were used in these studies. Mice were maintained under pathogen-free conditions according to NIH guidelines. The use of animals in these studies was approved by the Cleveland Clinic Institutional Animal Care and Use Committee.

Renal Ischemia and Reperfusion

Renal I/R injury was performed in mice as previously detailed [12,27]. Briefly, mice were given 20 U sodium heparin i.p. 20 minutes before surgery. The mice were anesthetized with phenobarbital and kept warm under a 60-W light bulb until surgery. Under aseptic conditions, the abdominal cavity was opened with a midline incision and the bilateral renal pedicle was occluded non-traumatically with a microvascular clamp (World Precision Instruments, Sarasota, FL) and the wound was temporarily closed with 4-0 silk suture. Mice were placed on a heat pad under a 60-W light bulb to maintain intra-peritoneal temperature at either 32 or 37°C. A sensor tip of the Traceable™ Certificate Memory Monitoring Thermometer (Fisher Scientific) was placed into the abdominal cavity during surgery to ensure maintenance temperature at 32 or 37°C during the imposition of renal ischemia. Kidneys were subjected to ischemia for periods of 15–60 min. Immediate and complete renal reperfusion was confirmed visually and the peritoneal cavity was closed. After recovery from anesthesia, mice were given access to food and water. Sham-operated mice were treated in an identical manner except for the bilateral clamp of the renal pedicle.

In Vivo Depletion of Leukocyte Populations

Rat anti-mouse Gr-1 monoclonal antibody (RB6-8C5), anti-mouse CD4 mAb (GK1.5) and anti-mouse CD8 mAb (YTS 191) were purified from spent culture supernatant using protein G-Sepharose. To deplete mice of neutrophils, animals received 150 µg of RB6-8C5 i.p. on days -1, 0, and 1 of I/R. To deplete mice of CD4⁺ T cells or CD8⁺ T cells, animals received 250 µg of anti-CD4 mAb or anti-CD8 mAb i.p. on days -3, -2 and -1 before imposition of ischemia on day 0. Analysis of lymph nodes of treated sentinel mice indicated ≥ 96% depletion of the target T cell population. Control animals received equivalent amounts of rat IgG (Sigma Aldrich, St. Louis, MO).

Renal Function Measurement

Sham operated and mice subjected to bilateral renal I/R injury were anesthetized with isoflurane and bled from the postorbital plexus using a heparin-coated microcapillary tube at 24 hr intervals. The serum was stored at -80°C until measurement. Serum creatinine levels were measured using the Creatinine Kit (Sigma Diagnostics, Inc., St. Louis, MO).

Quantification of Leukocyte Populations in Kidneys

To determine the number of neutrophils, macrophages, CD4⁺ T cells and CD8⁺ T cells in ischemic kidneys during reperfusion, one quarter pieces of the retrieved kidneys were weighed. The kidneys were incubated in RPMI 1640 culture medium with 2% fetal calf serum for 1 hr and then were pushed through a 70 µm cell strainer using a syringe plunger. The cells were collected and the erythrocytes lysed using ACK Lysing Buffer (GIBCO, Grand Island, NY). After 2 washes, viable cells were counted using Trypan blue exclusion. Aliquots of the cells were preincubated with anti-CD16/CD32 Fc receptor antibody (BD Pharmingen, San Diego, CA) for 5 min to block nonspecific antibody binding and then samples were incubated with FITC-conjugated anti-CD45 mAb and PE-conjugated antibody to detect macrophages or CD8

T cells and APC-conjugated antibody to detect neutrophils or CD4 T cells (all antibodies from BD Pharmingen) for 30 min at 4°C. Cells were analyzed using three-color flow cytometry on a FACSCalibur (BD Biosciences, San Jose, CA). The forward scatter and FL1 (CD45⁺) channels were used to gate the leukocytes in the kidney tissue followed by analysis of the specific leukocyte populations. For each sample, 200,000 events were accumulated. The data were analyzed using CellQuest software (BD Biosciences). Total numbers of each leukocyte population were calculated by: (the total number of leukocytes counted) × (% of the leukocyte population counted in the CD45⁺ cells)/100. The data are reported as number of each leukocyte population/g kidney tissue from sham and I/R animals.

Immunohistochemistry

For immunohistochemistry, retrieved kidneys were halved, embedded in OCT compound (Sakura Finetek U.S.A., Torrance, CA), and immediately frozen in liquid nitrogen. Coronal sections were cut (7 µm), mounted onto slides, dried for 1 hr, and then fixed in acetone for 10 minutes. Slides were immersed in PBS for 10 min and in 3% hydrogen peroxide/methanol for 5 min at room temperature to eliminate endogenous peroxidase activity. Endogenous biotin activity was blocked with Biotin Blocking System (DAKO, Carpinteria, CA). After treating with normal rat serum (1:100), rat anti-mouse Gr-1 mAb RB6.8C5, diluted 1:100 in PBS with 1% bovine serum albumin (BSA) to detect neutrophils, or 1:50 dilutions of rat anti-CD4 mAb GK1.5 to detect CD4 T cells, rat anti-CD8α mAb 53–6.7 to detect CD8 T cells, or rat anti-mouse anti-macrophage F4/80 mAb was added to the sections. Control sections were incubated with rat IgG.

After 1 hr, slides were washed 3X with PBS and incubated for 20 min with biotinylated rabbit anti-rat IgG antiserum (Sigma Aldrich) diluted 1:100 in PBS/1% BSA. After 3 washes in PBS, slides were incubated with streptavidin-horseradish peroxidase (DAKO) for 20 min. The DAB (3,3'-diaminobenzidine) substrate-chromagen solution (Vector Laboratories, Inc., Burlingame CA) was applied to the slides for 0.5–3 min. After rinsing in dH₂O, slides were counterstained with hematoxylin, washed with dH₂O, cover-slipped, and viewed with a light microscope. Images were captured using Image Pro Plus (Media Cybernetics, Silver Spring, MD). Numbers of positively staining cells were counted in 10 random fields per slide and four slides per kidney for four different kidneys at X200 magnification.

Measurement of Tissue Cytokines by ELISA

Kidneys samples stored in liquid nitrogen were dissolved in 500 µl of PBS with 0.01 M EDTA and a proteinase inhibitor cocktail (10 µg/ml phenylmethyl sulfonyl fluoride, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 100 µg/ml Pefabloc SC, and 100 µg/ml chymostatin), and then 1 ml of 1.5% Triton X-100 in PBS was added. After incubation with agitation for 1 hr at 4°C, samples were centrifuged, the supernatant was collected, and the total protein concentration was determined using the BCATM Protein Assay Kit (Pierce, Rockford, IL). KC/CXCL1, MIP-2/CXCL2 and MCP-1/CCL2 concentrations were measured by sandwich ELISA using Quantikine M kits (R&D Systems, Minneapolis, MN). To determine the activation of neutrophils during reperfusion of ischemic kidneys, the concentration of myeloperoxidase (MPO) was measured using the Mouse MPO ELISA test kit (Cell Sciences, Canton, MA). Results are reported as protein per mg of total tissue protein ± SD.

RNA extraction and quantitative analysis of chemokines in kidney

One-quarter pieces of harvested kidney were frozen in liquid nitrogen. Total tissue RNA was extracted using the RNeasyTM Mini Kit (QIAGEN, Valencia, CA) and reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Real time PCR was performed on a Prism 7700 Sequence Detection System (Applied Biosystems, Foster City,

CA) with test KC/CXCL1, MIP-2/CXCL2 and MCP-1/CCL2 primers and Mrpl 32 used as the control (Applied Biosystems, Foster City, CA).

Transferase dUTP Nick-End Labeling (TUNEL) Analysis

Apoptotic cells were visualized with the terminal deoxynucleotide transferase FragEL DNA fragmentation kit (Oncogene, Boston, MA), analogous to terminal deoxynucleotide transferase mediated nick end-labeling. Numbers of apoptotic cells were counted in a blinded manner in 10 random fields per slide and four slides per kidney for four different kidneys at x200 magnification.

Determination of Renal Vascular Permeability

Mice were injected with 1% Evan's blue dye in PBS (2.5 µl/g b.w.) i.v. 30 min before kidney harvest. Just before harvest, the circulation was cleared by transcardiac perfusion using 10 ml HBSS. After harvest, one-quarter of the kidney was weighed, cut into 8 pieces and placed in 300 µl formamide. Samples were incubated at 60°C for four days and then centrifuged for 30 minutes at 14,000 rpm at 4°C to remove tissue debris. The quantity of extracted Evan's blue dye in the supernatant was determined by measuring absorbance at 610 nm. Another one-quarter of the kidney was dried at 60°C for 4 days and weighed before and after drying, the concentration of Evan's blue per gram dry weight kidney was calculated using the wet/dry weight ratio. A standard curve of Evans blue in formamide was used to convert absorbances into µg Evans blue/g of dried tissue ± SD.

Statistical Analyses

Significant differences in survival of animals subjected to renal I/R injury were determined by Kaplan-Meier survival analysis and log-rank test analysis and $p < 0.05$ was considered a significant difference between treatment groups. Data for quantitation of infiltrating cells, and levels of cytokine production are reported as mean ± SD for each group of 4–6 animals and the significance of differences in means were analyzed by the Mann-Whitney *U* test using Prism (Graph Pad Software, San Diego, CA) and $p < 0.05$ was considered a significant difference.

Results

Ischemic maintenance temperature influences the level of injury following reperfusion

Groups of wild type C57BL/6 mice were subjected to 30 min of bilateral renal ischemia with body temperature maintained at either 32 or 37°C. Control (sham) animals were subjected to the same surgical procedure with the omission of renal pedicle occlusion and were maintained at 37°C for the 30 min. All animals maintained at 37°C during renal ischemia expired within 4 days while sham-operated animals and animals maintained at 32°C during renal ischemia all survived longer than 30 days after reperfusion with no overt indication of injury (Figure 1a). Coincident with the loss of viability, animals maintained at 37°C during renal ischemia had high levels of serum creatinine 24 hr after reperfusion and these levels continued to increase until the time of expiration (Figure 1b). In contrast, animals maintained at 32°C during renal ischemia had a slight rise in serum creatinine levels 24 hr after reperfusion and these levels quickly declined to or near the levels observed in the sham-operated animals.

The influence of ischemic maintenance temperature on the presence of leukocyte populations in the ischemic kidneys during reperfusion was then determined. This was performed by retrieving and digesting the ischemic kidney at three different time points after reperfusion, staining the cells with antibodies to identify specific leukocyte populations, and using flow cytometry analysis to directly count the numbers of the specific leukocytes present per gram

of renal tissue. Maintaining the ischemic temperature at 32°C resulted in a small increase in neutrophil infiltration 3 hr after reperfusion and this fell to the level observed in kidneys from the sham-operated animals thereafter (Figure 2a). Ischemia performed at 37°C resulted in a similar increase in neutrophil infiltration at 3 hr post-reperfusion as ischemia performed at 32°C. Unlike the latter group of animals, however, this infiltration continued to increase at 9 and 24 hr post-reperfusion in animals subjected to ischemia at 37°C. Maintenance of ischemia at 32°C resulted in a moderate decrease in the number of macrophages in the kidneys at the post-reperfusion times monitored whereas ischemia at 37°C resulted in 3–4-fold decreases in the number of macrophages following reperfusion (Figure 2b). Ischemia imposed at either 32 or 37°C resulted in decreases in CD4 T cell numbers with a more significant decrease observed in kidneys subjected to ischemia at 37°C and these decreased numbers were maintained throughout the 24 hr observation period (Figure 2c). The numbers of CD8 T cells in the kidneys during reperfusion also decreased in the ischemic kidneys during reperfusion with an earlier decrease observed in the 37°C ischemic group but in each of the ischemic groups these numbers rose to the levels observed in the sham-operated group by 24 hr post-reperfusion (Figure 2d).

The numbers of infiltrating leukocyte populations assessed using flow cytometry analyses was reflected by the intensity of neutrophil, macrophage, CD4 and CD8 T cell infiltration into prepared sections of the ischemic kidneys at the times post-reperfusion examined (Figure 3a and data not shown). The tissue location of neutrophil infiltration into the ischemic kidneys was also examined at 9 and 24 hr post-reperfusion. Infiltration of neutrophils into the cortex of kidneys subjected to ischemia at either 32 or 37°C was low 9 and 24 hrs after reperfusion (data not shown). In kidneys subjected to ischemia at 32°C, low intensity neutrophil infiltration was detected in the medulla and at the cortical-medullary junction at 9 hr post-reperfusion but infiltration into the medulla decreased at 24 hr post-reperfusion (Figure 3b). In contrast, neutrophils infiltrating the kidneys subjected to ischemia at 37°C were present at greater numbers in both the medulla and the cortical-medullary junction 9 hr after reperfusion and the intensity of this infiltration was maintained at 24 hr post-reperfusion. When entire cross-sections of individual kidneys were examined, injury to tubules including cast formation and loss of nuclei in tubular epithelial cells was more marked in kidneys subjected to 30 min of ischemia at 37°C compared to ischemia imposed at 32°C (Table 1). Furthermore, this injury increased to the most severe grade (+4) for both cast formation and loss of epithelial nuclei by 24 hrs post-reperfusion in the kidneys subjected to ischemia at 37°C.

The levels of myeloperoxidase (MPO) produced in the ischemic kidneys after reperfusion were consistent with the more intense neutrophil infiltration into kidneys subjected to ischemia at 37°C (Figure 4a). At the 32°C ischemic temperature, MPO levels reached peak at 6 hr after reperfusion and declined thereafter. In contrast, these levels continued to rise after the 6 hr time point and significantly greater levels of MPO were evident at 9, 24 and 48 hrs after reperfusion of kidneys subjected to ischemia at the higher temperature. Furthermore, marked differences in vascular permeability at 9 hrs reperfusion of kidneys subjected to different ischemic times at each of the two maintenance temperatures were observed (Figure 4b). Low increases in Evan's blue dye permeability were observed in kidneys subjected to 45 and 60 min of ischemia at 32°C whereas imposition of 30 and 60 min of renal ischemia at 37°C resulted in high levels of Evan's blue dye in the kidneys at 9 hrs post-reperfusion. Apoptotic cells were evident as early as 9 hr after reperfusion in kidneys subjected to ischemia at 37°C and the number of apoptotic cells more than doubled by 24 hr after reperfusion (Figure 5a and b). Apoptotic cells in kidneys subjected to ischemia at 32°C were not evident until 24 hr after reperfusion and were at low numbers.

Since these results documented an increased neutrophil infiltration that correlated with increased renal dysfunction including increases in vascular leak, tubular cell apoptosis, serum creatinine and loss of viability, the effect of neutrophil depletion prior to imposition of ischemia

at each maintenance temperature was tested. Depletion of neutrophils protected mice subjected to 30 min of bipedicle renal ischemia at 37°C and decreased serum creatinine levels to those observed in mice subjected to renal ischemia at 32°C (Figure 1c and d).

Absence of CD4 and/or CD8 T cells does not affect renal ischemia/reperfusion injury

A series of reports has implicated CD4 T cells in mediating tubular injury following reperfusion of ischemia kidneys [22–24] and low numbers of CD4 and CD8 T cells were clearly present during reperfusion when renal ischemia was imposed at either 32 or 37°C. Therefore, the role of these T cell populations in the injury was directly tested. First, groups of wild type C57BL/6 mice were treated with rat IgG or CD4 depleting antibodies just prior to imposing bilateral renal I/R injury. In each group of treated wild-type animals as well as in a group of B6.CD4^{-/-} mice, ischemia for 30 min at 37°C resulted in loss of viability by day 4 post-reperfusion (Figure 6a) with corresponding high serum creatinine levels (Figure 6b). Ischemia imposed at 32°C did not result in loss of viability of any of the groups of animals and post-reperfusion serum creatinine levels were virtually identical to those observed in wild type animals subjected to the ischemia. Similarly, the absence of CD8 T cells did not attenuate the loss of viability or renal dysfunction when ischemia was imposed at 37°C (Figure 6c and d).

A second approach was used by comparing viability and renal dysfunction following bilateral renal occlusion at 32 and 37°C in mice without both B and T lymphocytes, RAG-1^{-/-} mice (Figure 6e and f). Both loss of viability and serum creatinine levels in RAG-1^{-/-} mice was identical to wild type mice when the 30 min ischemia was imposed at 37°C and ischemia imposed at 32°C had little effect on the RAG-1^{-/-} mice other than a short increase in serum creatinine levels at 24 hr post-reperfusion which quickly fell to control levels thereafter.

Ischemic temperature influences levels of neutrophil chemoattractants produced during reperfusion

Since ischemia at 37°C resulted in a much greater infiltration of neutrophils into the kidneys during reperfusion, the effect of ischemic temperature on the gene expression and production of chemokines directing the infiltration of these and other leukocyte populations was investigated. Expression of CXCL1/KC and CXCL2/MIP-2 mRNA was induced at peak levels 6 hrs after reperfusion of kidneys subjected to 30 min of ischemia at 37°C (Figure 7). These levels were 4–5 fold higher than the mRNA levels observed following reperfusion of kidneys subjected to ischemia at 32°C and then fell quickly to background levels by 24 hr post-reperfusion. The levels and patterns of mRNA for these neutrophil chemoattractants were reflected by the temporal production of chemokine protein in the kidneys. In contrast to the neutrophil chemoattractants, mRNA and protein levels of the macrophage chemoattractant CCL2 did not appear until approximately 48 hrs after reperfusion of kidneys subjected to ischemia at 37°C and levels induced in kidneys during reperfusion following ischemia at 32°C were much lower. Consistent with the observed absence of a role for T cells in renal IRI in these studies, expression levels of mRNA encoding the T cell chemoattractant CXCL10/IP-10 were low and identical during the first 24 hrs after reperfusion of ischemic kidneys maintained at 32 or 37°C (data not shown).

In contrast to the induction of the neutrophil chemoattractants, TNFα mRNA peaked at high levels at 24 hrs after reperfusion in kidneys subjected to ischemic maintenance temperatures of either 32 or 37°C (Figure 8). Renal ischemia imposed at 37°C induced high levels of mRNA encoding IL-1β and IL-6 at 9 h and these dropped quickly to background levels thereafter whereas ischemia at 32°C did not induce detectable increases in levels of mRNA encoding these proinflammatory cytokines throughout the first 48 hrs after reperfusion (Figure 7).

Finally, the effect of ischemic time and temperature on the production of the neutrophil chemoattractants was tested at 9 hrs after reperfusion. During a renal ischemic maintenance temperature of 32°C, production of CXCL1/KC and CXCL2/MIP-2 in the kidneys did not increase above background levels until 45 min of ischemia was imposed and these levels rose thereafter with increasing ischemic time (Figure 9). In contrast, ischemia imposed for as little as 15 min at 37°C induced high levels of CXCL1/KC and CXCL2/MIP-2 after reperfusion although increased ischemia time at this maintenance temperature did not alter the production of these much beyond these levels.

Discussion

The initial intent of this study was to investigate mechanisms of CD4 T cell mediated tissue injury following renal IRI. Studies from several investigators had indicated the presence of CD4 T cells in ischemic kidneys during reperfusion and the contribution of these cells to the acute tissue injury [22–24]. Neither the kidney tissue ligands activating these CD4 T cells nor the functions expressed to mediate the injury have been identified. We considered the possibility that studies failing to observe a role for CD4 T cells in renal IRI might be due to the ischemic conditions imposed to induce the injury [25,26]. Therefore, two different ischemic maintenance temperatures were compared to test the potential role for CD4 T cells, as well as CD8 T cells and B cells, in contributing to renal IRI. Consistent with these latter studies, no evidence supporting a pathogenic role for CD4 T cells in renal IRI was observed. Use of several different experimental approaches indicated that both renal dysfunction and viability were equivalent when wild type mice and mice that had no CD4 or CD8 T cells were subjected to ischemia at 37°C. While a small number of CD4 T cells are present in mouse kidneys, IRI induced a decrease in these numbers with a more rapid decrease observed in kidneys subjected to the higher ischemic maintenance temperature. Such decreases have been previously reported and have led to the proposal that CD4 T cells infiltrate ischemic kidneys within the first 3 hrs after reperfusion and then emigrate from the kidney thereafter [28,29]. While the current study has not directly addressed early movement of the CD4 T cells, the CD4 and CD8 T cells in the kidney clearly decrease in numbers by 9 hrs post-reperfusion.

It is worth noting that a critical role for CD4 T cell mediated injury has been clearly shown in rodent models of liver ischemia where CD4 T cell recruitment is directed by CXCL10/IP-10 that is induced during reperfusion [30]. We have also observed CD4 T cells infiltrating heterotopically transplanted cardiac allografts within 24 hrs post-transplant but have not observed a function (e.g. IFN- γ production) expressed by these T cells (A. Schenk, manuscript submitted). In the current studies, CXCL10 was induced during reperfusion of ischemic kidneys but at similar and low levels following the non-lethal 32 and lethal 37°C ischemic maintenance temperatures. Similarly, reperfusion induced levels of the T cell and macrophage/monocyte chemoattractant CCL2/MCP-1 were at low levels following ischemia at either of the test maintenance temperatures. Moreover, ischemia at the higher temperature mediated rapid and sustained decreases in kidney macrophage numbers during reperfusion. These decreases suggest that the role of macrophages in contributing to injury following renal ischemia/reperfusion in this model is dispensable and raise the possibility that the macrophages may afford some protection during the injury. A pathogenic role for macrophages during reperfusion of ischemic kidneys has been suggested but remains to be definitely tested [29]. In contrast, infiltrating macrophages clearly have protective functions, in part by producing HO-1 and anti-inflammatory cytokines [23,31]. A critical function of macrophages infiltrating into tissue sites of inflammation is the phagocytosis and removal of apoptotic cells [32]. The low number of macrophages and high numbers of apoptotic renal tubular cells present during reperfusion of kidneys are likely to contribute to the injury observed following ischemia-reperfusion in these studies.

The higher metabolic activities occurring at higher temperatures are predictive of increased tissue injury when ischemia is imposed at higher temperatures. A recent study reported that more severe injury was induced at higher ischemic maintenance temperatures in a rat model of IRI although mechanisms underlying the increased injury were not investigated [33]. In the current study, the ischemic maintenance temperature had a critical effect on the renal dysfunction and viability following IRI. At the 37°C ischemic temperature, 5–10 fold higher levels of CXCL1/KC and CXCL2/MIP-2 were transcribed and produced than when the renal ischemic temperature was maintained at 32°C. This increased production of neutrophil chemoattractants was followed by a 10-fold increase in the number of neutrophils infiltrating the ischemic kidneys during reperfusion and increased neutrophil activation as reflected by increased MPO release and vascular permeability. Previous studies from this and other laboratories have demonstrated the ability of neutralizing CXCL1 and CXCL2 antibodies to attenuate renal IRI in rodent models implicating a key role for CXCL1 and/or CXCL2 directed neutrophil infiltration and activation in renal acute ischemic injury [12,19].

TNF α has been implicated as a proinflammatory cytokine playing a major role in tubular injury following IRI including the induction of tubular cell apoptosis [34,35]. Recent studies by Dong and colleagues [36] have indicated that renal resident dendritic cells are the primary source of TNF α at early times after reperfusion. Surprisingly, the temporal expression of TNF α as well as the levels expressed in ischemic kidneys was virtually identical whether ischemia was imposed at 32 or 37°C. These results suggest that the role of TNF α in mediating tubular cell apoptosis and other pathology in renal ischemia-reperfusion is likely to be indirect and must be exacerbated by an additional factor(s) that is increased by ischemia at the higher maintenance temperature. In contrast to the expression of TNF α , expression of IL-1 β and IL-6 was much higher during reperfusion of kidneys subjected to ischemia at the higher temperature and peaked much earlier after reperfusion than TNF α . Both IL-1 and IL-6 are potent inducers of adhesion molecules and various cytokines, including CXCL1/KC and CXCL2/MIP-2 [37–40]. The high levels of IL-1 β and IL-6 expression induced when ischemia is imposed at 37°C are likely to underlie the increased neutrophil chemoattractant production and subsequent injury observed at the higher ischemic maintenance temperature observed in these studies. Decreased injury is observed in IL-1 deficient mice following renal IRI whereas mice with a genetically targeted deletion in IL-1 receptor antagonist have increased injury [39].

In addition to ischemic temperature, the results of the current study also indicate an important effect of the time of ischemia duration. At the lower ischemic maintenance temperature, longer ischemia times resulted in time dependent up-regulation of CXCL1/KC and CXCL2/MIP-2 production. Recent studies from this laboratory have reported that IL-8 mRNA expression levels in biopsies from renal grafts taken 30 min after reperfusion correlate with the ischemic time imposed on the graft [41]. Prolonged ischemic times clearly have an adverse effect on renal graft outcome [3–6]. However, a recent multicenter study has reported that survival of cadaver donor kidney grafts was not influenced by cold ischemic times up to 18 hrs but ischemic times after that resulted in poorer graft survival [42].

The results of this study have failed to identify a role for CD4 T cells in mediating tissue pathology in renal IRI. The results strongly support a pathogenic role for chemokine-directed infiltration and activation of neutrophils that is influenced by the maintenance temperature imposed during ischemia. The results in the current study demonstrating a protective effect of neutrophil depletion are confirmatory of those from this and many other labs documenting the pathologic role for neutrophils during reperfusion of ischemic tissues [12,14,18,19,21]. Overall, these results predict that strategies targeting neutrophil infiltration and activation rather than adaptive immune components will be particularly efficacious in attenuating acute ischemic injury of kidneys.

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Figure 1.

Renal ischemia at 37 but not 32°C results in renal dysfunction and loss of viability following reperfusion. (a, b) Groups of 8 C57BL/6 mice were subjected to bilateral renal occlusion for 30 min with the temperature maintained at either 37 (–◆–) or 32°C (–□–). Sham operated animals were maintained at 37°C (–X–) during the 30 min. The viability and serum creatinine levels of each animal were assessed on the indicated day post-reperfusion. (c, d) Groups of 6 C57BL/6 mice were treated with control rat IgG (–◆–) or with neutrophil-depleting mAb (–X–, –□–) and then subjected to 30 min of bilateral renal occlusion with the temperature maintained at either 32 (–□–) or 37°C (–◆–, –X–) and the viability and serum creatinine levels were assessed. Serum creatinine levels on each indicated day post-reperfusion are shown as mean for each group of animals $\pm$ SD. * $p < 0.001$.

Figure 2.

Enumeration of leukocyte populations in ischemic kidneys during reperfusion. Groups of 4 C57BL/6 mice were subjected to 30 min of bilateral renal occlusion with the temperature maintained at either 32 (shaded bars) or 37°C (black bars) and sham operated animals were maintained at 37°C (white bars) during the 30 min. At the indicated time after reperfusion, kidneys were retrieved, digested, and cells were stained for flow cytometry analysis to determine the numbers of the indicated leukocyte populations in the kidneys. *p < 0.05.

Figure 3a

Figure 3.

Neutrophil infiltration into kidneys subjected to 30 min of ischemia at 32 vs. 37°C. C57BL/6 mice were subjected to bilateral renal occlusion for 30 min at different temperatures. (a) The kidneys were retrieved 9 hrs after reperfusion and prepared frozen sections were stained to detect infiltrating neutrophils. (b) Frozen sections were prepared of ischemic kidneys 9 and 24 hrs after reperfusion and stained to detect neutrophils. The presence of neutrophils in the cortex/medulla junction and medulla at each time point was assessed. + signs indicate the relative intensity of neutrophils in the renal location. (Magnification, ×200).

Figure 4.

Increases in myeloperoxidase and vascular leak following ischemia at 32 vs. 37°C. a) Groups of 6 C57BL/6 mice were subjected to bilateral renal occlusion for 30 min at different temperatures. The kidneys were retrieved at the indicated time after reperfusion and the quantity of myeloperoxidase in tissue homogenates was tested by ELISA. * $p < 0.02$. (b) Bilateral renal occlusion was imposed for 30, 45 or 60 min at 32 (white bars) or 37°C (black bars). Evan's blue dye was injected i.v. 8.5 hrs post-reperfusion and 30 min later the kidneys were retrieved. The amount of dye in tissue extracts was determined. * $p < 0.05$ when compared to group subjected to renal ischemia for same time at 32°C.

Figure 5a

Figure 5b

Figure 5.

Increased apoptosis in kidneys subjected to ischemia at 37 but not 32°C. Groups of 4 C57BL/6 mice were subjected to bilateral renal occlusion for 30 min at different temperatures and the kidneys were retrieved 9 and 24 hrs after reperfusion. (a) Prepared formalin-fixed sections were stained by TUNEL to detect apoptotic cells. Magnification, $\times 200$. (b) Numbers of TUNEL positive cells in 10 randomly selected high power fields for four slides/kidney and four kidneys per group were determined. The mean number of TUNEL positive cells per field for each group $\pm$ SD is shown. * $P < 0.05$.

Figure 6.

The absence of CD4 and CD8 T cells does not alter renal ischemia-reperfusion injury. (a, b) Groups of 4 wild-type C57BL/6 mice (-□-, -◆-) were treated with CD4 depleting mAb and these mice and groups of 4 B6.CD4^{-/-} mice (-X-, ---) were subjected to bilateral renal occlusion for 30 min with temperature maintained at either 32 (-□-, -X-) or 37°C (-◆-, ---). (c, d) Groups of 4 wild-type C57BL/6 mice (-□-, -◆-) were treated with CD8 depleting mAb and these mice and groups of 4 B6.CD8^{-/-} mice (-X-, ---) were subjected to bilateral renal occlusion for 30 min with temperature maintained at either 32 (-□-, -X-) or 37°C (-◆-, ---). (e, f) Groups of 4 RAG-1^{-/-} mice were subjected to bilateral renal occlusion for 30 min with temperature

maintained at either 32 (-□-) or 37°C (-◆-). The viability and serum creatinine levels were assessed. Serum creatinine levels on each indicated day post-reperfusion are shown as mean for each group of animals $\pm$ SD. * $p < 0.001$.

Figure 7.

Induction of chemokine mRNA and protein following reperfusion of kidneys subjected to ischemia at 32 vs. 37°C. Groups of C57BL/6 mice were subjected to 30 min of bilateral renal occlusion with the temperature maintained at either 32 (■) or 37°C (○). Kidneys were retrieved at the indicated time points after reperfusion. Renal tissue homogenates were prepared and the temporal expression of chemokine mRNA and proteins were determined by qRT-PCR and ELISA, respectively. Data indicate mean levels for 6 kidneys assayed at each time point $\pm$ SE. * $p < 0.05$.

Figure 8.

Induction of proinflammatory cytokine mRNA following reperfusion of kidneys subjected to ischemia at 32 vs. 37°C. Groups of C57BL/6 mice were subjected to 30 min of bilateral renal occlusion with temperature maintained at either 32 (■) or 37°C (○). Kidneys were retrieved at the indicated time points after reperfusion. Renal tissue homogenates were prepared and the temporal expression of cytokine genes was determined by qRT-PCR. Data indicate mean levels for 6 kidneys assayed at each time point $\pm$ SE. * $p < 0.05$.

Figure 9.

Induction of neutrophil chemoattractant mRNA expression is influenced by ischemic time and maintenance temperature. Groups of C57BL/6 mice were subjected to bilateral renal occlusion for the indicated times with the temperature maintained at either 32 (white bars) or 37°C (black bars) and 9 hrs after reperfusion renal tissue homogenates were prepared and the temporal expression of CXCL1 and CXCL2 mRNA was determined by qRT-PCR. Data indicate mean levels for 6 kidneys assayed at each time point $\pm$ SE. * $p < 0.05$.

Table 1
Histopathologic grading of kidneys during reperfusion after imposition of ischemia at 32 or 37°C

Ischemia at 32°C/30 min Harvest after 9h		Ischemia at 37°C/30 min (n=2) Harvest after 9h		Ischemia at 32°C/30 min Harvest after 24h		Ischemia at 37°C/30 min (n=3) Harvest after 24h	
Tubular casts	Loss of nuclei in tubular epithelial cells	Tubular casts	Loss of nuclei in tubular epithelial cells	Tubular casts	Loss of nuclei in tubular epithelial cells	Tubular casts	Loss of nuclei in tubular epithelial cells
3+	2+	3+	2+	1+	1+	4+	4+
2+	2+	3+	3+	1+	1+	4+	4+
1+	1+			1+	1+	4+	4+

Entire longitudinal sections of kidneys retrieved 9 or 24 hours after ischemia imposed at 32 or 37°C were examined and the tubules in the cortico-medullary junction scored for injury. Each cross-section was examined for two criteria, debris/casts in tubules and nuclear loss in epithelial tubule cells, and graded for mild damage (+1) to most severe damage (+4). All longitudinal sections from individual kidneys looked identical so grades for one individual representative section was scored. The number of individual kidneys examined for the two criteria is indicated (n); individual kidneys are listed in the columns.