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Interleukin-6 mediates lung injury following ischemic acute kidney injury or bilateral nephrectomy

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Patients with acute kidney injury frequently have pulmonary complications. Similarly ischemic acute kidney injury or bilateral nephrectomy in rodents causes lung injury characterized by pulmonary edema, increased pulmonary capillary leak and interstitial leukocyte infiltration. Interleukin-6 is a pro-inflammatory cytokine that is increased in the serum of patients with acute kidney injury and predicts mortality. Here we found that lung neutrophil infiltration, myeloperoxidase activity, the neutrophil chemokines KC and MIP-2 and capillary leak all increased within 4 h following acute kidney injury in wild-type mice. These pathologic factors were reduced in interleukin-6-deficient mice following acute kidney injury or bilateral nephrectomy. The lungs of mutant mice had reduced KC but MIP-2 was similar to that of wild type mice. Wild-type mice, treated with an interleukin-6 inactivating antibody, had decreased lung myeloperoxidase activity and KC levels following acute kidney injury. Our study shows that interleukin-6 contributes to lung injury following acute kidney injury.

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KEYWORDS: acute renal failure; interleukin-6; KC; CXCL1; MIP-2; CXCL2

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Acute kidney injury (AKI) is associated with an increased incidence of respiratory failure requiring mechanical ventilation, which greatly increases mortality.[1–4] The cause of respiratory failure in patients with AKI is incompletely understood. Using rodent models, we and others have previously demonstrated that lung injury occurs following ischemic AKI.[5–11] Approximately 50% of AKI in hospitalized patients is caused by renal ischemia;[12] therefore, understanding the remote organ effects of ischemic AKI is clinically relevant. However, lung injury also occurs after ischemia–reperfusion injury of other organs such as the liver, gut, and hind limb.[13–17] Bilateral nephrectomy is increasingly being utilized to isolate the effects of renal failure (for example, impaired clearance and metabolism) from those of renal ischemia; significant lung injury following bilateral nephrectomy has been reported by our group and others.[5,10,18] Interestingly, pulmonary edema, increased pulmonary capillary leak, and increased interstitial leukocytes are hallmarks of lung injury after bilateral nephrectomy as well as after ischemic AKI. In this study, we sought to identify facilitators of AKI-mediated lung injury using these two different models of AKI.

We have demonstrated previously that serum IL-6 increases after ischemic AKI and bilateral nephrectomy in mice.[5] In patients with AKI, IL-6 is significantly increased and is predictive of mortality.[19] Likewise, in patients with acute lung injury (ALI), circulating IL-6 is elevated and predicts mortality.[20,21] Impaired neutrophil migration in IL-6-deficient mice has been described in infectious and noninfectious models of lung inflammation.[22–24] Given this background, we hypothesized that circulating IL-6 plays a role in lung injury following AKI. Using methods to inhibit IL-6, we demonstrate that IL-6 contributes to lung injury following ischemic AKI and bilateral nephrectomy.

RESULTS

Lung myeloperoxidase activity after AKI

To determine the time course of lung injury after ischemic AKI and bilateral nephrectomy, myeloperoxidase (MPO) activity was measured in lung tissue from wild-type mice at baseline and at 1, 2, 4, and 24 h after sham operation, ischemic AKI, or bilateral nephrectomy. MPO activity

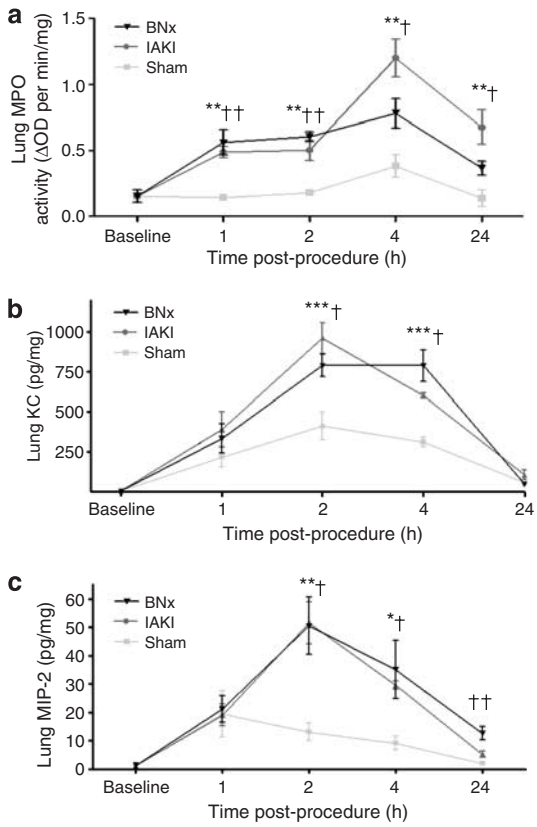

Figure 1 | Lung MPO activity, KC, and MIP-2 after ischemic AKI and bilateral nephrectomy. MPO activity (a marker for neutrophils) and KC and MIP-2 (neutrophil chemokines) were measured in lung tissue of wild-type mice at baseline and at 1, 2, 4, and 24 h after sham operation (Sham), ischemic AKI (IAKI), and bilateral nephrectomy (BNx). (**a**) MPO activity significantly increased at 1, 2, 4, and 24 h following IAKI and BNx compared with sham operation ($N = 3$–11). Chemokines (**b**) KC and (**c**) MIP-2 significantly increased at 2 and 4 h following IAKI and BNx compared with sham operation as measured by ELISA ($N = 3$–9). *$P < 0.05$ IAKI vs Sham, **$P < 0.01$ IAKI vs Sham, ***$P < 0.001$ IAKI vs Sham, $^{\dagger}P < 0.05$ BNx vs Sham, $^{\dagger\dagger}P < 0.01$ BNx vs Sham.

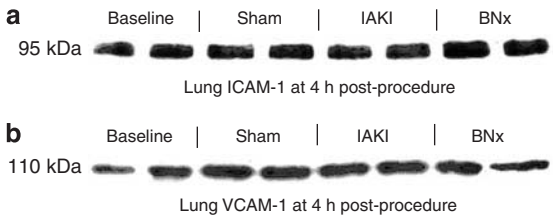

Figure 2 | Lung adhesion molecules after ischemic AKI and bilateral nephrectomy. Immunoblotting for the adhesion molecules ICAM-1 and VCAM-1 in lung tissue was performed at baseline and at 1, 2, 4, and 24 h after sham operation (Sham), ischemic AKI (IAKI), and bilateral nephrectomy (BNx). Both lung (**a**) ICAM-1 and (**b**) VCAM-1 were detected at baseline and at 4 h after sham operation, ischemic AKI, and bilateral nephrectomy; no detectable differences were observed among groups. Immunoblots are representative of at least three separate experiments.

significantly increased after ischemic AKI and bilateral nephrectomy compared with sham operation at all time points, peaking at 4 h (Figure 1a). MPO, the most abundant protein in neutrophils, is released by activated neutrophils after entry into sites of inflammation and is a key component of their microbicidal activity. MPO activity is widely used as a biochemical marker for tissue neutrophil content. Monocytes may contain a small amount of MPO due to synthesis or uptake of neutrophil-derived MPO.[25] MPO may have direct deleterious pulmonary effects, as severe acute lung injury resulting from intratracheal administration of MPO has been reported.[26]

Lung chemokines and adhesion molecules after AKI

To examine potential mediators of lung neutrophil recruitment, the neutrophil chemokines keritinocyte-derived chemokine (KC) (CXCL1) and macrophage inflammatory protein-2 (MIP-2) (CXCL2) were measured by ELISA in lung tissue of wild-type mice at baseline and at 1, 2, 4, and 24 h after sham operation, ischemic AKI, or bilateral nephrectomy. As shown in Figure 1b and c, both lung KC and MIP-2 increased at 2 and 4 h after ischemic AKI and bilateral nephrectomy compared with sham operation.

To further investigate potential mediators of lung neutrophil recruitment, immunoblotting for the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) was performed in lung tissue obtained from wild-type mice at baseline and at 1, 2, 4, and 24 h after sham operation, ischemic AKI, or bilateral nephrectomy. Both ICAM-1 and VCAM-1 were present in the lung at all time points after sham operation, ischemic AKI, and bilateral nephrectomy; however, expression was similar to that observed at baseline (no surgery) (Figure 2a and b). In addition, expression of lung ICAM-1 and VCAM-1 did not vary among the different time points in all surgical groups (data not shown). Equal protein loading was verified by membrane staining for all immunoblots (not shown).

Lung histology after AKI

To determine whether peak MPO activity 4 h after AKI corresponded to an increase in lung neutrophil recruitment and lung injury, lung histology was examined 4 h after sham operation, ischemic AKI, and bilateral nephrectomy. In contrast to the normal lung histology seen after sham operation, pulmonary histology after ischemic AKI and bilateral nephrectomy was characterized by increased vascular congestion, septal edema, and neutrophil infiltration (Figure 3). Interstitial neutrophil counts (per 50 high powered fields, 40× original magnification) were 17 ± 4 in sham, 98 ± 13 in ischemic AKI, and 150 ± 38 in bilateral nephrectomy ($P < 0.05$ ischemic AKI vs sham, $P < 0.05$ bilateral nephrectomy vs sham, $P =$ NS ischemic AKI vs bilateral nephrectomy, $N = 3$).

Pulmonary capillary leak after AKI

To determine whether increased lung MPO activity and lung neutrophil infiltration observed 4 h after ischemic AKI and

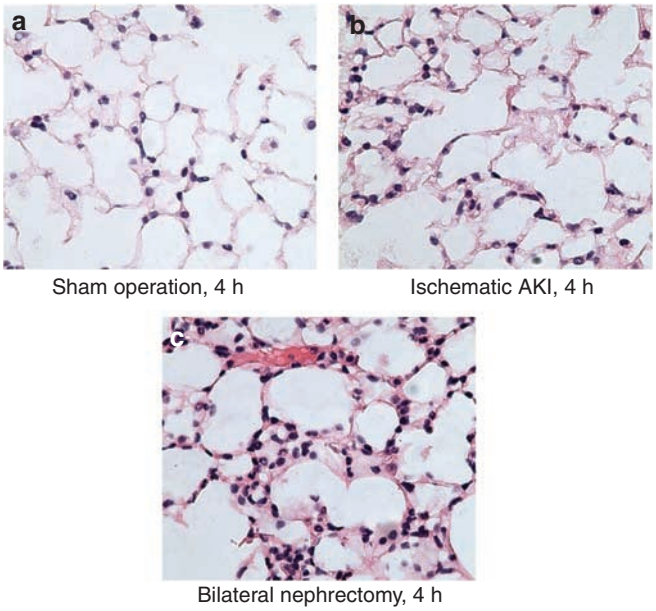

Figure 3 | Lung histology after ischemic AKI and bilateral nephrectomy. Pulmonary histology (hematoxylin and eosin, ×40) is normal 4 h after (**a**) sham operation in wild-type mice. Pulmonary histology 4 h after (**b**) ischemic AKI and (**c**) bilateral nephrectomy in wild-type mice is characterized by septal edema and neutrophil infiltration. Figures are representative of at least three separate experiments.

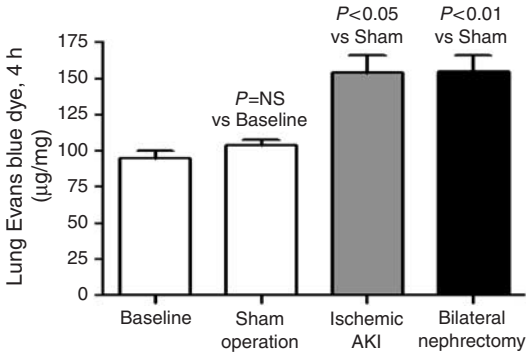

Figure 4 | Pulmonary capillary leak after ischemic AKI and bilateral nephrectomy. Evans blue dye (EBD) was administered via tail vein injection, and lung EBD content (an indicator of pulmonary capillary leak) was determined at baseline and at 4 h after sham operation, ischemic AKI, and bilateral nephrectomy in wild-type mice. EBD content increased after ischemic AKI and bilateral nephrectomy, indicating increased capillary leak ($N = 4$ for baseline, 7 for sham operation, 6 for ischemic AKI, and 10 for bilateral nephrectomy).

bilateral nephrectomy was associated with increased capillary leak, lung content of Evans blue dye (EBD) was determined at baseline and at 4 h after sham operation, ischemic AKI, and bilateral nephrectomy in wild-type mice. EBD was administered via tail vein injection, and lung EBD content was determined as an indicator of lung capillary leak. Lung EBD significantly increased in ischemic AKI and bilateral nephrectomy compared with sham operation (Figure 4).

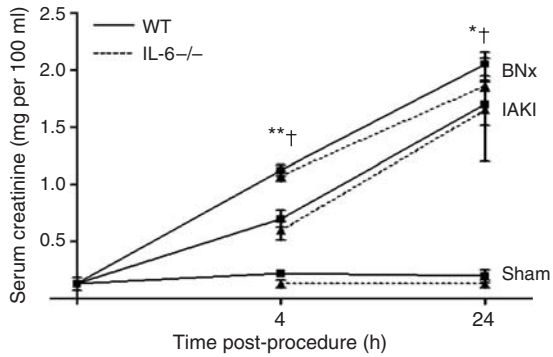

Figure 5 | Time course of renal failure after ischemic AKI and bilateral nephrectomy in wild-type (WT) and IL-6-deficient (IL-6−/−) mice. Serum creatinine was determined at baseline and at 4 and 24 h after sham operation (Sham), ischemic AKI (IAKI), and bilateral nephrectomy (BNx). Serum creatinine was unchanged following sham operation, but was increased at 4 and 24 h after ischemic AKI and bilateral nephrectomy. No significant difference in serum creatinine was observed between WT and IL-6−/− mice at any time point ($N = 3$–6). *$P < 0.01$ IAKI vs Sham, **$P < 0.001$ IAKI vs Sham, †$P < 0.001$ BNx vs Sham.

Wild-type and IL-6-deficient mice (IL-6−/−) develop AKI at similar rates after ischemic AKI or bilateral nephrectomy

To test the hypothesis that lung injury after AKI is interleukin-6 (IL-6) dependant, IL-6−/− mice were studied. As the peak of lung injury in wild-type mice was at 4 h, end points of lung injury were examined 4 h after ischemic AKI and bilateral nephrectomy in IL-6−/− mice.

To determine the rate of development of renal dysfunction in wild-type compared with IL-6−/− mice, serum creatinine was measured at baseline and at 4 and 24 h after sham operation, ischemic AKI, or bilateral nephrectomy. Serum creatinine significantly increased 4 and 24 h after ischemic AKI or bilateral nephrectomy in wild-type and IL-6−/− mice (Figure 5). There was no significant difference in the timing or degree of renal dysfunction between wild-type and IL-6−/− mice. Likewise, blood urea nitrogen was similar in wild-type and IL-6−/− mice after sham operation, ischemic AKI and bilateral nephrectomy (data not shown).

Serum IL-6 increases after ischemic AKI and bilateral nephrectomy in wild-type mice

Serum IL-6 was measured by ELISA 4 h after sham operation, ischemic AKI, and bilateral nephrectomy in wild-type and IL-6−/− mice. IL-6 in wild-type mice was significantly elevated after both ischemic AKI and bilateral nephrectomy compared with sham and was not detected in IL-6−/− mice (Figure 6).

Lung injury after AKI in IL-6−/− mice

Increased lung MPO activity demonstrated after ischemic AKI and bilateral nephrectomy in wild-type mice was significantly attenuated in IL-6−/− mice (Figure 7a). To determine whether improved MPO activity in IL-6−/− mice was associated with improved capillary leak, lung content of EBD was determined in wild-type and IL-6−/− mice 4 h after

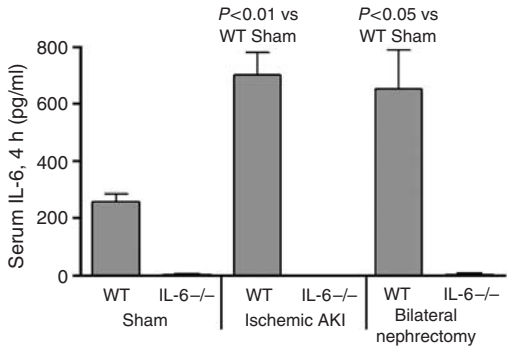

Figure 6 | Serum IL-6 after ischemic AKI and bilateral nephrectomy. Serum IL-6 was determined 4 h after sham operation, ischemic AKI, and bilateral nephrectomy in wild-type (WT) and IL-6-deficient (IL-6 −/−) mice. IL-6 was elevated after both ischemic AKI and bilateral nephrectomy compared with sham ($N = 3$–5). As expected, IL-6 was not detected in IL-6−/− mice ($N = 3$–5).

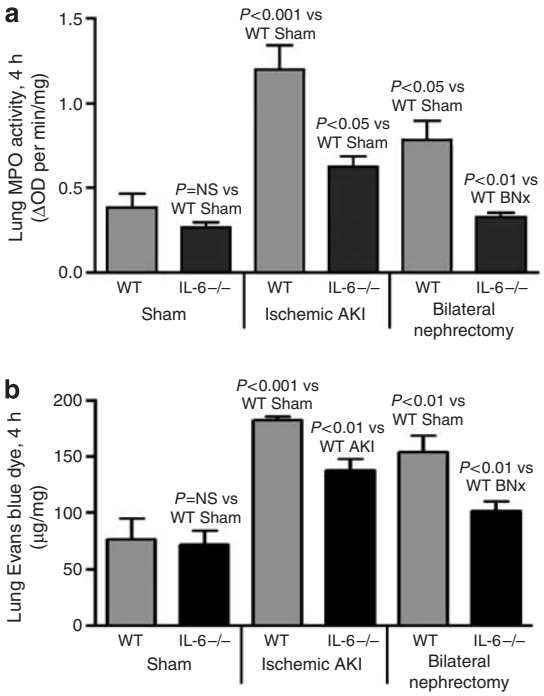

Figure 7 | Lung injury after AKI in IL-6−/− mice. (**a**) Lung MPO activity (a marker for neutrophils) was increased 4 h after ischemic AKI and bilateral nephrectomy in wild-type (WT) mice, and this increase was ameliorated in IL-6-deficient (IL-6−/−) mice ($N = 4$–6). (**b**) Lung EBD content (a marker of capillary leak) was attenuated in IL-6−/− mice with ischemic AKI or bilateral nephrectomy compared with wild type ($N = 5$–9).

ischemic AKI and bilateral nephrectomy. Reduced lung EBD, indicating reduced capillary leak, occurred in IL-6−/− mice after both ischemic AKI and bilateral nephrectomy (Figure 7b).

Lung chemokines after AKI in IL-6−/− mice

To investigate whether decreased lung MPO activity observed in IL-6−/− mice was associated with altered lung chemokine

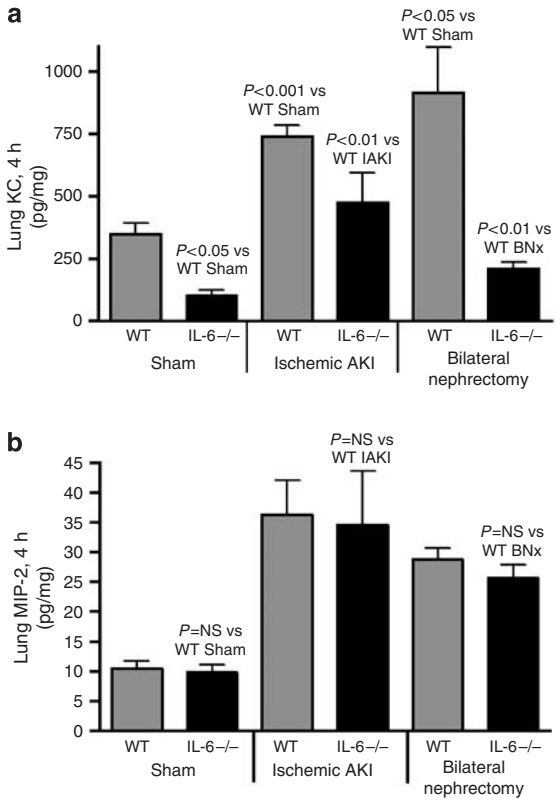

Figure 8 | Lung chemokines after AKI in IL-6−/− mice. Lung KC and MIP-2 were measured by ELISA 4 h after ischemic AKI and bilateral nephrectomy. (**a**) Lung KC was significantly reduced in IL-6-deficient (IL-6−/−) mice compared with wild-type (WT) after ischemic AKI and bilateral nephrectomy ($N = 3$–6). (**b**) Lung MIP-2 was similar in IL-6-deficient and wild-type mice after ischemic AKI and bilateral nephrectomy ($N = 4$–6).

expression, the neutrophil chemokines KC and MIP-2 were measured by ELISA in lung tissue 4 h after sham operation, ischemic AKI, and bilateral nephrectomy in wild-type and IL-6−/− mice. Lung KC was significantly reduced in IL-6−/− compared with wild-type mice after ischemic AKI and bilateral nephrectomy (Figure 8a). In contrast, no differences in lung MIP-2 were found in IL-6−/− compared with wild-type mice after AKI (Figure 8b).

Lung injury after AKI in wild-type mice administered with anti-IL-6 antibody

To test whether an intervention targeting IL-6 would ameliorate lung injury after AKI, 20 μg anti-IL-6 antibody vs IgG control was administered to wild-type mice 1 h before surgery, at the time of clamp removal (ischemic AKI) or nephrectomy (bilateral nephrectomy) and 1 h following surgery (60 μg total). Administration of anti-IL-6 antibody to wild-type mice significantly reduced pulmonary MPO activity 4 h after bilateral nephrectomy; a trend toward decreased MPO activity was observed in ischemic AKI (Figure 9a). Additionally, increases in lung KC after ischemic AKI and bilateral nephrectomy were significantly attenuated in wild-type mice treated with anti-IL-6 antibody vs IgG

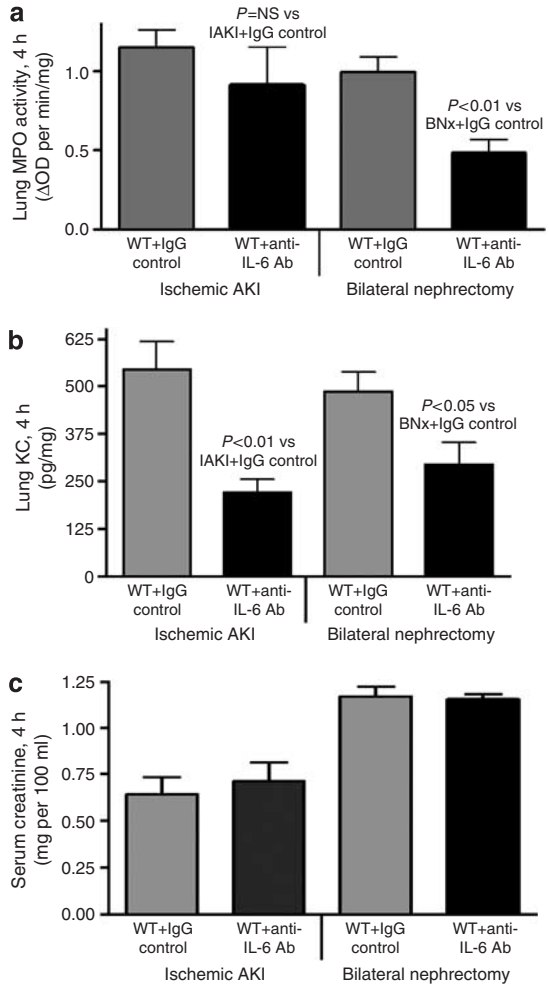

Figure 9 | Lung MPO activity and KC after administration of anti-IL-6 antibody to wild-type mice in AKI. (**a**) Pulmonary MPO activity 4 h after bilateral nephrectomy was decreased with anti-IL-6 antibody treatment. (**b**) The increase in lung KC 4 h after ischemic AKI or bilateral nephrectomy was significantly attenuated in wild-type (WT) mice administered with anti-IL-6 antibody vs IgG control ($N = 6$). (**c**) Serum creatinine was similar in wild-type mice treated with anti-IL-6 antibody or IgG control 4 h after ischemic AKI and bilateral nephrectomy ($N = 5$–7).

control (Figure 9b); no difference in lung MIP-2 was noted (data not shown). To determine if the improvement in lung injury was independent from effects of anti-IL-6 antibody on kidney function, serum creatinine was measured 4 h after ischemic AKI or bilateral nephrectomy and was found to be similar in mice administered with perioperative anti-IL-6 antibody vs IgG control (Figure 9c).

Lung MPO activity after intravenous administration of IL-6 to wild-type mice

To test whether intravenous administration of IL-6 could directly cause lung injury, 1 μg of recombinant murine IL-6 was administered by tail vein injection to wild-type mice (no surgery), which resulted in increased pulmonary MPO activity at 4 h compared with vehicle administration

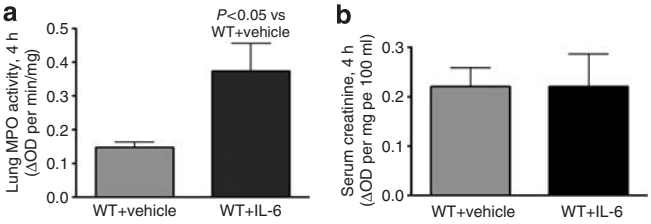

Figure 10 | Lung MPO activity after intravenous administration of IL-6 to wild-type mice. A total of 1 μg of recombinant murine IL-6 vs vehicle (sterile PBS) was administered via tail vein injection to wild-type (WT) mice. (**a**) At 4 h, lung MPO activity was significantly increased in mice administered with IL-6 vs vehicle. (**b**) Serum creatinine was not increased in wild-type mice 4 h after the intravenous administration of IL-6 ($N = 5$).

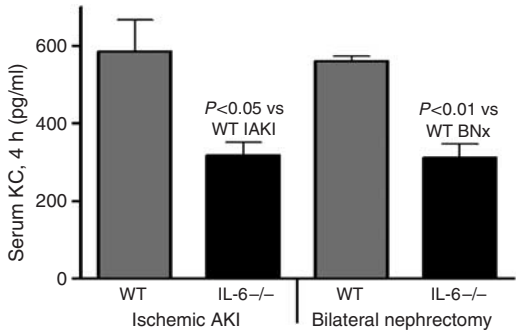

Figure 11 | Serum KC in IL-6-deficient mice. A total of 22 serum cytokines/chemokines were measured 4 h after sham operation, ischemic AKI, and bilateral nephrectomy in wild-type (WT) and IL-6-deficient (IL-6−/−) mice. Serum KC, a neutrophil chemokine, was significantly decreased after ischemic AKI and bilateral nephrectomy in IL-6-deficient mice ($N = 3$–6).

(Figure 10a). Neither KC nor MIP-2 was detected in lungs of wild-type mice 4 h following the intravenous administration of IL-6 (data not shown). Serum creatinine 4 h after intravenous administration of IL-6 was similar to creatinine in mice administered with vehicle (saline) (Figure 10b).

Serum cytokines in IL-6−/− mice

A total of 22 serum cytokines/chemokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-9, IL-12(p40), IL-12(p70), IL-13, IL-17, IL-19, MIP-1α, MIP-1β, granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor, KC, monocyte chemotactic protein-1, eotaxin, regulated on activation normal T cell expressed and secreted, tumor necrosis factor-α, interferon-γ) were measured 4 h after sham operation, ischemic AKI, and bilateral nephrectomy in wild-type and IL-6−/− mice. KC was significantly reduced in IL-6−/− mice compared with wild type after both ischemic AKI and bilateral nephrectomy (Figure 11). IL-12 (p40) was significantly reduced in IL-6−/− mice compared with wild type after bilateral nephrectomy alone (data not shown). No significant differences between wild-type and IL-6−/− mice after ischemic AKI or bilateral nephrectomy were noted in the remaining 20 serum cytokines/chemokines measured (data not shown).

DISCUSSION

Acute kidney injury is an independent contributor to mortality[27–30] regardless of degree of renal impairment.[27,29,31] Interventions to enhance kidney recovery in patients with AKI have been largely unsuccessful, limiting treatment to supportive measures such as dialysis. An attractive approach to reducing the significant morbidity and mortality associated with AKI is to specifically target the deleterious systemic complications that result from AKI.

Clinical human and experimental animal studies demonstrate that pulmonary dysfunction is an important systemic consequence of AKI. Respiratory failure requiring mechanical ventilation develops more frequently in patients with AKI,[2] and patients with AKI have impaired ability to wean from the ventilator.[3] The need for mechanical ventilation is an independent predictor of mortality in patients with AKI, with mortality rates of 81% in those requiring vs 29% in those not requiring mechanical ventilation.[1] Animal models of AKI including ischemic AKI and bilateral nephrectomy have been utilized to study the pulmonary effects of AKI. Although anti-inflammatory therapies reduce lung injury after AKI in rodents,[5,8] specific mediators of lung injury after AKI have not been identified. In this study, we found that IL-6 contributes to lung injury after AKI.

Multiple independent investigators have demonstrated that lung injury occurs after ischemic AKI or bilateral nephrectomy in rodents, characterized by increased interstitial leukocytes,[5,8,10] lung MPO activity,[5,8] BAL fluid cellularity,[10] pulmonary edema,[5] and capillary leak as measured by either Evans blue dye[6,10] or BAL fluid protein.[5] Importantly, these lung findings are similar to clinical and autopsy studies in patients who demonstrate lung injury consisting of pulmonary edema and leukocyte infiltration after AKI.[32–34]

The study of lung injury following AKI in rodents has been limited to few time points;[5,6,8–11] therefore, we examined multiple early time points after both ischemic AKI and bilateral nephrectomy to detect the onset of injury. We found that increased lung MPO activity, neutrophil infiltration, septal edema, and capillary leak occurred as early as 4 h after ischemic AKI as well as bilateral nephrectomy. This corroborates the findings of Deng *et al.*,[8] who reported lung injury 4 h after ischemic AKI. The use of the ischemic AKI model is clinically relevant, as half of the cases of AKI in hospitalized patients are due to ischemia. However, as lung injury occurs following ischemia/reperfusion injury of other organs, the bilateral nephrectomy model has been employed to examine the systemic effects of renal failure without ischemia. We confirm previous reports that pulmonary injury also occurs following bilateral nephrectomy[5,10,11,18] and demonstrate that timing of lung injury following bilateral nephrectomy appears to parallel that observed in ischemic AKI despite a recent report that the lung genomic response is strikingly different in these two models.[7]

As lung injury after AKI is characterized by lung neutrophil infiltration, and neutrophils are key pathogenic mediators of ALI, we examined the effect of AKI on potential mediators of neutrophil recruitment including the CXC neutrophil chemokines KC and MIP-2, as well as the adhesion molecules ICAM-1 and VCAM-1. Lung KC and MIP-2 were significantly increased as early as 2 h after AKI, preceding the peak in lung MPO activity and histological injury observed at 4 h. Increased lung[8] and cardiac[35] ICAM-1 mRNA 4 h after ischemic AKI has been reported. However, we found that although ICAM-1 and VCAM-1 were present in lung following ischemic AKI and bilateral nephrectomy, protein levels in the AKI groups did not differ from that expressed constitutively. Although lung protein expressions of ICAM-1 and VCAM-1 were not altered by AKI, this does not exclude these adhesion molecules as important mediators of lung neutrophil recruitment. Indeed, anti-ICAM-1 antibody administration reduced cardiac neutrophil recruitment following ischemic AKI.[35]

The signal(s) arising from AKI that results in increased pulmonary capillary leak and lung neutrophil recruitment, potentially via an effect on neutrophil chemokines, has not been identified. We have previously reported that serum cytokine profiles are different after ischemic AKI and bilateral nephrectomy;[5] however, serum IL-6 is elevated after both ischemic AKI and bilateral nephrectomy in mice. Remarkably, significant increases in serum IL-6 are also observed in hospitalized patients with AKI,[19] ALI, and adult respiratory distress syndrome[21,36] and are predictive of mortality. Neutrophil recruitment in various inflammatory models has been shown to be facilitated by IL-6 using IL-6-deficient mice and antibodies against IL-6 and its receptors.[37,38] Given this background, we hypothesized that IL-6 plays a role in AKI-mediated lung injury. In this study, we demonstrate that IL-6 contributes to lung injury after AKI, as IL-6-deficient mice as well as wild-type mice administered with anti-IL-6 antibodies have decreased lung MPO activity and reduced capillary leak after AKI.

We determined that strategies targeting IL-6 are associated with reduced lung KC, but not MIP-2, after AKI. In addition, 22 cytokines/chemokines were measured in the serum of IL-6-deficient mice after AKI and only KC was significantly reduced after both ischemic AKI and bilateral nephrectomy. Previous studies have demonstrated that serum KC is increased after ischemic AKI in mice.[5,39] As has been demonstrated with IL-6, IL-8 (a human analog of KC) is elevated in the serum of patients with AKI and ALI,[19,36] elevated in the BAL fluid of patients with ALI,[20] and predictive of complications and mortality in AKI and ALI.[19,21] Previous studies have suggested a link between IL-6 and IL-8. In a murine model of inflammation, impaired neutrophil accumulation in IL-6-deficient mice was reversed with the administration of IL-8.[37] Furthermore, IL-6-dependant endothelial cell production of KC has been described.[37,40]

In a recent study, functional genomic analysis demonstrated increased IL-6 signaling effects in the lung after ischemic AKI.[41] IL-6 signaling requires a specific IL-6 receptor (IL-6R) as well as a ubiquitously expressed signal-transducing protein, gp130. IL-6 signaling can occur in cells expressing the transmembrane IL-6R, but can also occur in cells lacking

the membrane-bound IL-6R in a process termed as *trans*-signaling. In *trans*-signaling, IL-6 binds to circulating soluble IL-6 receptor (sIL-6R) and the IL-6/sIL-6R complex binds to membrane bound gp130. Endothelial cells lack membrane-bound IL-6R; however, endothelial cell production of KC in response to IL-6 *trans*-signaling has been reported.[37,40] Increased serum sIL-6R in mice with $HgCl_2$-induced AKI was recently reported,[42] and sIL-6R is increased in patients with chronic renal failure.[43] As alveolar macrophages express the IL-6 receptor, they are an alternative potential source of lung KC in response to increased circulating IL-6.

Other investigators have reported renal protection after ischemic AKI in IL-6-deficient mice.[44] One could question whether the improvement in AKI-mediated lung injury achieved using methods targeting IL-6 is simply due to reduced kidney injury. We demonstrate that IL-6 contributes to lung injury independent of its potential effects on kidney function, as no difference in the degree of renal dysfunction after ischemic AKI was detected in IL-6-deficient mice or wild-type mice treated with anti-IL-6 antibodies compared with controls. As renal function is eliminated entirely after bilateral nephrectomy, the protection against lung injury in this model cannot be due to an effect on the kidney.

The use of the bilateral nephrectomy model also illustrates other important effects of renal failure independent of renal ischemia. The kidney itself can be a source of IL-6 after ischemia/reperfusion injury;[44] however, the elevation in serum IL-6 after bilateral nephrectomy indicates that the kidney is not the major source of increased IL-6 after AKI. As the kidney has been demonstrated to contribute to IL-6 clearance,[5,45] increased serum IL-6 after AKI likely results from renal and extrarenal IL-6 production combined with reduced renal IL-6 elimination.

In conclusion, we identify IL-6 as an important contributor to lung injury after AKI. As inflammatory serum cytokines have been shown to be elevated and predictive of mortality in critically ill AKI patients, anti-inflammatory strategies including cytokine removal via renal replacement modalities have been explored in the hopes of improving morbidity and mortality. These strategies are nonspecific, however. By elucidating direct pathogenic effects of particular cytokines, targeted therapies can be designed. Multiple clinical studies of patients with AKI and ALI have consistently demonstrated significant elevations in IL-6 and IL-8, which are predictive of mortality, but have not revealed a direct role of IL-6 and IL-8 in kidney and/or lung injury. We demonstrate that IL-6 contributes to AKI-mediated lung injury, potentially via an effect on lung production of KC. Importantly, increased serum IL-6 and lung KC after AKI precedes detectable lung injury; therefore, a therapeutic window for specific cytokine-directed therapies may exist. We demonstrate that administration of anti-IL-6 antibody was associated with reduced lung KC and improved lung injury after AKI. Therefore, specific therapies targeting IL-6 or KC merit further investigation as potential strategies to reduce the significant mortality associated with AKI and ALI.

MATERIALS AND METHODS

Animals

Eight- to ten-week-old male C57BL/6 mice (wild-type) and IL-6-deficient mice backcrossed over eight generations on a C57BL/6 background were used (Jackson Labs, Bar Harbor, ME, USA). Mice were maintained on a standard diet and water was made freely available. All experiments were conducted with adherence to the NIH Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the Animal Care and Use Committee of the University of Colorado (Protocol no. 81102007(06)1D).

Surgical protocol

Three surgical procedures were performed as described previously:[5] (1) sham operation, (2) ischemic AKI, and (3) bilateral nephrectomy. Mice were anesthetized with IP Avertin (2,2,2-tribromoethanol: Aldrich, Milwaukee, WI, USA), a midline incision was made, and the renal pedicles identified. For ischemic AKI, pedicles were clamped for 22 min. The bilateral renal pedicle clamp model is established in our laboratory.[46,47] After clamp removal, kidneys were observed for restoration of blood flow by the return to their original color. The abdomen was closed in one layer. Sham surgery consisted of the same procedure except that clamps were not applied. For bilateral nephrectomy, renal pedicles were tied off with suture and then cut distally. The ureters were pinched off with forceps and the kidneys removed.

Serum blood urea nitrogen and creatinine measurement

Serum was collected as described previously.[5] Blood urea nitrogen and creatinine were measured using an autoanalyzer (Beckman Instruments, Fullerton, CA, USA).

Serum IL-6 measurement

Serum IL-6 was measured by ELISA according to assay instructions (R&D Systems, Minneapolis, MN, USA).

Flow cytometry determination for serum cytokines

Serum IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-9, IL-12(p40), IL-12(p70), IL-13, IL-17, IL-19, MIP-1α, MIP-1β, granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor, KC, monocyte chemotactic protein-1, eotaxin, regulated on activation normal T cell expressed and secreted, tumor necrosis factor-α, and interferon-γ were determined using a bead-based multiplex cytokine kit (Bio-Rad, Hercules, CA, USA) in conjunction with flow-based protein detection and the Luminex LabMAP multiplex system (Luminex, Austin, TX, USA) according to the manufacturer's directions (detection limit for each cytokine = 1.95 pg/ml).

Tissue processing of lungs for histology

The right hilum was tied off, the right lobes removed, rinsed with PBS and frozen in liquid nitrogen. The left lung was expanded with 0.5% low melting agarose at a constant pressure of 25 cm H_2O, allowing for homogenous expansion of lung parenchyma as described previously,[48] removed, fixed in 10% formalin for 24 h, and paraffin-embedded.

Lung neutrophils

Five-micrometer sections of paraffin-embedded lung tissue were stained with hematoxylin and eosin using standard protocols. Neutrophils were counted on the basis of morphological criteria; at least 50 high-powered fields (× 40) were counted per slide.

Lung KC and MIP-2

Frozen lung was prepared for ELISA as described previously.[5] Supernatants were analyzed for protein content using a Bio-Rad DC protein assay kit (Hercules, CA, USA). KC and MIP-2 were determined by ELISA (R&D Systems, Minneapolis, MN, USA).

Evans blue dye

A total of 250 µl of EBD (5 mg/ml) was injected via tail vein 1 h before killing. Lungs were perfused with 5 ml PBS via the right ventricle to remove EBD within the vasculature, excised, weighed, and homogenized in 2 ml formamide. The homogenate was incubated in a 37 °C water bath overnight, then centrifuged at 10,000 g for 30 min. The optical density of supernatant was determined at 620 nm, and EBD concentration was calculated against a standard curve (µg EBD per gram of lung tissue).

MPO activity

One-fourth lung was used to determine MPO activity as described previously.[5]

ICAM-1 and VCAM-1 immunoblotting

Frozen lung was homogenized in radioimmunoprecipitation assay buffer with protease inhibitor; western blotting was performed as described previously.[49] Goat anti-murine ICAM-1 polyclonal antibody (R&D Systems, Minneapolis, MN, USA; 1:2000) or rat anti-murine VCAM-1 monoclonal antibody (R&D Systems; 1:1000) were used.

Anti-IL-6 antibody administration

A total of 20 µg anti-IL-6 antibody vs IgG control (eBioscience, San Diego, CA, USA) was administered to wild-type mice by tail vein injection 1 h before surgery, intraperitoneally at the time of clamp removal (ischemic AKI) or nephrectomy (bilateral nephrectomy) and intraperitoneally 1 h following surgery (60 µg total).

IL-6 administration

A total of 1 µg recombinant murine IL-6 in sterile PBS (R&D Systems) was administered by tail vein injection to wild-type mice.

Statistical analysis

Data were analyzed by one-way analysis of variance comparing the three conditions (sham operation, ischemic AKI, and bilateral nephrectomy) at each time point. If significant F-statistic from analysis of variance existed, this test was followed by Dunnett *post hoc* multiple comparison procedure with sham operation as the control group. For all other comparisons, Student's *t*-test was used. A *P*-value of <0.05 was considered as statistically significant.

DISCLOSURE

All the authors declared no competing interests.

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