TNFR1-dependent pulmonary apoptosis during ischemic acute kidney injury

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White LE, Santora RJ, Cui Y, Moore FA, Hassoun HT. TNFR1-dependent pulmonary apoptosis during ischemic acute kidney injury. Am J Physiol Lung Cell Mol Physiol 303: L449–L459, 2012. First published June 22, 2012; doi:10.1152/ajplung.00301.2011.—Despite advancements in renal replacement therapy, the mortality rate for acute kidney injury (AKI) remains unacceptably high, likely due to remote organ injury. Kidney ischemia-reperfusion injury (IRI) activates cellular and soluble mediators that incite a distinct pulmonary proinflammatory and proapoptotic response. Tumor necrosis factor receptor 1 (TNFR1) has been identified as a prominent death receptor activated in the lungs during ischemic AKI. We hypothesized that circulating TNF-α released from the postischemic kidney induces TNFR1-mediated pulmonary apoptosis, and we aimed to elucidate molecular pathways to programmed cell death. Using an established murine model of kidney IRI, we characterized the time course for increased circulatory and pulmonary TNF-α levels and measured concurrent upregulation of pulmonary TNFR1 expression. We then identified TNFR1-dependent pulmonary apoptosis after ischemic AKI using TNFR1−/− mice. Subsequent TNF-α signaling disruption with Etanercept implicated circulatory TNF-α as a key soluble mediator of pulmonary apoptosis and lung microvascular barrier dysfunction during ischemic AKI. We further elucidated pathways of TNFR1-mediated apoptosis with NF-κB (Complex I) and caspase-8 (Complex II) expression and discovered that TNFR1 proapoptotic signaling induces NF-κB activation. Additionally, inhibition of NF-κB (Complex I) resulted in a proapoptotic phenotype, lung barrier leak, and altered cellular flice inhibitory protein (C-FLIP) isoforms, suggesting that the balance between NF-κB and C-FLIP is important in mediating lung apoptosis after ischemic AKI. We hypothesized that TNFR1 triggers ischemic AKI-induced pulmonary apoptosis and aimed to elucidate specific molecular signals leading to programmed cell death. Here, we describe a time course for circulatory TNF-α activation and pulmonary TNFR1 expression and provide evidence for TNFR1-dependent apoptotic signaling in the lungs after kidney IRI. Additionally, by competitive inhibition of circulatory TNF-α with Etanercept, we characterize the critical role of TNF-α-mediated TNFR1 activation in mediating lung apoptosis and microvascular barrier dysfunction after ischemic AKI. In the pursuit of specific mechanisms of TNFR1-mediated apoptosis, we assayed for Complex I (NF-κB) and Complex II (Caspase-8) expression, which mediate prosurvival and proapoptotic pathways, respectively. We identified activation of NF-κB (Complex I) after ischemic AKI-induced pulmonary apoptosis without the induction of caspase-8 (Complex II) expression. Direct inhibition of NF-κB with Bay 11–7082 resulted in an apoptotic phenotype, lung microvascular barrier dysfunction, and altered expression of cellular flice inhibitory protein (C-FLIP) isoforms, suggesting that the balance between NF-κB (Complex I) and caspase-8 (Complex II) signaling may regulate ischemic AKI-induced pulmonary apoptosis and injury.

MATERIALS AND METHODS

Animal care. All procedures were approved by The Institutional Animal Care and Use Committee at The Methodist Hospital Research Institute for reprint requests and other correspondence: H. T. Hassoun, Johns Hopkins Medicine International, 1300 Thames St., Suite 200, Baltimore, MD 21231 (e-mail: hhassou1@jhmi.edu).

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Animals recovered with free access to food and water. In some IP, and the incision was closed in two layers with 4–0 silk suture. Atraumatic vascular clamp application. After 60 min, the clamps (if present) were removed. Animals underwent midline laparotomy with bilateral renal pedicle isolation, and, for those assigned to IRI, an atrumatic vascular clamp was applied across both renal pedicles for 60 min. Sham animals underwent identical procedures without vascular clamp application. After 60 min, the clamps (if present) were removed, animals were resuscitated with 1 l of warm sterile saline IP, and the incision was closed in two layers with 4–0 silk suture. Animals recovered with free access to food and water. In some experiments, a competitive inhibitor of soluble TNF-α, Etanercept (Enbrel; Immunex, Thousand Oaks, CA) 100 μg (0.2 ml, reconstituted in PBS per manufacturer’s instructions) was administered IP 15 h and 1 h before surgery. Etanercept dosing was determined based on prior murine models, which demonstrated TNF-α neutralization (1, 6, 10, 35). In another set of experiments, animals received 20 mg/kg Bay 11–7082 (Enzo Life Sciences, Plymouth Meeting, PA), an irreversible inhibitor of IκBα phosphorylation that prevents NF-κB activation and translocation into the nucleus, dissolved in 0.5% DMSO administered IP 30 min before IRI or sham laparotomy (29). Animals were killed at 4 or 24 h after the experimental procedure by exsanguination under general anesthesia, and tissues were collected for analysis.

Renal function. Upon death, inferior vena cava blood samples (~0.5 ml) were obtained from each animal and centrifuged at 3,000 revolution/min for 15 min for serum. Serum creatinine levels (mg/dl) were measured as a marker of renal function using an Ortho-Clinical Diagnostics VITROS 5.1 Fusion (Ortho-Clinical Diagnostics, Raritan, NJ). Peroxidase in situ lavage. Bronchoalveolar lavage (BAL) fluid analysis was performed as a surrogate measurement of pulmonary microvascular permeability as previously described (8, 9, 31). 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 stored on ice. Recovered BAL fluid underwent BCA total protein assay (Bio-Rad, Hercules, CA) according to the manufacturer’s protocol. Optical density readings of samples were converted to micrograms per milliliter using values obtained from standard curves generated with serial dilutions of BSA.

Assessment of lung apoptosis. Upon death, the right main bronchus was isolated and ligated. The left lung was filled with 0.5% low-melting agarose in 10% formalin at a constant pressure of 25 mmHg, allowing for homogeneous expansion of lung parenchyma. Inflated lungs were then fixed in 10% formalin for 48 h and embedded in paraffin blocks. Sections of paraffin-embedded tissues (5 μm) were obtained, and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays were performed with an ApopTag Peroxidase In Situ Apoptosis Detection Kit following manufacturer’s instructions (Millipore, Billerica, MA). Briefly, after deparaffinization and rehydration, slides were treated with 20 μg/ml proteinase K for 15 min, immersed in equilibration buffer (75 μL/5 cm2) and incubated at room temperature for 30 min. Slides were then treated with 55 μL/5 cm2 of TdT enzyme at 37°C for 1 h, washed with buffer, and incubated with anti-digoxigenin conjugate (65 μL/5 cm2) for 30 min at room temperature. Color was developed with peroxidase substrate (75 μL/5 cm2), methyl green counterstain (0.5% wt:vol) was applied, and coverslips were mounted on slides. Measurements of TUNEL-positive nuclei were performed by counting the average number of positive cells on 10 images/slide (40× magnification) captured by an independent observer blinded to the experiment using a Zeiss microscope, and digital images were saved.

Active (cleaved) caspase-3 immunohistochemistry (IHC) was performed on paraffin-embedded, formalin-fixed mouse lungs. Deparaffinization was performed with xylene, sections were rehydrated with distilled water, and then antigen retrieval was performed with high-pH unmasking solution (Vector Laboratories, Burlingame, CA) by steaming for 20 min followed by cooling at room temperature for another 20 min. Specimens were incubated with cleaved caspase-3 (Asp175) antibody (Cell Signaling Technology, Danvers, MA) in a 1:100 dilution at room temperature for 1 h followed by ImmPRESS anti-rabbit IgG polymer detection kit secondary antibody (Vector Laboratories) for 20 min at room temperature. Staining was completed by 2-min incubation with ImmPACT diaminobenzidine peroxidase substrate at room temperature. Measurements of active caspase-3-positive cells were performed on 10 images/slide (40× magnification) captured by an independent observer blinded to the experiment. Results for both TUNEL and caspase-3 IHC are represented as mean fold change (FC) relative to wild-type (WT) sham.

Measurement of serum TNF-α levels. Circulatory and whole lung homogenate TNF-α production was analyzed with the ELISA MAX Deluxe Set (BioLegend, San Diego, CA) per manufacturer’s instructions. Briefly, lung tissue was homogenized after isolation as previously described, and serum and lung homogenate samples underwent ELISA assay for TNF-α determination (9). Absorbance was measured with a Bio-TEK Synergy HT plate reader (Bio-TEK Instruments, Winooski, VT), and results are expressed in pg/ml.

Protein expression by immunoblot. Caspase-8 is the initiator caspase activated by Complex II formation in the cytosol; therefore, protein expression of cytosolic caspase-8 (Complex II) was determined with Western immunoblotting. Lung tissue homogenates were fractionated into nuclear and cytosolic portions using the Nuclear/Cytosol Fractionation Kit (BioVision, Mountain View, CA) per manufacturer’s instructions. Total protein was determined in these cytosolic fractions using the Bradford assay and then quantitated by the Bio-Rad Laboratories) according to the manufacturer’s instructions. The primer sets were tested over a temperature gradient for efficiency and specificity and verified over three orders of magnitude of a dilution series of mRNA for linearity. The primers used, designed by PrimerBank, and the RT-PCR conditions were as
follows: TNFR1 primers: 5′-CCG GGA GAA GAG GGA TAG CTT-3′ and 5′-TCG GAC AGT CAC TCA CCA AGT-3′, Tm=59.9°C; β-actin primers: 5′-GGCTGTATTTCCCTCCATCG-3′ and 5′-CCAGTGGTAAACATGCGCATG-3′, Tm=65.5°C. The relative expression levels of each mRNA were calculated using the 2−ΔΔCt method normalizing to β-actin and relative to the control samples. The presence of one product of the correct size was verified by 1.5% agarose gel electrophoresis.

Determination of NF-κB activation. Complex I formation leads to NF-κB activation and translocation of p65 into the nucleus. The total protein in the nuclear fraction of lung tissue homogenate was measured using the Bradford protein assay and quantitated by Western blot as described above utilizing the NF-κB p65 rabbit monoclonal IgG (1:10,000 dilution in tPBS; Santa Cruz Biotechnology).

Statistical analysis. For comparison within groups, a paired Student’s t-test was performed between sham vs. IRI. P values <0.05 were considered significant, with data expressed as means ± SE. To compare creatinine, TUNEL, and cleaved caspase-3 IHC results across groups, data were analyzed by SPSS Statistics 17 software (17.0.0; SPSS, Somers, NY) with the Mann-Whitney U-test and 95% confidence interval (data did not meet criteria for ANOVA). Data with P < 0.05 (2-tailed) were considered statistically significant, with Western blot analyses, TUNEL, and cleaved caspase-3 IHC values expressed as mean FC ± SE and absolute values (AV) listed in the figure legends.

RESULTS

Renal function after experimental ischemic AKI. We measured serum creatinine (mg/dl) at 4 and 24 h after sham and kidney IRI to correlate local ischemic kidney damage with distant organ pulmonary effects. Compared with sham, creatinine was increased at 4 h (0.2 ± 0 vs. 0.72 ± 0.1, P = 9.95 × 10−7) and 24 h (0.18 ± 0.02 vs. 1.94 ± 0.17, P = 0.01) after kidney IRI in WT mice (Fig. 1).

Effect of ischemic AKI on lung apoptosis. TUNEL assays and cleaved (active) caspase-3 IHC staining techniques were used to assess cellular apoptosis and caspase-3 activation after ischemic AKI (Fig. 1). Kidney IRI did not produce a change in the number of TUNEL-positive cells at 4 h compared with sham (FC = 1.0 ± 0.15 vs. 1.69 ± 0.36, P = 0.13) controls. However, there were more TUNEL-positive cells after IRI at 24 h (FC = 1.46 ± 0.31 vs. 12.9 ± 2.1, P = 0.008) compared with sham. Of note, there was no difference between sham groups at either 4 or 24 h after kidney IRI.

Cleaved (active) caspase-3, the effector caspase in the pro-apoptotic caspase cascade, was measured at 24 h to corroborate with the onset of TUNEL positivity (Fig. 1). Ischemic AKI increased lung cleaved caspase-3-positive cells (FC = 1 ± 0.11 vs. 2.96 ± 0.64, P = 0.02) compared with sham, indicating that induction of apoptosis after ischemic AKI correlates with the proapoptotic pulmonary phenotype seen by TUNEL staining.

Effect of ischemic AKI on TNF-α and TNFR1 expression. Cytokine TNF-α regulates many biological processes after injury and inflammation, and, because our previous work
Fig. 2. Effect of ischemic acute kidney injury (AKI) on TNF-α levels and pulmonary TNF receptor (TNFR) 1 expression. A: serum TNF-α levels measured by ELISA (pg/ml) increased after kidney IRI at 2 h (20.1 ± 2.3 vs. 47.5 ± 6.9*, P = 0.04) and at 4 h (18.5 ± 2.5 vs. 56.4 ± 5.4*, P = 9.16 × 10⁻⁴), but not at 24 h (23.7 ± 4.3 vs. 38.6 ± 9.0, P = 0.16) compared with sham. B: lung tissue TNF-α levels did not increase after kidney IRI at either 4 h (51.6 ± 12.0 vs. 72.4 ± 7.2, P = 0.16) or 24 h (71.1 ± 23.5 vs. 51.6 ± 7.3, P = 0.47) compared with sham controls. C: RT-PCR confirmed no increase in lung TNF-α expression at either 4 (1 ± 0.1 vs. 0.98 ± 0.1, P = 0.5) or 24 h (1 ± 0.3 vs. 1.1 ± 0.09, P = 0.6) h of ischemic AKI. n = 5/group. D: relative fold change for WT lung TNFR1 gene expression by quantitative RT-PCR demonstrated increased expression at 4 h (FC = 1 ± 0.05 vs. 1.34 ± 0.11*, AV = 0.48 ± 0.02 vs. 0.65 ± 0.05*, P = 0.03) and 24 h (FC = 1 ± 0.19 vs. 1.81 ± 0.25*, AV = 0.41 ± 0.08 vs. 0.75 ± 0.1*, P = 0.03) compared with sham. *P < 0.05 vs. sham.

**Fig. 2.**

**Effect of ischemic acute kidney injury (AKI) on TNF-α levels and pulmonary TNF receptor (TNFR) 1 expression.**

**A:** Serum TNF-α levels measured by ELISA (pg/ml) increased after kidney IRI at 2 h (20.1 ± 2.3 vs. 47.5 ± 6.9*, P = 0.04) and at 4 h (18.5 ± 2.5 vs. 56.4 ± 5.4*, P = 9.16 × 10⁻⁴), but not at 24 h (23.7 ± 4.3 vs. 38.6 ± 9.0, P = 0.16) compared with sham. **B:** Lung tissue TNF-α levels did not increase after kidney IRI at either 4 h (51.6 ± 12.0 vs. 72.4 ± 7.2, P = 0.16) or 24 h (71.1 ± 23.5 vs. 51.6 ± 7.3, P = 0.47) compared with sham controls. **C:** RT-PCR confirmed no increase in lung TNF-α expression at either 4 (1 ± 0.1 vs. 0.98 ± 0.1, P = 0.5) or 24 h (1 ± 0.3 vs. 1.1 ± 0.09, P = 0.6) h of ischemic AKI. **D:** Relative fold change for WT lung TNFR1 gene expression by quantitative RT-PCR demonstrated increased expression at 4 h (FC = 1 ± 0.05 vs. 1.34 ± 0.11*, AV = 0.48 ± 0.02 vs. 0.65 ± 0.05*, P = 0.03) and 24 h (FC = 1 ± 0.19 vs. 1.81 ± 0.25*, AV = 0.41 ± 0.08 vs. 0.75 ± 0.1*, P = 0.03) compared with sham. *P < 0.05 vs. sham.

**Fig. 3.**

**Effect of TNFR1−/− on pulmonary apoptosis after kidney IRI.** **A:** Kidney IRI produced lower lung TUNEL-positive cells at 24 h for TNFR1−/− mice compared with WT mice (FC = 4 ± 1.23 vs. 8.84 ± 1.44*, AV = 0.76 ± 0.23 vs. 1.68 ± 0.27*, P = 0.047), but no difference was seen during sham laparotomy (FC = 2 ± 0.39 vs. 1 ± 0.2, AV = 0.38 ± 0.07 vs. 0.19 ± 0.04, P = 0.056). **B:** Representative lung micrographs of TUNEL staining demonstrating decreased lung apoptosis after IRI in TNFR1−/− mice at 24 h.

**TNF-α signals primarily through TNFR1,** and we measured TNFR1 gene expression in whole lung tissue homogenate at 4 and 24 h to correlate the proapoptotic pulmonary phenotype with TNFR1 receptor activation (Fig. 2). Kidney IRI produced increased lung TNFR1 gene expression by relative FC at 4 h (1 ± 0.05 vs. 1.34 ± 0.11, P = 0.03) and at 24 h (1 ± 0.19 vs. 1.81 ± 0.25, P = 0.03) compared with sham.

**TNFR1-dependent pulmonary apoptosis after ischemic AKI.** TNFR1 activation by TNF-α initiates both proapoptotic (Complex II) and antiapoptotic/proinflammatory (Complex I) pathways (28). Because WT animals exhibit increased pulmonary TNFR1 transcription after kidney IRI, we examined the pulmonary response in TNFR1−/− animals after AKI.

Pulmonary apoptosis after ischemic AKI was measured with TUNEL staining (Fig. 3). Kidney produced fewer TUNEL-positive cells at 24 h in TNFR1−/− mice compared with WT mice (FC = 4 ± 1.23 vs. 8.84 ± 1.44, P = 0.047); however,

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**Effect of TNFR1−/− on pulmonary apoptosis after kidney IRI.** **A:** Kidney IRI produced fewer lung TUNEL-positive cells at 24 h for TNFR1−/− mice compared with WT mice (FC = 4 ± 1.23 vs. 8.84 ± 1.44*, AV = 0.76 ± 0.23 vs. 1.68 ± 0.27*, P = 0.047), but no difference was seen during sham laparotomy (FC = 2 ± 0.39 vs. 1 ± 0.2, AV = 0.38 ± 0.07 vs. 0.19 ± 0.04, P = 0.056). **B:** Representative lung micrographs of TUNEL staining demonstrating decreased lung apoptosis after IRI in TNFR1−/− mice at 24 h.
there was no difference between TNFR1−/− and WT mice following sham laparotomy (FC = 2 ± 0.39 vs. 1 ± 0.2, P = 0.056).

Renal function was worse after 24 h of kidney IRI for both WT (0.18 ± 0.02 vs. 1.94 ± 0.17, P = 0.007) and TNFR1−/− mice (0.22 ± 0.02 vs. 1.22 ± 0.38, P = 0.009) compared with sham. Of note, TNFR1−/− mice provided no local protection from ischemic AKI, with a similar extent of kidney injury in TNFR1−/− and WT mice after IRI (1.94 ± 0.17 vs. 1.22 ± 0.38, P = 0.17). There was no difference between WT and TNFR1−/− sham groups (0.18 ± 0.02 vs. 0.22 ± 0.02, P = 0.18).

Lung TNFR1 signaling after ischemic AKI. Lung homogenates were fractionated and protein expression of cytosolic caspase-8 (Complex II) and nuclear NF-κB (Complex I) was determined with Western blot (Fig. 4). No difference in caspase-8 (Complex II) expression was demonstrated at 24 h for WT (1 ± 0.03 vs. 1.04 ± 0.06, P = 0.21) or TNFR1−/− (1 ± 0.13 vs. 1.2 ± 0.32, P = 0.37) groups after kidney IRI compared with sham. However, WT mice exhibited an increase in nuclear p65 (activated) NF-κB expression (1 ± 0.2 vs. 1.9 ± 0.1, P = 0.005) at 24 h after kidney IRI. TNFR1−/− mice demonstrated decreased NF-κB expression compared with WT mice (1.9 ± 0.1 vs. 1.07 ± 0.11†, P = 0.047), suggesting that Complex I activation is favored by TNFR1-mediated signaling after kidney IRI.

Inhibition of TNF-α-TNFR1 binding attenuates pulmonary apoptosis and lung injury after kidney IRI. Animals receiving Etanercept, a competitive inhibitor of soluble TNF-α, or vehicle underwent either 60 min of bilateral renal pedicle ischemia or sham laparotomy as described, and assays for renal function, pulmonary apoptosis, lung barrier leak, and Complex I vs. Complex II formation were performed.

Kidney IRI produced TUNEL-positive cells at 24 h in mice treated with Etanercept (FC = 0.63 ± 0.16 vs. 2.47 ± 0.53, P = 0.008) although the extent of TUNEL positivity was less than vehicle-treated mice (FC = 2.47 ± 0.53 vs. 8.84 ± 1.44, P = 0.009) (Fig. 5). The increase in cleaved caspase-3 expression seen in vehicle mice (FC = 1 ± 0.11 vs. 2.96 ± 0.64, P = 0.02) was not evident in Etanercept mice, with no difference compared with sham (FC = 0.84 ± 0.10 vs. 1.78 ± 0.37, P = 0.12). Similar to vehicle animals, Etanercept groups demonstrated an increase in creatinine after 24 h of ischemic AKI (FC = 0.20 ± 0.03 vs. 2.12 ± 0.17, P = 0.008).

Focusing on molecular pathways of TNFR1-mediated apoptosis, we aimed to determine whether TNF-α-TNFR1 signaling promoted Complex I or Complex II formation after ischemic AKI. Lung tissue homogenate from Etanercept-treated animals was analyzed for nuclear Complex I (NF-κB) and cytosolic Complex II (Caspase-8) activation. Lung NF-κB expression was decreased in Etanercept-treated mice compared with vehicle-treated mice during kidney IRI (1.17 ± 0.08 vs. 1.9 ± 0.1, P = 0.008), suggesting that pulmonary apoptosis after ischemic AKI is associated with Complex I activation initiated by TNF-α-TNFR1 signaling. Kidney IRI produced no change in caspase-8 (Complex II) expression in Etanercept-treated mice compared with vehicle (1.42 ± 0.03 vs. 1.57 ± 0.16, P = 0.41).

To establish a potential direct role for TNF-α/TNFR1 signaling-induced pulmonary apoptosis and lung microvascular barrier dysfunction, we measured BAL protein levels (μg/ml) in vehicle-treated and Etanercept-treated mice 24 h after ische-

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**Fig. 4.** Lung TNFR1 signaling after ischemic AKI. A and B: no difference in caspase-8 expression (Complex II) was demonstrated after kidney IRI in WT vs. TNFR1−/− animals (FC = 1.04 ± 0.06 vs. 1.2 ± 0.32, P = 0.37). C and D: lung NF-κB p65 (Complex I) expression was decreased in TNFR1−/− animals compared with WT (FC = 1.9 ± 0.1 vs. 1.07 ± 0.11†, P = 0.047) after ischemic AKI. Compared with sham, WT animals had an increased in lung NF-κB expression after ischemic AKI (FC = 1 ± 0.2 vs. 1.9 ± 0.1*; AV = 0.26 ± 0.05 vs. 0.51 ± 0.03*; P = 0.005). *P < 0.05 for IRI vs. sham, †P < 0.05 for WT vs. TNFR1−/−.
mic AKI. Vehicle-treated mice demonstrated an increase in BAL protein after IRI compared with sham (48.7 ± 1.7 vs. 75.8 ± 6.6, \( P = 0.01 \)), whereas Etanercept-treated mice had BAL protein levels after IRI, which did not differ from sham controls (35.7 ± 5.6 vs. 54.6 ± 8.4, \( P = 0.08 \)). These results suggest that TNFRI-dependent pulmonary apoptosis is an important mediator of lung microvascular barrier injury during ischemic AKI.
NF-κB inhibition augments lung apoptosis. WT mice were treated with Bay 11–7082 (Bay 11), an irreversible inhibitor of NF-κB activation, or vehicle to correlate Complex I activation with the proapoptotic pulmonary response. Efficacy of NF-κB inhibition with Bay-11 was confirmed with p65 Western immunoblotting, which demonstrated a decrease in nuclear NF-κB expression in Bay-11 groups compared with vehicle (1.63 ± 0.06 vs. 0.08 ± 0.05, \( P = 2.8 \times 10^{-4} \)) during ischemic AKI (Fig. 6). Caspase-8 (Complex II) activation remained equivocal, with no change in caspase-8 expression after 24 h of kidney IRI in Bay-11 groups (1.04 ± 0.06 vs. 0.98 ± 0.28, \( P = 0.96 \)) compared with vehicle.

Whereas inhibition of TNF-α/TNFR1 signaling with Etanercept decreased lung apoptosis and NF-κB expression during ischemic AKI, direct NF-κB (Complex I) inhibition produced an apoptotic phenotype (Fig. 7). Similar to vehicle controls, Bay-11-treated groups demonstrated a significant increase in TUNEL-positive lung cells after 24 h of kidney IRI (FC = 0.79 ± 0.12 vs. 6.2 ± 1.7, \( P = 0.009 \)). Additionally, mice receiving Bay-11 expressed more cleaved caspase-3-positive cells after 24 h of ischemic AKI compared with vehicle (FC = 6.13 ± 0.70 vs. 2.96 ± 0.64, \( P = 0.03 \)). Renal function was significantly worse after 24 h of kidney IRI for both vehicle-treated (0.18 ± 0.02 vs. 1.94 ± 0.17, \( P = 0.007 \)) and Bay-11-treated mice (0.14 ± 0.02 vs. 1.94 ± 0.07, \( P = 0.007 \)) compared with sham.

To correlate the apoptotic phenotype with lung functional injury, we measured BAL protein in vehicle-treated and Bay-11-treated mice 24 h after ischemic AKI. There was no difference between Bay-11-treated and vehicle-treated mice after IRI (75.8 ± 6.6 vs. 53.1 ± 8.4, \( P = 0.09 \)), again correlating the apoptotic phenotype to lung microvascular barrier dysfunction after ischemic AKI. Similar to vehicle-treated mice (48.7 ± 1.7 vs. 75.8 ± 6.6, \( P = 0.01 \)), Bay-11-treated mice demonstrated an increase in BAL protein after IRI compared with sham (21.1 ± 2.4 vs. 53.1 ± 8.4, \( P = 0.006 \)). However, Bay-11-treated animals also had decreased BAL protein after sham laparotomy compared with vehicle-treated groups (48.7 ± 1.7 vs. 21.1 ± 2.4, \( P = 6 \times 10^{-4} \)).

**DISCUSSION**

Despite advancements in renal replacement therapy, AKI remains a significant predictor of mortality. 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 postischemic kidney (16, 21, 33). Despite a clear clinical correlation between AKI and ALI, little is known about the pathophysiology of kidney-lung crosstalk after AKI. We have previously identified caspase-dependent pulmonary endothelial cell apoptosis resulting in microvascular barrier dysfunction after 24 h of ischemic AKI (3, 8, 9). Our laboratory is presently characterizing the pulmonary endothelial cell-specific phenotypical, functional, and transcriptional response to ischemic AKI both in vivo and in vitro in an attempt to understand how this cell-specific response can influence lung dysfunction after kidney IRI.

In this present study, we have taken a mechanistic approach to determining the predominant pathway to pulmonary apoptosis activated after ischemic AKI. In a murine model of kidney IRI-induced lung dysfunction, we have identified that 1) ischemic AKI induces circulatory TNF-α activation and pulmonary TNFR1 expression, 2) pulmonary apoptosis after ischemic AKI occurs in a TNFR1-dependent manner, 3) TNFR1 signaling disruption diminishes the onset of pulmonary apoptosis and microvascular barrier dysfunction, 4) TNFR1 proapoptotic signaling disruption diminishes the onset of pulmonary apoptosis and microvascular barrier dysfunction, 5) TNFR1 proapoptotic signaling disruption diminishes the onset of pulmonary apoptosis and microvascular barrier dysfunction, 6) TNFR1 proapoptotic signaling disruption diminishes the onset of pulmonary apoptosis and microvascular barrier dysfunction.
Fig. 7. Effect of NF-κB inhibition on pulmonary apoptosis and injury. A: kidney IRI induced TUNEL positivity in lungs of both vehicle-treated and Bay 11-treated mice (FC = 8.84 ± 1.44 vs. 6.2 ± 1.7, AV = 1.68 ± 0.27 vs. 1.18 ± 0.33, P = 0.402), both of which were increased compared with sham. B: representative lung micrographs of TUNEL staining at 24 h exhibit increased apoptosis in both vehicle and Bay-11 groups. C: kidney IRI induced more caspase-3 positivity in Bay-11-treated groups compared with vehicle-treated groups (FC = 2.96 ± 0.64 vs. 6.13 ± 0.70†, AV = 1.63 ± 0.35 vs. 3.37 ± 0.38, P = 0.03), both of which were increased compared with sham. D: representative lung micrographs of cleaved caspase-3 immunohistochemistry show increased cleaved caspase-3-positive cells in both vehicle and Bay-11 groups after IRI. E: BAL protein was measured at 24 h in vehicle-treated and Bay-11-treated mice following sham or ischemic AKI. Similar to vehicle-treated mice (48.7 ± 1.7 vs. 75.8 ± 6.6*, P = 0.01), Bay-11-treated mice demonstrated increased BAL protein leak during kidney IRI compared with sham (21.1 ± 2.4 vs. 53.1 ± 8.4*, P = 0.006). Whereas there was no difference in BAL protein leak following IRI between vehicle-treated and Bay-11-treated mice (75.8 ± 6.6 vs. 53.1 ± 8.4, P = 0.09), Bay-11 mice had decreased BAL protein after sham compared with vehicle-treated groups (48.7 ± 1.7 vs. 21.1 ± 2.4†, P = 6.005 × 10⁻⁵), n ≥ 5/group, *P < 0.05 for IRI vs. sham, †P < 0.05 for vehicle vs. Bay-11.
signaling induces NF-κB (Complex I) activation, and 5) direct NF-κB inhibition augments caspase-3 activation, the proapoptotic phenotype, and lung microvascular injury.

The potential mediators implicated in this distant organ response remain unknown but may include the downregulation of pulmonary epithelial Na-K-ATPase and aquaporin 5 (25), the activation of proinflammatory cytokines such as IL-6 (14), and macrophage trafficking and activation that may confer proinflammatory pulmonary damage after ischemic AKI (19). Prior research identified TNF-α, a key cytokine that regulates numerous biological pathways including inflammation, proliferation, differentiation and cell death, in models of both local kidney damage and distant organ dysfunction after IRI (2, 13, 24, 27). Increased serum TNF-α after ischemic AKI has previously been reported by other investigators (13), and we have demonstrated the early activation of serum TNF-α with concurrent early and sustained activation of the lung TNFR1 receptor. This implicates the release of TNF-α from the postischemic kidney as a soluble mediator of organ crosstalk.

The role of local pulmonary TNF-α production after ischemic AKI remains unknown. We have previously developed techniques to isolate lung microvascular endothelial cells in vivo, and RT-PCR arrays identified an increase in TNF-α production after kidney IRI compared with sham (3). However, in this study, we found no increase in TNF-α of whole lung homogenate by ELISA or RT-PCR (data not identified). Activation of CD3+ T cells in vivo, and RT-PCR arrays identified an increase in TNF-α production after kidney IRI compared with sham (3). However, in this study, we found no increase in TNF-α of whole lung homogenate by ELISA or RT-PCR (data not included). Infiltrating lymphocytes could be another potential source of local TNF-α production in the lungs, and our laboratory has previously investigated the role of T cells as potential mediators of distant organ pulmonary injury after ischemic AKI (3, 15). These preliminary studies have identified trafficking of activated CD3+ T cells into the lungs after ischemic AKI, with a predominant CD8+ T cell population (22). Although these activated T cell populations were necessary for kidney IRI-induced lung apoptosis, we did not find increased expression of T cell TNF receptors or TNF-α, suggesting that T cell-mediated lung apoptosis may occur independently from local T cell TNF-α production after ischemic AKI (22).

TNF-α signaling in many organ injury models can trigger both a regenerative and cell-death response, and these pleiotropic effects are attributed to its ability to activate both apoptotic and survival pathways through the TNFR1 receptor (7). TNFR1 is a prominent death receptor pathway activated after inflammation and apoptosis, and we have previously identified robust AKI-induced TNFR1 pathway activation by global gene expression profiling (9). The TNFR1 receptor is critical to pulmonary apoptosis after ischemic AKI, with significantly fewer TUNEL-positive cells at 24 h in TNFR1−/− mice compared with sham. We hypothesize that the increase in caspase-3-positive cells in TNFR1−/− mice results from TNFR1-independent activation of caspase-3, perhaps to serve in a nonapoptotic function such as cellular inflammation (20). Inhibition of TNF-α-TNFR1 binding with Etanercept produced an attenuated proapoptotic phenotype that was not as complete as in TNFR1−/− mice. This would imply either incomplete TNF-α-TNFR1 binding blockade by Etanercept or potential TNFR1 receptor activation independent of TNF-α; further studies are needed to clarify this discrepancy. Despite these confounding data, pretreatment with Etanercept attenuated lung microvascular barrier dysfunction measured by BAL protein. We have previously correlated pulmonary apoptosis with lung microvascular barrier injury (9); however, this study highlights specific molecular mechanisms and implicates the critical role of TNFR1-mediated lung apoptosis in mediating lung injury following ischemic AKI.

TNFR1 signaling includes activation of two pathways: Complex I (TNFR1, TRADD, RIP, TRAF2, and c-IAP1) at the membrane, which results in NF-κB activation and prosurvival signals, and Complex II (FADD, procaspases 8 and 10) in the cytosol, which promotes the apoptotic cascade. TNFR1 activation initiates a complex signaling caspase between these two prosurvival and proapoptotic pathways, and the current paradigm suggests that Complex II (apoptotic) activation occurs depending on the balance between levels of NF-κB and C-FLIP isoform expression (28). Complex I activation produces TRAF1, TRAF2, and c-IAP1 and c-IAP2 induction, which suppress caspase-8 activation and thus apoptosis (32). In our present study, we have shown NF-κB activation independent of caspase-8 activation, yet this occurred concurrently with cellular apoptosis and activation of caspase-3, the executioner caspase. In both TNFR1−/− mice and in WT mice after treatment with Etanercept, ischemic AKI was associated with fewer TUNEL-positive cells and also decreased NF-κB activation. Furthermore, direct inhibition of NF-κB with Bay-11 resulted in an apoptotic phenotype with TUNEL-positive cells and activation of caspase-3. Therefore, the role of NF-κB activation in TNFR1-mediated lung apoptosis remains unknown, prompting the exploration of further downstream regulators of Complex I/Complex II signaling after ischemic AKI. This proapoptotic phenotype was associated with lung microvascular barrier dysfunction, further implicating lung apoptosis as a critical mediator of lung injury after ischemic AKI.

Caspase-8 serves as an initiator caspase in the caspase cascade and, when activated, cleaves (and activates) caspase-3, the main effector caspase that stimulates the caspase cascade and subsequent apoptosis. However, in our model, we have demonstrated signs of apoptosis and caspase-3 activity in the absence of caspase-8 activation. Several plausible explanations for caspase-8-independent caspase-3 activation include 1) difficulty in detecting initiator caspases by immunoblotting (12), 2) concurrent nondeath receptor-dependent pathways to apoptosis (such as the mitochondrial pathway) occurring after IRI, which also employ caspase-3, and 3) additional newly identified mediators of caspase-8-independent caspase-3 activation after TNFR1 proapoptotic signaling (4). Alternatively, caspase enzymes also serve nonapoptotic functions, such as processing of cytokines after inflammation and the proliferation of T cells. Therefore, the inability of Etanercept to completely suppress caspase-3 activation despite decreased phenotypic evidence of apoptosis may be due to nonapoptotic functions of caspase-3 (17).

The present study is limited without cell-specific assays to directly link apoptosis and lung injury with a certain cell type. However, we have previously identified lung microvascular endothelial cells undergoing apoptosis in vivo following ischemic AKI and hypothesize that these microvascular endothelial cells, with their critical role in maintaining the lung semipermeable barrier, are specific targets of lung apoptosis and injury after kidney IRI. In an effort to advance our understanding of
the pathophysiology of indirect lung injury after ischemic AKI, our laboratory has recently focused on identifying lung microvascular endothelial cell-specific changes in ischemic AKI utilizing parallel in vivo and in vitro methods. Ischemic AKI incites specific transcriptional and phenotypic alterations in lung microvascular endothelial cells, including activation of proinflammatory and proapoptotic genes, along with cytoskeletal rearrangement and apoptosis in vitro (3). RT-PCR arrays performed on isolated lung microvascular endothelial cells in vivo at 24 h of kidney IRI identified activation of genes related to the TNF superfamily, apoptosis, and TNF-α, which follows the same pattern as our data performed on whole lung tissue (3). Future endeavors of our laboratory remain focused on understanding the lung endothelial cell-specific response to ischemic AKI.

In summary, our research has identified a critical role for the TNFR1 receptor in pulmonary apoptosis after ischemic AKI. Kidney-lung crosstalk after kidney IRI represents a complex biological process activated by several key soluble and cellular mediators. Future endeavors to characterize the intricate balance between programmed cell death and cell survival in organ crosstalk may improve outcomes in this formidable clinical challenge.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


