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Lung T Lymphocyte Trafficking and Activation during Ischemic Acute Kidney Injury

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Despite advances in renal replacement therapy, the mortality rate for acute kidney injury (AKI) remains unacceptably high, likely owing to extrarenal organ dysfunction. Kidney ischemia–reperfusion injury (IRI) activates cellular and soluble mediators that facilitate organ crosstalk and induce caspase-dependent lung apoptosis and injury through a TNFR1-dependent pathway. Given that T lymphocytes mediate local AKI in the kidney and are known to drive TNFR1-mediated apoptosis, we hypothesized that T lymphocytes activated during kidney IRI would traffic to the lung and mediate pulmonary apoptosis during AKI. In an established murine model of kidney IRI, we identified trafficking of CD3+ T lymphocytes to the lung during kidney IRI by flow cytometry and immunohistochemistry. T lymphocytes were primarily of the CD3+CD8+ phenotype; however, both CD3+CD4+ and CD3+CD8+ T lymphocytes expressed CD69 and CD25 activation markers during ischemic AKI. The activated lung T lymphocytes did not demonstrate an increased expression of intracellular TNF-α or surface TNFR1. Kidney IRI induced pulmonary apoptosis measured by caspase-3 activation in wild-type controls, but not in T cell-deficient (Tnu/nu) mice. Adoptive transfer of murine wild-type T lymphocytes into Tnu/nu mice restored the injury phenotype with increased cellular apoptosis and lung microvascular barrier dysfunction, suggesting that ischemic AKI–induced pulmonary apoptosis is T cell dependent. Kidney–lung crosstalk during AKI represents a complex biological process, and although T lymphocytes appear to serve a prominent role in the interorgan effects of AKI, further experiments are necessary to elucidate the specific role of activated T cells in modulating pulmonary apoptosis.

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A acute kidney injury (AKI) affects 2–5% of hospitalized patients and ≥67% of the critically ill, yet despite advances in renal replacement therapy, critically ill patients with AKI have a nearly 60% risk of death, with minimal improvement in recent decades (1–3). Clinical observations suggest an association between AKI and dysfunction of extrarenal organs, such as the heart, lung, and brain, and new experimental data have emerged that focus on the interactive effects of concomitant kidney and distant organ dysfunction (4, 5). These studies have highlighted (1) the pathophysiological importance of proinflammatory and pro-apoptotic pathways as contributors to the complex nature of this interorgan crosstalk, and (2) the involvement of both soluble and cellular-based mediators in this altered immune response during AKI.

Given the complexity of interorgan crosstalk and the need to discover novel pathways that are functionally important, we initiated a genome-based discovery approach in a murine model of experimental AKI. In prior studies, we have discovered that kidney ischemia–reperfusion injury (IRI) induces functional and transcriptional changes in the mouse lung that are distinct from those induced by uremia alone, and have identified potential inflammation-associated mediators that connect local and systemic injury and apoptosis (6–8). Our prior studies indicate the lung microvascular endothelial cell (EC) as the primary target of these kidney IRI-induced changes (8, 9). Detailed investigations of pulmonary vascular permeability have underscored the importance of the balance between complex tethering forces involved in cell–cell and cell-extracellular matrix interactions, and endothelial apoptosis leads to disruption of these complex interactions and a potential for loss of endothelial barrier function (10, 11).

Although the effector mechanisms leading to distant organ endothelial injury and apoptosis during AKI remain unknown, several recent studies have discovered that lymphocytes play an important role in the immediate response to ischemia–reperfusion in various organs, thus challenging traditional concepts of innate and adaptive immunity (12, 13). In an effort to better understand the altered immune response during AKI, the distinct roles of kidney resident and infiltrating B and T lymphocyte populations have been closely examined, and CD4+ T lymphocytes are now recognized as important mediators of the early local immune response during AKI (14, 15). In fact, T cell trafficking during kidney IRI can occur within 1 h of the insult and incites the release of proinflammatory mediators that increase renal microvascular permeability through leukocyte–EC interactions (16, 17).

Given the importance of pulmonary endothelial apoptosis as a mechanism for distant organ lung dysfunction during kidney IRI, and that the latter has been shown to be modulated by T cells, we investigated a potential role for T cells in the context of kidney–lung crosstalk. We characterized the timing, pattern, and activation of pulmonary T cell subsets during kidney IRI in a well-established mouse AKI model, and studied the effects of IRI on distant organ
lungs apoptosis in T cell-deficient (T<sup>−/−</sup>) mice prior to and following adoptive transfer of T cells. On the basis of prior in vivo observations of the importance of the TNFR in mediating distant organ lung apoptosis, T cells were analyzed for TNFR1 (CD120) expression and intracellular TNF-α staining. In this study, we demonstrate that activation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells and pulmonary trafficking of primarily CD8<sup>+</sup> T lymphocytes are sentinel events during kidney IRI, and although T cells appear necessary for AKI-induced lung apoptosis, these effects may be independent of local TNF-α production.

Materials and Methods

Animal care

All procedures were approved by the Institutional Animal Care and Use Committee and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Briefly, 6- to 8-wk-old male C57BL/6J wild-type (WT) or T cell-deficient nu/nu (B6.Cg-Foxn1<sup>−/−</sup>) mice, weighing ∼25–30 g, were obtained from The Jackson Laboratory (Bar Harbor, ME) or Taconic (Germantown, NY), respectively, and housed under pathogen-free conditions according to National Institutes of Health guidelines for at least 5 d prior to any operative procedures.

Kidney injury model

Prior to the procedure, all animals were placed on a heating pad and anesthetized using sodium pentobarbital (50 mg/kg i.p.) and received buprenorphine for analgesia (0.01 mg/kg s.c.). The adequacy of anesthesia was assessed by paw and tail pinching. For mice assigned to undergo experimental IRI, a midline laparotomy was performed, and the right and left renal pedicles were bluntly dissected. An atrumatic microvascular clamp was placed on each pedicle for an allotted ischemic time of 60 min. After gently removing the clamps, animals were resuscitated with 1 ml warm sterile saline i.p. The incision was closed in two layers, using a 4-0 silk suture. Sham controls underwent identical procedures as above, without placement of a microvascular clamp. All animals were allowed to recover with free access to food and water. At 4 or 24 h following IRI or sham procedures, mice were euthanized by exsanguination under general anesthesia, and tissues were collected and processed for the assays described below.

Renal function during experimental IRI

Blood samples were obtained from both sham and IRI-treated animals at the time of euthanasia and centrifuged at 8000 rpm for 10 min to isolate the serum. Serum creatinine levels were measured to identify ischemic acute kidney injury, using a 557A Creatinine Kit (Sigma Diagnostics, St. Louis, MO) and analyzed on a Cobas Mira S Plus automated analyzer (Roche Diagnostics, Indianapolis, IN).

Lung immunohistochemistry

After completion of the allotted treatment time, the animals were reanesthetized, and the right main bronchus was isolated and suture ligated. Low-melting agarose (0.5%) was instilled into the left main bronchus through a 20-gauge angiocatheter via a tracheotomy. After complete expansion of the left lung, it was excised and placed on ice. The inflated lungs were fixed in 10% formalin for 48 h and embedded into paraffin blocks.

Immunohistochemistry (IHC) staining for T cells was performed on formalin-fixed tissue, as previously described (8). Briefly, equal amounts of protein (17 μg) were loaded into each well of a 15% Tris-glycine gel (Bio-Rad). After electrophoresis for 1 h at 100 V, the gel was blotted onto a nitrocellulose membrane by electrophoretic transfer at 30 V overnight. The membrane was washed, blocked with 5% normal goat serum and incubated for 1 h at 37°C. The remaining NEN was quenched with FACS buffer and stained with fluorochrome-conjugated mAbs for FACS analysis. The fluorochrome-conjugated mAbs to mouse IgG were used for flow cytometry analysis as was the following: anti-mCD16/CD32 (2.4G2), anti-mCD3e APC (145-2C11), anti-mCD4 PerCP (RM4-5), anti-mCD8<sup>b</sup> FITC (53-5.8), anti-mCD19 PE (1D3), anti-mCD69 PE (H1.2F3), anti-mNK1.1 PE (PK136), anti-mCD44 FITC (IM7), and anti-mCD8 (53.6.72; BD Pharmingen).

Flow cytometry analysis

Lung-cell suspension was preincubated with anti-mCD16/CD32 FeR for 10 min to minimize nonspecific Ab binding. Cells were then incubated with various combinations of mAbs for 30 min at 4°C, washed twice with FACS buffer, and fixed with 1% paraformaldehyde.

Three-color immunofluorescence staining was analyzed using a FACS-Calibur instrument (BD Biosciences). The lymphocytes were gated using forward and side scatter parameters to exclude debris and dead cells; thus, 10,000 events were acquired in each assay for analysis. Data were analyzed using CellQuest software (BD Biosciences).

Intracellular cytokine staining

FACS analysis was used to determine intracellular TNF-α expression on T lymphocyte subsets at 4 and 24 h after ischemic AKI. Briefly, samples were first stained with the appropriate fluorochrome-labeled Abs for cell surface markers CD3, CD4, and CD8. Following surface staining, cells were washed and permeabilized with perm/wash solution for 20 min and stained with PE-conjugated mAb anti-TNF-α. Finally, samples were analyzed for the presence of intracellular TNF-α, using FACS analysis.

Isolation of splenic lymphocytes

Spleens were removed from C57BL/6J mice and collected in RPMI 1640 buffer on ice. Splenocytes were processed into a single-cell suspension, using sterile technique in RPMI 1640 buffer. The cell suspension was spun and rehydrated with serial alcohol solutions submitted to heat-steam treatment for 30 min in a 10 mM citric acid monohydrate solution. The endogenous peroxidase activity was quenched by incubating the slides with Peroxidase Block for 5 min. Specimens were incubated with anti-active caspase 3 primary Ab overnight at 4°C. Slides were labeled with a polymer for 1 h, followed by incubation with a 3,3’-diaminobenzidine substrate chromogen for 10 min at room temperature, generating a brown precipitate at the Ag site. (Dako kit, HRP-conjugated secondary Ab). Quantification of caspase–3–positive cells was performed by analysis of 10 random fields per slide by a blinded observer.

Caspase-3 activity was measured by fluorometric assay (BD Biosciences), as described previously (8). Briefly, lung protein homogenate was obtained by sonicating lung tissue samples in a cell lysis buffer (Cell Signaling Technology) containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, and 1 mM Na<sub>3</sub>VO<sub>4</sub> and leupeptin. Lung tissue homogenates were centrifuged at 10,000 g for 15 min, and the supernatants stored at −80°C until used for caspase-3 activity assay. Direct protein quantitation was performed using the DC Protein Assay Kit (Bio-Rad, Hercules, CA). Equal amounts of protein were added to each well in a 96-well plate in 200 μl 1× HEPES buffer. For each reaction, 5 μl reconstituted Ac-DEVD-AMC was added, followed by 1 ml of 10 μM AMC buffer on ice. The remaining 37°C was measured using a plate reader with an excitation wavelength of 380 nm and an emission wavelength range of 420–460 nm and expressed as International Units per milligram of protein × 10<sup>3</sup>.

Cleaved (activated) caspase-3 protein expression from lung tissue homogenates was measured by Western immunoblotting, as previously described (8). Briefly, equal amounts of protein (17 μg) were loaded into each well of a 15% Tris-glycine gel (Bio-Rad). After electrophoresis for 1 h at 100 V, the gel was blotted onto a nitrocellulose membrane by electrophoretic transfer at 30 V overnight. The membrane was washed, blocked with 5% blocking solution, and probed with cleaved caspase-3 Ab (Cell Signaling, Danvers, MA), which detects both the 17- and 19-kDa lengths of activated caspase-3. The immunoreactive bands were visualized using a secondary Ab conjugated to HRP and a Bio-Rad Gel Doc (Bio-Rad) and analyzed by Quantity One software (Bio-Rad). β-Actin was used as a loading control, and results are normalized to corresponding sham.

Isolation of lymphocytes from mouse lungs

Lungs were collected from euthanized mice and homogenized in RPMI 1640 buffer into a single-cell suspension. Samples were incubated with ammonium chloride red cell lysis buffer for 5 min at room temperature and neutralized with RPMI 1640. Cellular suspensions were passed through 30-μm nylon filters and washed with PBS. The remaining pellet was resuspended with FACS buffer and stained with fluorochrome-conjugated mAbs for FACS analysis. The fluorochrome-conjugated mAbs to mouse Ags used for flow cytometry analysis were as follows: anti-mCD16/CD32 (2.4G2), anti-mCD3e APC (145-2C11), anti-mCD4 PerCP (RM4-5), anti-mCD8<sup>b</sup> FITC (53-5.8), anti-mCD19 PE (1D3), anti-mCD69 PE (H1.2F3), anti-mNK1.1 PE (PK136), anti-mCD44 FITC (IM7), and anti-mCD8 (53.6.72; BD Pharmingen).

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Spleens were removed from C57BL/6J mice and collected in RPMI 1640 buffer on ice. Splenocytes were processed into a single-cell suspension, using sterile technique in RPMI 1640 buffer. The cell suspension was spun

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1800 rpm for 5 min, and the supernatant was discarded. RBCs were lysed in 1 ml ammonium chloride RBC lysis buffer and neutralized with RPMI 1640. After washing, cells were resuspended in PBS. The population was purified using the Pan T Cell Isolation Kit (Miltenyi Biotec, Cat. no. 130-090-861). The purity and viability of the isolated population were confirmed with FACS analysis.

Adoptive transfer of T cells

Isolated T cells from WT C57BL6/J mice were injected into T
nu/nu mice via the tail vein. Cells were injected at doses of 6 x 10^6 cells per mouse, and 1 wk was allowed for T lymphocyte adaptation. Recipient mice underwent sham laparotomy or bilateral kidney IRI, as described above, and were sacrificed at 24 h by exsanguination. The kidney–lung phenotype was assessed by measuring serum creatinine and cleaved (activated) caspase-3 protein expression in whole-lung tissue, as described in the preceding sections. Adequacy of transfer was analyzed by collecting the spleens from transferred animals and processing whole spleens into a single-cell suspension, as described above, which was analyzed by FACS. Specimens were stained with fluorochrome-labeled Abs to identify the total population of CD3+, CD3+CD4+, and CD3+CD8+ lymphocytes.

Assessment of lung microvascular barrier dysfunction

Bronchoalveolar lavage (BAL) fluid analysis was performed in WT, T
nu/nu and adoptively transferred T
nu/nu mice (T
nu/nu + T cells) as a surrogate measure for pulmonary microvascular permeability, as previously described (6, 8, 18). BAL fluid was obtained by slow delivery of 0.75 ml warm (~37˚C) PBS via a tracheotomy. The fluid was withdrawn by gentle suction, the process was repeated twice, and solutions were combined and

FIGURE 2. IHC of CD3+ T lung lymphocytes. Representative lung CD3+ IHC micrographs (polyclonal rabbit anti-mouse CD3 [Calbiochem] with isotype primary Ab background) (original magnification ×40) of C57BL6 mice at 24 h following sham or kidney IRI show increased percentage of total CD3+ T lymphocytes (arrows) in IRI-treated mice.

FIGURE 3. Characterization of infiltrating lung T lymphocytes during ischemic AKI. FACS analysis of T lymphocyte subsets showed (A) no difference in CD3+CD4+ (17.8 ± 1.03 versus 26.1 ± 4.1), CD3+CD8+ (20.2 ± 3.7 versus 26.7 ± 5.4), or CD3+NK1.1+ (0.65 ± 0.3 versus 0.42 ± 0.2) T lymphocytes at 4 h in IRI versus sham controls and (B) a predominant influx of pulmonary CD3+CD8+ (15.3 ± 3.3 versus 24.9 ± 2.7) T lymphocytes at 24 h in IRI-treated mice, compared with sham controls, with no difference in CD3+CD4+ (23.6 ± 4.8 versus 19.9 ± 1.6) or CD3+NK1.1+ (0.86 ± 0.18 versus 1 ± 0.03) populations. Representative FACS analyses of (C) CD3+CD4+ and CD3+CD8+ and (D) CD3+NK1.1+ lymphocyte populations at 24 h are provided. *p < 0.05 versus sham, n = 6 per group.
stored on ice. Recovered BAL fluid underwent a bicinchoninic acid total protein assay (Bio-Rad, Hercules, CA) according to the manufacturer’s protocol. OD readings of samples were converted to micrograms per milliliter, using values obtained from standard curves generated with serial dilutions of BSA.

Statistical analysis

Data are expressed as means ± SE and were analyzed with one-way ANOVA for data varying by treatment group and phenotype. Individual group means were compared using a Tukey multiple comparison test. A p value < 0.05 was considered significant.

Results

Phenotype of lung lymphocytes during ischemic AKI

At 4 or 24 h during experimental IRI, WT mice were euthanized, and whole-lung tissue was collected for FACS analysis of lymphocyte trafficking. Whole-lung tissue was processed into a single-cell suspension, and samples were stained with FITC-, PE-, or PerCP-labeled anti-CD3, -CD19, -NK1.1, -CD4, and -CD8 (BD Pharmingen). At 24 h, FACS analysis showed an increase in CD3+ T cells (expressed as percentage of total lymphocytes) in IRI-treated mice compared with sham controls (25.01 ± 0.4 versus 36.5 ± 4; p ≤ 0.05), which was not observed at 4 h following IRI (41.02 ± 4.7 versus 52.9 ± 4.4; p = 0.09) (Fig. 1). CD19+ B cells were decreased at 4 h during IRI (43.5 ± 2.5 versus 31.02 ± 2.9; p ≤ 0.05) but demonstrated no change at 24 h, compared with sham (38.1 ± 3.9 versus 27.2 ± 5.2; p = 0.14). Using IHC staining in paraffin-embedded lung tissue, we confirmed an increase in lung CD3+ T lymphocytes in IRI-treated mice compared with sham controls (Fig. 2).

T cell subset analysis demonstrated an increase in lung CD3+ CD8+ T cells in IRI versus sham controls at 24 h (15.3 ± 3.3 versus 24.9 ± 2.7; p ≤ 0.05) that was not present at 4 h (20.2 ± 3.7 versus 26.7 ± 5.4; p = 0.35) (Fig. 3). The percentages of lung CD3+CD4+ T cells and CD3+NK1.1+ cells did not differ between sham and IRI groups at either time point.

Activation of CD4+ and CD8+ lung T lymphocytes during ischemic AKI

FACS analysis was also used to identify the presence of T cell activation markers on pulmonary CD4+ and CD8+ T lymphocytes during experimental AKI (Fig. 4). After 4 h of kidney IRI, the expression of CD69 on pulmonary CD3+CD4+ lymphocytes (0.3 ± 0.07 versus 0.9 ± 0.03; p ≤ 0.05) increased, compared with that in the sham group, although the number of positive cells was small in both treatment groups. However, at 24 h, we observed greater expression of activation markers and nearly a 3-fold increase in

FIGURE 4. Lung T lymphocyte activation during ischemic AKI. FACS analysis of early and late activation markers on T lymphocyte subsets showed (A) increased CD69 expression in CD3+CD4+ (0.3 ± 0.07 versus 0.9 ± 0.03*), but not in CD3+CD8+ (0.1 ± 0.04 versus 0.1 ± 0.03), lymphocytes versus sham at 4 h during kidney IRI, and increased expression of CD69 on CD3+CD4+ (3.3 ± 1.53 versus 15.8 ± 0.9*) and CD3+CD8+ T lymphocytes (4.9 ± 2 versus 15.6 ± 1*) in IRI-treated mice at 24 h, compared with sham controls. (B) No differences in CD25 expression were seen in CD3+CD4+ (0.21 ± 0.05 versus 0.05 ± 0.01) or CD3+CD8+ (0.9 ± 0.2 versus 0.05 ± 0.03) lymphocytes at 4 h; however, at 24 h, increased CD25 expression was seen in CD3+CD8+ (23.3 ± 3 versus 41.5 ± 1.1*), but not CD3+CD4+ (28.9 ± 2.9 versus 31.3 ± 1.7), lymphocytes during ischemic AKI. Representative FACS analyses of activation markers CD69 (C) and CD25 (D) in CD4+ and CD8+ T lymphocyte populations at 24 h are provided. *p < 0.05 for IRI versus sham, n = 3/group; *p < 0.05 for IRI versus sham.
CD69 expression on both CD4+ (3.3 ± 1.53 versus 15.8 ± 0.9; \( p = 0.05 \)) and CD8+ (4.9 ± 2 versus 15.6 ± 1; \( p = 0.05 \)) lymphocytes in IRI compared with sham animals. CD25 expression was negligible on both T cell subsets at 4 h following kidney IRI; however, its expression was significantly increased on pulmonary CD8+ (23.3 ± 3 versus 41.5 ± 1.1; \( p = 0.05 \)), but not CD4+ (28.9 ± 2.9 versus 31.3 ± 1.7; \( p = 0.52 \)), T lymphocytes at 24 h following IRI. These findings suggest that kidney IRI induces early and sustained trafficking and activation of pulmonary infiltrating T lymphocytes during AKI.

Lung T lymphocyte TNF-α production and TNFR expression during ischemic AKI

Our previous studies have suggested a prominent role for TNFR1 during ischemic AKI at 24 h during sham and IRI are provided. FACS analysis during IRI on CD3+CD4+ (0.35 for intracellular TNF-α-dependent apoptosis, infiltrating T lymphocytes were analyzed to determine whether infiltrating T cells are a source for TNF-α-dependent pulmonary apoptosis following kidney IRI (8, 19). To test the hypothesis that distant organ effects of AKI are directly T cell mediated, we analyzed whole-lung tissue for the presence of caspase-3-positive cells by IHC and caspase-3 activity in T

FIGURE 5. Intracellular TNF-α and TNFR expression on lung lymphocytes during ischemic AKI. FACS analysis of intracellular cytokine expression showed (A) no increase in intracellular TNF-α in CD3+CD4+ (0.39 ± 0.06 versus 1.5 ± 0.7) or CD3+CD8+ (0.3 ± 0.16 versus 0.7 ± 0.4) T lymphocytes in IRI-treated mice compared with sham controls. There was also no increase in TNFR1 expression (B) during IRI on CD3+CD4+ (0.35 ± 0.07 versus 0.5 ± 0.09) or CD3+CD8+ (0.14 ± 0.02 versus 0.17 ± 0.03) T lymphocytes. Representative FACS analyses of TNF-α (C) and TNFR1 (D) expression in CD3+CD4+ and CD3+CD8+ lymphocytes at 24 h during sham and IRI are provided.

Kidney function during ischemic AKI

In prior studies, we have defined the time- and dose-dependent kidney and lung injury response in our model and found that 60 min of bilateral renal ischemia produces a proapoptotic pulmonary phenotype and functional lung injury in WT animals at 24 h (6). On the basis of those studies, we measured serum creatinine (milligrams per deciliter) levels 24 h following sham or kidney IRI in C57BL/6 WT and Tnu/nu mice. Serum creatinine was increased during kidney IRI in both WT (0.46 ± 0.1 versus 2.7 ± 0.2; \( p = 0.05 \)) and Tnu/nu (0.35 ± 0.04 versus 2.1 ± 0.13; \( p = 0.05 \)) mice when compared with respective sham controls (Fig. 6).

T lymphocyte-dependent lung apoptosis during ischemic AKI

To evaluate the hypothesis that AKI-induced pulmonary apoptosis is T cell mediated, we analyzed whole-lung tissue for the presence of caspase-3-positive cells by IHC and caspase-3 activity in T

Adoptive transfer of T lymphocytes and confirmation of reconstitution in T cell-deficient mice

To test the hypothesis that distant organ effects of AKI are directly T cell mediated, T lymphocytes were isolated from whole spleens of C57BL/6 mice and transferred into T cell-deficient mice to reproduce the injury phenotype. A total of 6 × 10⁸ pooled cells were transferred into each recipient mouse by tail vein injection. The pooled lymphocytes consisted of 57% CD4+ and 33% CD8+ T lymphocytes. At 1 wk after T cell transfer, Tnu/nu mice underwent sham and IRI procedures, as previously described, and were observed for evidence of AKI-induced pulmonary apoptosis. At the time of sacrifice, T cell reconstitution was confirmed by FACS analysis of single-cell spleen suspensions obtained from recipient mice. Total CD3+ lymphocytes from sham and IRI-treated Tnu/nu mice were determined to be 13.9 ± 0.9% and 13.5 ± 0.8% of total lymphocytes, respectively (Fig. 7). Similar to WT mice, adoptively transferred Tnu/nu mice, compared with sham, demonstrated worsened kidney function, as indicated by increased serum creatinine (0.1 ± 0 versus 1.8 ± 0.51; \( p = 0.05 \)) during IRI (Fig. 8).

Effects of T lymphocyte adoptive transfer on lung apoptosis during ischemic AKI

As described in the preceding sections, we observed decreased markers of pulmonary apoptosis in Tnu/nu mice during kidney IRI, compared with WT controls. Adoptively transferred Tnu/nu mice demonstrated an increase in lung cleaved (activated) caspase-3 protein expression during kidney IRI, compared with sham (1 ± 0.2 versus 1 ± 1.77 ± 0.1; \( p = 0.05 \)), and similar to WT controls (1 ± 0.1 versus 1.84 ± 0.2; \( p = 0.05 \)), suggesting T lymphocyte-dependent lung apoptosis during ischemic AKI.

T lymphocyte-dependent lung microvascular barrier dysfunction during kidney IRI

We have previously described caspase-dependent lung apoptosis during ischemic AKI, which results in pulmonary microvascular barrier dysfunction (8). To determine the role of
T lymphocyte-mediated lung barrier leak during kidney IRI, we measured BAL total protein as a surrogate marker for lung injury in WT, T\textsuperscript{nu/nu}, and adoptively transferred T\textsuperscript{nu/nu} mice, compared with sham, during ischemic AKI. WT mice demonstrated an increase in BAL protein leak during IRI, compared with sham (48.7 ± 1.7 versus 75.8 ± 6.6; p < 0.05); however, this injury was not observed in T\textsuperscript{nu/nu} mice (23 ± 4 versus 42.3 ± 9.4; p = 0.1). Adoptive transfer of T lymphocytes restored lung microvascular barrier dysfunction with increased BAL protein leak (48 ± 5.4 versus 87.8 ± 3.3; p < 0.05), implicating T lymphocytes as important mediators of lung functional injury during ischemic AKI.

**Discussion**

Despite advances in renal replacement therapy, AKI remains a significant predictor of mortality, likely because of its deleterious effects on distant organs. AKI alters the host innate and adaptive immune response, and experimental data have identified both soluble and cellular mediators of organ crosstalk activated by the posts ischemic kidney (4, 20). Organ crosstalk involves a complex interplay between numerous biochemical, cellular, and tissue-specific factors that incite remote pulmonary proinflammatory and proapoptotic signaling (6, 8, 14). Focusing on molecular pathways, we have identified caspase-dependent pulmonary apoptosis occurring through a TNFR1-dependent pathway during ischemic AKI.
AKI (8). Considerable data also implicate the innate immune response in local kidney IRI, stemming from the production of oxygen free radicals, secretion of inflammatory cytokines, activation of ECs, and recruitment of polymorphonuclear cells (21). Recent studies also identify T lymphocytes, particularly of the CD4+ phenotype, as early modulators of the injury response to IRI in the kidney, challenging their traditional role as mediators of adaptive immunity and “innocent bystanders” during acute inflammation (13–15).

In this study, we sought to characterize specific populations of infiltrating T lymphocytes in the lung and identify their potential role in mediating pulmonary apoptosis during ischemic AKI. In a murine model of kidney IRI-induced distant organ dysfunction, we have identified (1) pulmonary infiltration of activated CD3+ T lymphocytes; (2) specific subpopulations of T cells, primarily of the CD8+ phenotype, trafficking to the lungs; and (3) T lymphocyte-dependent pulmonary apoptosis and microvascular barrier dysfunction, which may occur independently of local TNF-α production; and finally, we found that adoptive transfer of CD3+ T lymphocytes restored the pulmonary proapoptotic and injury phenotype after ischemic AKI.

T lymphocytes have previously been implicated in various models of direct organ ischemic injury, including the brain (22), lung (23), heart (24), intestine (25), liver (26), and kidney (15). Previous research has implicated CD3+CD4+ T lymphocytes of the Th1 phenotype in mediating renal injury during direct IRI. CD4+ knockout mice, but not CD8+ knockout mice, were protected from kidney injury during IRI, and adoptive transfer of CD4+ T lymphocytes restored phenotypic and functional injury (15). The pathogenic effect is mediated by INF-γ-producing CD4+ cells, and adenosine 2A receptor agonists mediate their renal-protective effect through this specific T lymphocyte population (27). Additional investigations in STAT4-deficient mice have also identified CD4+ T lymphocytes of the Th1 phenotype as pathogenic, whereas CD4+ cells of the Th2 phenotype are renal protective in models of direct kidney IRI (28).

We identified infiltration of activated CD3+CD8+ T lymphocytes into the lungs at 24 h during kidney IRI, which correlates with the onset of microvascular barrier dysfunction and pulmonary injury previously described (8). These infiltrating cells were primarily of the CD3+CD8+ phenotype; however, both CD3+ and CD8+ T lymphocytes displayed increased CD69 and CD25 activation markers. As CD69 is typically induced within 2–24 h of activation on the surface of T cells, we expected an increase within our 24-h time point (29). Prior research has shown that CD69 increases early (within 4 h) during sham and IRI in CD3+ T lymphocytes infiltrating into the kidney; and in the lung, we found infiltrating T lymphocytes express CD69 as well, though specifically in animals undergoing IRI at 24 h (14). This activated population of CD3+CD8+ lymphocytes differs from those infiltrating the kidney during IRI, presenting an intriguing topic for future research.

Further investigative studies are needed to understand the function of CD8+ T lymphocytes in mediating pulmonary injury during ischemic AKI. CD8+ T cells are generally implicated in direct cytotoxicity; however, they typically require costimulation by either dendritic cells or CD4+ T cells to differentiate from naive to activated cytotoxic CD8+ T cells (30). In our model, we found infiltration of primarily CD8+ T lymphocytes into the lungs, but both CD4+ and CD8+ T cells expressed markers of activation, suggesting that CD4+ T cells may serve an important role in crosstalk by activating cytotoxic T lymphocytes in the lungs during ischemic
AKI. We identified increased CD69 expression on CD3+CD4+ lymphocytes early during IRI, suggesting that this population may serve as an important initial stimulant to activate cytotoxic T cells. Future experiments focusing on these specific roles may delineate mechanisms of crosstalk between T lymphocyte subsets during ischemic AKI.

Cytotoxic CD8+ T lymphocytes are also known to express TNF superfamily members such as TNF-α and FasL when activated. Although the current study did not identify increases in CD3+ CD8+ T cell expression of TNFR1 or TNF-α, our prior research has identified a prominent role for the TNF superfamily in the distant organ effects of kidney IRI (8, 19). In addition, we have identified TNFRI-dependent pulmonary apoptosis and increased levels of circulatory TNF-α during ischemic AKI (19). Despite no evidence of increased TNFR1 or TNF-α expression on infiltrating T cells, Tnu/nu mice demonstrated significant protection from pulmonary apoptosis and functional injury during ischemic AKI. Previous research has identified an increase in intracellular TNF-α on infiltrating T lymphocytes in the kidney during IRI, but the percentage of total cells expressing TNF-α constituted a mere 3.8% of total CD3+ cells (14). These low numbers could reflect low sensitivity of this assay by flow cytometry, or this finding may suggest that although circulatory TNF-α plays a predominant role in mediating TNFR-dependent pulmonary apoptosis, T cell-dependent apoptosis occurs through an alternative death receptor pathway. For example, Fas ligand has been identified as a key mediator of renal injury, and FasL-deficient mice exhibited fewer infiltrating TNF-α-producing T lymphocytes in the kidney and renal lymph nodes (31). Alternatively, TNFR1 may be selectively upregulated in pulmonary ECs independent of T cell TNFR1 expression.

To correlate lung apoptosis with pulmonary functional injury, we found that Tnu/nu mice were protected from lung microvascular barrier leak, and adoptive transfer of T lymphocytes restored the lung injury phenotype, implicating the T lymphocyte as an important mediator of lung microvascular barrier dysfunction during kidney IRI. The BAL protein results are limited in their significance, however, owing to small study groups in these animal experiments. Specifically, although Tnu/nu mice, compared with sham, demonstrated no significant increase in BAL protein leak during IRI. BAL leak was lower than in WT and adoptively transferred Tnu/nu mice, which may reflect the limited numbers of animals available for use in this experiment. The mechanisms behind lung protein leak during kidney IRI remain incompletely understood, but experimental data implicate the lung microvascular EC as a key target for ischemic AKI-induced inflammation, apoptosis, and microvascular barrier dysfunction. Our laboratory has shown that kidney IRI incites a distinct transcriptional and phenotypic response in lung microvascular ECs, resulting in cytoskeletal derangements, expression of proinflammatory and proapoptotic signals, and phenotypic apoptosis and microvascular barrier dysfunction (9). Potential crosstalk between activated T lymphocytes and lung microvascular ECs remains an intriguing area of research, and further experimental studies are needed to elucidate the mechanisms behind T lymphocyte-mediated lung microvascular barrier dysfunction.

In summary, our research has identified a unique and complex role for activated T lymphocytes in pulmonary trafficking and the induction of apoptosis during kidney IRI. Kidney–lung crosstalk during ischemic AKI represents a complex biological process involving a milieu of activated cellular and soluble mediators that incite remote organ injury. Future endeavors to characterize the intricate pathway to programmed cell death and the role that T lymphocytes play in this process may improve outcomes in this formidable clinical challenge.

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References


