

CD4⁺ T lymphocytes mediate acute pulmonary ischemia–reperfusion injury

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Objective: Postischemic reperfusion of the lung triggers proinflammatory responses that stimulate injurious neutrophil chemotaxis. We hypothesized that T lymphocytes are recruited and activated during reperfusion and mediate subsequent neutrophil-induced lung ischemia–reperfusion injury.

Methods: An in vivo mouse model of lung ischemia–reperfusion injury was used. C57BL/6 mice were assigned to either the sham group (left thoracotomy) or 7 study groups that underwent 1-hour left hilar occlusion followed by 1 to 24 hours of reperfusion. After in vivo reperfusion, the lungs were perfused ex vivo with buffer whereby pulmonary function was assessed. Lung vascular permeability, edema, neutrophil accumulation, and cytokine/chemokine production (tumor necrosis factor α , interleukin 17, CCL3, and CXCL1) were assessed based on Evans blue dye leak, wet/dry weight ratio, myeloperoxidase level, and enzyme-linked immunosorbent assay, respectively.

Results: A preliminary study showed that 2 hours of reperfusion resulted in greater pulmonary dysfunction than 1 or 24 hours of reperfusion. The 2-hour reperfusion period was thus used for the remaining experiments. Comparable and significant protection from ischemia–reperfusion injury–induced lung dysfunction and injury occurred after antibody depletion of neutrophils or CD4⁺T cells but not CD8⁺T cells ($P < .05$ vs immunoglobulin G control). Lung ischemia–reperfusion injury was proportional to the infiltration of neutrophils but not T cells. Moreover, pulmonary neutrophil infiltration and the production of CXCL1 (KC) were significantly diminished by CD4⁺T-cell depletion but not vice versa.

Conclusions: Both CD4⁺T lymphocytes and neutrophils accumulate during reperfusion and contribute sequentially to lung ischemia–reperfusion injury. The data suggest that neutrophils mediate ischemia–reperfusion injury; however, CD4⁺T cells play a critical role in stimulating chemokine production and neutrophil chemotaxis during ischemia–reperfusion injury.

Respiratory failure remains the most common complication in the perioperative period after lung transplantation. One of the major causes of respiratory failure and complications acutely observed after transplantation is ischemia–reperfusion injury (IRI),¹ which has been reported to be responsible for up to 30% of patient mortality within 30 days.² An increasing body of evidence has shown that IRI is associated with enhanced inflammatory responses during reperfusion. Our previous animal experiments have shown that alveolar macrophages and circulating leukocytes contribute importantly to lung IRI, with macrophages serving as triggers and leukocytes (mainly neutrophils) serving as end-effectors.^{3–6} Furthermore, we recently reported that alveolar epithelial cells, especially type II cells, interact with alveolar macrophages to initiate inflammatory responses during IRI.⁷

However, the signaling pathways between alveolar macrophages and neutrophils remain to be defined.

There is growing evidence that T cells might also participate in the pathogenesis of lung IRI.^{8–10} T cells are found to infiltrate the lung and are activated during reperfusion earlier than neutrophils.¹⁰ Lymphocyte-deficient rats or mice have decreased IRI.^{9,10} Cytokines and chemokines that stimulate T-cell chemotaxis and activation, such as interleukin (IL) 8, IL-12, IL-18, CCL5, and CCL2, are increased during lung IRI.^{7,9,11–13} T cells are known to amplify inflammatory responses through the secretion of lymphokines, including interferon γ (IFN- γ), IL-2, IL-4, IL-17, and granulocyte–macrophage colony-stimulating factor.^{9,14} These stimulate the chemotaxis of neutrophils and monocytes to sites of injury.

Whether T cells participate importantly in the inflammatory cascade that results in lung IRI is unclear. In the current study we used an in vivo mouse model of lung IRI to examine the role of T cells in lung IRI. Because neutrophils are end-effectors of lung IRI, we also examined the effect of lymphocyte depletion of neutrophil trafficking into the lung. Monoclonal antibodies (mAbs) were used to render mice deficient in neutrophils, CD4⁺T cells, or CD8⁺T cells.

MATERIALS AND METHODS

Animals

This study used a total of 74 (8- to 12-week-old) male C57BL/6 mice (Jackson Laboratory, Bar Harbor, Me), which were assigned to 7 IRI study

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Abbreviations and Acronyms

BAL	= bronchoalveolar lavage
IFN- γ	= interferon γ
IgG	= immunoglobulin G
IL	= interleukin
IRI	= ischemia–reperfusion injury
LC	= lung compliance
mAb	= monoclonal antibody
MPO	= myeloperoxidase
TNF- α	= tumor necrosis factor α

groups and 1 sham group that underwent surgical intervention but not hilar clamping. This study conformed to the “Guide for the care and use of laboratory animals” published by the National Institute of Health (publication no. 85-23, revised 1985) and was conducted under protocols approved by the University of Virginia’s Institutional Animal Care and Use Committee.

In Vivo Depletion of Neutrophils

Rat anti-mouse Gr-1 mAb was used to deplete circulating neutrophils in mice, as reported by others.¹⁵ Briefly, 10 μ g of anti-Gr-1 mAb (eBioscience, San Diego, Calif) was injected through the tail vein 24 hours before lung ischemia. Perioperatively, blood (30–40 μ L) was obtained by puncturing the left external jugular vein, and leukocyte counts were performed with a HemaVet Hematology System (CDC Technologies, Oxford, Conn).

In Vivo Depletion of CD4⁺ or CD8⁺ T Lymphocytes

Depletion of CD4⁺ or CD8⁺ T cells was achieved by using selective antibodies, as reported previously.¹⁶ Anti-CD4 mAb (GK1.5) or anti-CD8a mAb (53-6.7, eBioscience) was injected intraperitoneally on 2 consecutive days at a dose of 0.2 mg per mouse per day. Two days after the second injection, these animals underwent lung ischemia.

In Vivo Model of Lung Ischemia and Reperfusion

Mice were anesthetized with inhalation of isoflurane, intubated with PE-60 tubing, and connected to a pressure-controlled ventilator (Harvard Apparatus Co, South Natick, Mass). Mechanical ventilation was performed with room air as adjusted to a rate of 150 strokes/min, a stroke volume of 1.0 mL, and a peak inspiratory pressure of less than 20 cm H₂O. Heparin (20 U/kg) was administered through external jugular injection. A left thoracotomy was performed by cutting the left fourth rib, and the left hilum was exposed. A 6-0 Prolene suture (Ethicon, Inc, Somerville, NJ) was placed around the hilum facilitated by a tip-curved (22-gauge) gavage needle. Both ends of the suture were then threaded through a 5-mm-long PE-50 tubing. Occlusion was achieved by pulling up on the suture and thus pushing the tube against the hilum to initiate ischemia. A small surgical clip was applied to the suture on top of the tube to maintain tension of the tube against the hilum. The thoracotomy was then closed with sutures, and the mouse was extubated, placed in a cage, and allowed to awaken during the 1-hour hilar occlusion period. Five minutes before reperfusion, the mouse was reanesthetized and reintubated. Reperfusion was achieved by removing the clip and the tube/suture. Again, the chest was closed with sutures. The mouse was extubated and returned to a cage until pulmonary function testing. The temperature was monitored during surgical intervention with an anal probe and maintained between 36.5°C and 37.5°C. Sham animals received only thoracotomy without hilar occlusion. To minimize pain and discomfort, an analgesic (buprenorphine, 0.2 mg/kg) was administered to all animals at the beginning of surgical intervention.

Measurement of Pulmonary Function

At the end of scheduled reperfusion, pulmonary function was evaluated by using an isolated, buffer-perfused mouse lung system (Hugo Sachs Elektronik, March-Huggstetten, Germany), as previously described by our laboratory.⁶ Briefly, mice were anesthetized with ketamine and xylazine. A tracheostomy was performed, and animals were ventilated with room air at 100 strokes/min and a tidal volume of 7 μ L/g body weight with a positive end-expiratory pressure of 2 cm H₂O. The animals were exsanguinated by means of inferior caval transection. The pulmonary artery was cannulated through the right ventricle, and the left ventricle was immediately tube vented through a small incision at the apex of the heart. The lungs were then perfused at a constant flow of 60 μ L per gram of body weight per minute with Krebs–Henseleit buffer containing 2% albumin, 0.1% glucose, and 0.3% N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (335–340 mOsm/kg H₂O). The perfusate buffer and isolated lungs were maintained at 37°C throughout the experiment by using a circulating water bath. Once properly perfused and ventilated, the lungs were maintained on the system for a 5-minute equilibration period before data were recorded for an additional 10 minutes. Hemodynamic and pulmonary parameters were recorded during this period by using the PULMODYN data acquisition system (Hugo Sachs Elektronik).

Bronchoalveolar Lavage

After pulmonary function measurements, the left lungs were lavaged with 0.4 mL of normal saline. A microclamp was used to occlude the right hilum before lavage. The bronchoalveolar lavage (BAL) fluid was immediately centrifuged at 4°C (500g for 5 minutes), and the supernatant was stored at –80°C until further analysis.

Lung Wet/Dry Weight Ratio

In separate groups the left lung was harvested, weighed, and then placed in a vacuum oven (at 58°C) until a stable dry weight was achieved. The ratio of lung wet weight to dry weight was then calculated.

Pulmonary Microvascular Permeability

IRI-induced microvascular permeability in the lungs was determined by using the Evans blue dye extravasation technique.¹⁷ Evans blue (20 mg/kg; Sigma–Aldrich, St Louis, Mo) was injected intravenously 30 minutes before death. The pulmonary vasculature was then perfused for 15 minutes by using the isolated, buffer-perfused lung system to remove intravascular dye. Lungs were then homogenized in phosphate-buffered saline to extract the Evans blue and centrifuged. The absorption of Evans blue was measured in the supernatant at 620 nm and corrected for the presence of heme pigments as follows:

$$A_{620} (\text{corrected}) = A_{620} - (1.426 \times A_{740} + 0.030).$$

Immunohistochemistry

A standard immunohistochemistry protocol for paraformaldehyde-fixed tissue was used, as detailed previously.¹⁶ Briefly, the left lung was harvested, cut into 4 short-axis slices, and immediately fixed in 1% paraformaldehyde in phosphate-buffered saline (pH 7.4) for paraffin embedding. Immunostaining was performed with rat anti-mouse neutrophil antibody (Serotec, Inc, Oxford, United Kingdom), anti-CD3 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif), or anti-Mac2 antibody (Accurate Chemical & Scientific Corp, Westbury, NY). Three lung tissue slides (1 slide per mouse) from each group were used for semiquantitative cell counts in peripheral lung tissue. These cell counts did not distinguish among cells in various components of the lung (eg, airspace, interstitial, or marginated) but included all cells in peripheral (alveolar) lung tissue. On each slide, the lung tissue was divided into 4 to 6 parallel zones, and 1 photo was taken at each zone where the target cells were found in the highest numbers at 100 \times magnification.

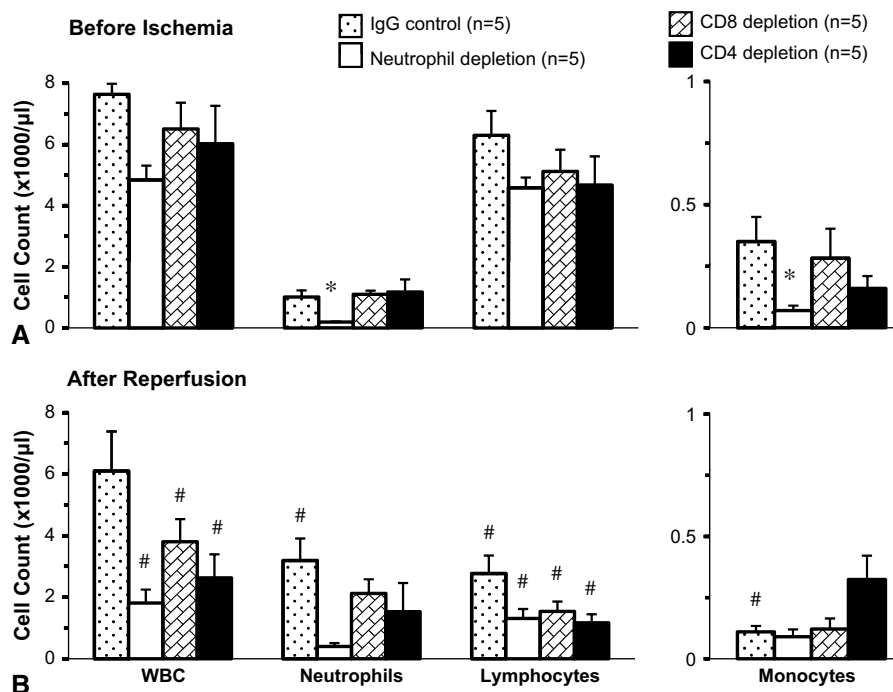


FIGURE 1. Complete blood cell counts in antibody-treated mice. A, Before hilar ligation, there was an 80% reduction in neutrophils in neutrophil-depleted mice compared with IgG control mice, a 19% reduction in lymphocytes in CD8-depleted mice, and a 26% reduction of lymphocytes in CD4-depleted mice. * $P < .05$ versus IgG control mice. B, Reperfusion caused a significant decrease in total circulating leukocytes and lymphocytes in all antibody-treated mice. However, circulating neutrophils also increased after reperfusion, but this was significant only in IgG control mice. # $P < .05$ versus the corresponding group in panel A. WBC, Total white blood cells.

Measurement of Myeloperoxidase

Myeloperoxidase (MPO) was measured in BAL fluid by using a mouse MPO enzyme-linked immunosorbent assay kit (Cell Sciences, Canton, Mass).

Measurement of Cytokines/Chemokines

Cytokines/chemokines in BAL fluid were quantified by using the Bioplex Bead Array technique with a multiplex cytokine panel assay (Bio-Rad Laboratories, Hercules, Calif), as previously done by our laboratory.⁷ The samples were analyzed as instructed with the Bioplex array reader, which is a fluorescent-based flow cytometer using a bead-based multiplex technology, each of which is conjugated with a reactant specific for a different target molecule.

Statistical Analysis

All data are presented as the mean \pm standard error of the mean. Data were compared with 1-way analysis of variance, followed by the Student's *t* test for unpaired data with the Bonferroni correction. Square roots of tissue cell counts were compared by using 1-way analysis of variance.

RESULTS

Time Course of Reperfusion Injury After 1 Hour of Hilar Ligation

To define the time point during reperfusion when lung injury was most significant, we evaluated pulmonary function after 1, 2, and 24 hours of reperfusion and after 1 hour of ischemia. At each time point, significant and roughly comparable elevations in pulmonary artery pressure and airway

resistance occurred (data not shown). Lung compliance (LC) was significantly worse after 2 and 24 hours of reperfusion versus that seen in sham mice (2.34 ± 0.19 and 4.02 ± 0.19 vs 5.61 ± 0.36 $\mu\text{L}/\text{cm H}_2\text{O}$, respectively; $P < .05$). LC was not significantly reduced after 1 hour of reperfusion (4.52 ± 0.65 $\mu\text{L}/\text{cm H}_2\text{O}$) but was significantly worse at 2 hours of reperfusion compared with that seen at 1 and 24 hours ($P < .05$). Based on these data, 1 hour of ischemia and 2 hours of reperfusion were used for the remainder of the study.

Changes in Circulating Leukocyte Numbers

Whole blood samples were collected from antibody-treated mice before ischemia and after 2 hours of reperfusion, and blood cells were counted with a HemaVet Hematology System (Figure 1). In neutrophil-depleted mice there was a greater than 80% reduction in the number of neutrophils ($P < .05$); however, there was also a significant reduction in monocytes (80%) compared with that seen in immunoglobulin G (IgG) isotype control mice (Figure 1, A). After reperfusion, the total number of circulating white blood cells and lymphocytes was significantly reduced in both IgG control and neutrophil-depleted mice compared with that seen before ischemia (Figure 1, B). Monocytes were further significantly reduced in IgG control mice after reperfusion but

TABLE 1. Counts of leukocyte cell infiltration in peripheral lung tissue

Groups (n = 3-5)	Sham	IgG control mice	Neutrophil-depleted mice	CD8 ⁺ T cell-depleted mice	CD4 ⁺ T cell-depleted mice
Neutrophils	4.2 ± 0.7	30.8 ± 2.7*	6.4 ± 0.9‡	24.5 ± 1.2*	17.5 ± 1.7*†
CD3 ⁺ T cells	6.4 ± 0.5	10.3 ± 0.9*	9.4 ± 0.7*	6.8 ± 0.5	2.7 ± 0.4‡
Macrophages	5.2 ± 0.6	3.7 ± 0.3	3.6 ± 0.5	5.2 ± 0.3	3.5 ± 0.4

*P < .05 versus sham mice. †P < .05 versus IgG control and CD8-depleted mice. ‡P < .05 versus all groups.

not in neutrophil-depleted mice. On the contrary, neutrophils were significantly increased by 2-fold after reperfusion in IgG control mice (compared with before ischemia) and not significantly increased in neutrophil-depleted mice (Figure 1, B).

In CD8- or CD4-depleted mice before ischemia, there were no significant changes in the number of total white blood cells, lymphocytes, neutrophils, or monocytes (Figure 1, A). Two hours after reperfusion (compared with before ischemia), there were significant reductions in total white blood cells (42%) and lymphocytes (70%) in CD8-depleted mice, and values were 56% and 75% in CD4-depleted mice, respectively (P < .05; Figure 1, B). Similar to what was observed in IgG isotype control mice, neutrophils were increased in CD8- and CD4-depleted mice after reperfusion, but these levels were not significant (Figure 1, B). There were no differences in the level of hemoglobin and platelets among all antibody-treated mice before or after reperfusion (data not shown).

Changes in Leukocyte Numbers and BAL MPO Levels

In sham and antibody-treated mice leukocytes in peripheral (alveolar) lung tissue were semiquantitatively evaluated in the left lung by means of immunohistochemistry. There were no significant differences in macrophage numbers between antibody-treated (after IRI) and sham mice (Table 1 and Figure 2, B, bottom row). Both CD4⁺ and CD8⁺ T cells express CD3 antigen, and thus immunohistochemistry with anti-CD3 antibody was used to assess combined CD4⁺ and CD8⁺ T cells. CD3⁺ T cells were significantly increased after IRI in IgG control and neutrophil-depleted mice compared with those seen in sham mice (Table 1). CD3⁺ T-cell counts were not altered in CD8-depleted mice but were significantly reduced in CD4-depleted mice (Table 1 and Figure 2, B, middle row). Neutrophil numbers were significantly increased in IgG control and CD8- and CD4-depleted mice but not in neutrophil-depleted mice compared with those seen in sham mice. However, neutrophil numbers were significantly lower in CD4-depleted mice compared with those seen in IgG control and CD8-depleted mice (Table 1 and Figure 2, B, top row). MPO levels in BAL fluid, an indicator of neutrophil infiltration into alveolar airspace, showed no difference between sham, neutrophil-depleted, and CD4-depleted mice but were significantly increased in IgG control and CD8-depleted mice (Figure 2, A).

Pulmonary Function During Reperfusion in Leukocyte-depleted Mice

As expected, pulmonary function was significantly impaired in lungs of IgG control mice after IRI compared with that seen in sham mice (Figure 3). Airway resistance was significantly increased, LC was significantly decreased, and pulmonary artery pressure was significantly increased in IgG control lungs. All parameters of pulmonary function were partially but significantly improved in neutrophil-depleted and CD4-depleted mice when compared with those seen in IgG control mice (Figure 3). No protection was observed in CD8-depleted mice.

Pulmonary Microvascular Permeability

Evans blue content in left lung tissue was measured to assess pulmonary microvascular leak. As expected, there was significantly higher Evans blue content after IRI (IgG control) versus that seen in sham mice (Figure 4, A). Evans blue content was partially but significantly reduced in both neutrophil-depleted and CD4-depleted mice compared with that seen in IgG control and CD8-depleted mice (Figure 4, A).

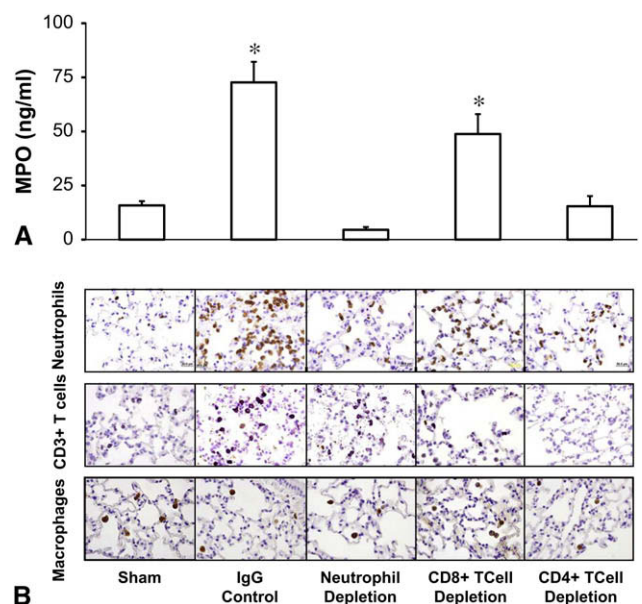


FIGURE 2. Myeloperoxidase (MPO) levels and leukocyte subtype infiltration into peripheral lung tissue after reperfusion. A, Myeloperoxidase levels in bronchoalveolar lavage fluid after reperfusion. *P < .05 versus sham mice. B, Examples of immunohistochemical staining for neutrophils, CD3⁺ T cells, and macrophages in the study groups.

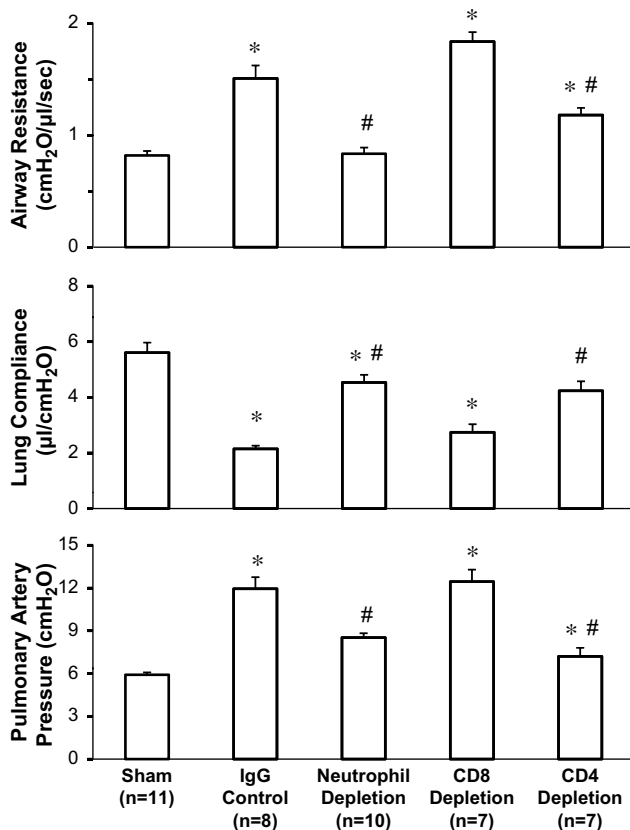


FIGURE 3. Effect of leukocyte subtype depletion on pulmonary function after reperfusion. Airway resistance, lung compliance, and pulmonary artery pressure were significantly improved in neutrophil-depleted and CD4⁺ T cell-depleted mice. No protection was observed in CD8⁺ T cell-depleted mice. **P* < .05 versus sham mice. #*P* < .05 versus IgG control and CD8-depleted mice.

Lung Wet/Dry Weight Ratio

Lung wet/dry weight ratio, used as an indicator of edema, was significantly increased in IgG control and CD8-depleted mice versus that seen in sham mice. Importantly, wet/dry weight ratio was significantly reduced in neutrophil- and CD4-depleted mice (Figure 4, B).

Changes in BAL Fluid Cytokine/Chemokine Expression

CCL3 (macrophage inflammatory protein 1), tumor necrosis factor α (TNF-α), IL-17, and CXCL1 (KC) levels in BAL fluid were significantly increased after IRI in IgG control mice and neutrophil- and CD8-depleted mice (Figure 5). No significant induction of IFN-γ or IL-12 was observed after IRI (data not shown). Neutrophil depletion significantly reduced expression of IL-17. Depletion of CD4⁺ T cells resulted in significantly reduced CCL3, TNF-α, IL-17, and CXCL1 levels versus those seen in IgG control mice. Depletion of CD8⁺ T cells did not significantly alter cytokine/chemokine expression versus that seen in IgG control mice.

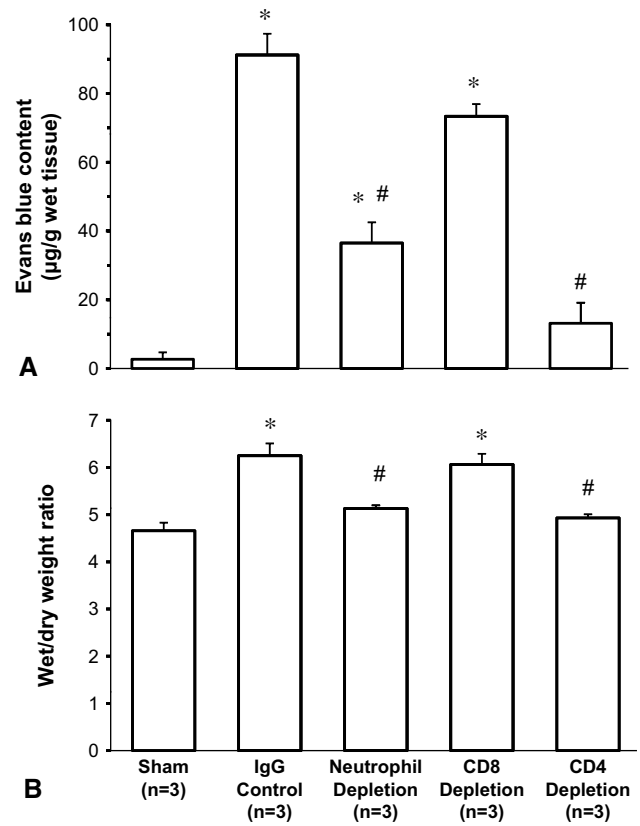


FIGURE 4. Microvascular permeability and pulmonary edema. A, Evans blue content in lung tissue as an indicator of microvascular leak. In antibody-treated mice Evans blue content was more significantly increased in IgG control and CD8-depleted mice than in neutrophil-depleted or CD4-depleted mice. B, Lung wet/dry weight ratio, as an indicator of edema, was significantly increased in IgG control and CD8-depleted mice but not in neutrophil-depleted or CD4-depleted mice (Figure 5, B). **P* < .05 versus sham mice. #*P* < .05 versus IgG control and CD8-depleted mice.

DISCUSSION

The current study used an in vivo mouse model to show that significant lung IRI occurs as early as 1 hour after left hilar ligation. Lung IRI was characterized by pulmonary dysfunction, edema, microvascular leak, and leukocyte infiltration. Lung IRI was significantly reduced in neutrophil- and CD4⁺ T cell-depleted mice but not in CD8⁺ T cell-depleted mice. Circulating T cells were found to be decreased after reperfusion, possibly because of vascular margination or redistribution to the lung or other tissues. Compared with sham mice, significant infiltration of CD3⁺ T cells occurred in control and neutrophil- and CD8-depleted mice but not in CD4-depleted mice. These results suggest that lung IRI is directly related to the level of infiltrating neutrophils but not T cells. The leukocyte counts performed in this study did not distinguish among cells in various components of the lung (eg, airspace, interstitial, or marginated) and thus encompassed all peripheral (alveolar) lung tissue. The results demonstrate that pulmonary neutrophil



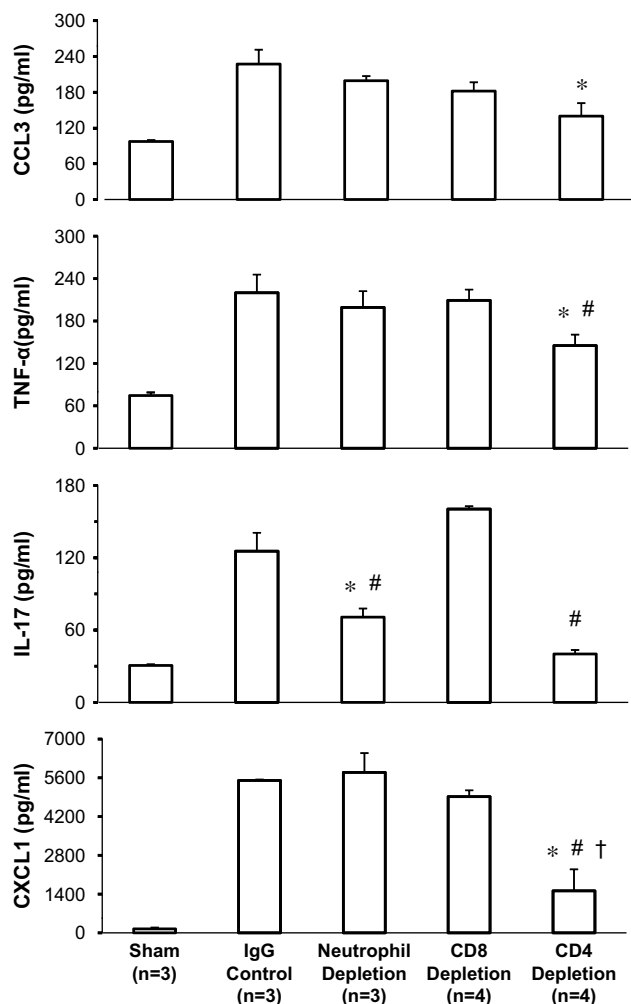


FIGURE 5. Expression of cytokines/chemokines in bronchoalveolar lavage fluid. CCL3 (macrophage inflammatory protein 1), tumor necrosis factor α (*TNF- α*), interleukin 17 (*IL-17*), and CXCL1 (KC) levels were all significantly increased in IgG control, neutrophil-depleted, and CD8-depleted mice compared with those seen in sham mice. In CD4-depleted mice there was no significant difference in CCL3, IL-17, and CXCL1 levels compared with those seen in sham mice. CXCL1 levels in CD4-depleted mice were significantly lower than in all other antibody-treated mice. **P* < .05 versus IgG control mice. #*P* < .05 versus CD8-depleted mice. †*P* < .05 versus neutrophil-depleted mice.

infiltration is dependent on CD4⁺ T cells but not vice versa. Cytokine/chemokine measurements revealed significantly increased CCL3 (macrophage inflammatory protein 1), TNF- α , IL-17, and CXCL1 (KC) production after reperfusion, which were all significantly reduced by depletion of CD4⁺ T cells. Taken together, these results indicate that both neutrophils and CD4⁺ T cells contribute importantly to acute lung IRI.

Mouse Model of Lung IRI

Inflammatory responses during lung IRI play a critical role in early graft failure after lung transplantation.^{1,2,4,9,11,14,18}

Most in vivo lung IRI models entail ligation of the left hilum for 60 to 90 minutes, followed by release of the ligature. Here the mice are typically maintained on ventilation until some point during reperfusion, and thus ventilator-induced lung injury could have significantly contributed to these models.¹⁹ The current study used a model with similar surgical intervention; however, the mechanical ventilation time was shortened to less than 20 minutes to minimize the potential of ventilator-induced lung injury. The absence of ventilator-induced injury is reflected in the current study by the sham mice, which display minimal lung injury and well-preserved lung function compared with that seen in the IRI group. In addition, lung function is relatively stable throughout 2 hours of continuous ventilation and perfusion in sham mice (data not shown), which would be expected to increase significantly over time if ventilator-induced injury was a significant component of this model.

Neutrophils Are End-effectors During Lung IRI

Studies have suggested that neutrophil accumulation appears to be the prime cellular mediator of pulmonary tissue destruction during IRI.¹⁻⁴ Lung IRI induces all the characteristics of an acute inflammatory response, such as oxidative stress; activation of complement, macrophages, and mast cells; elaboration of cytokines and chemokines; release of chemotactic factors; expression of cell adhesion molecules; neutrophil infiltration; and pulmonary necrosis.^{1,3,4,6,10,20,21} Compelling evidence from a variety of animal models and clinical studies indicates that neutrophils are the principle end-effectors of IRI.^{3,20,21} Significant numbers of activated neutrophils accumulate in the lung after reperfusion, as determined by means of tissue immunostaining¹⁰ or increased activity of MPO,²⁰ which is found almost exclusively within neutrophils.²² Neutrophils are generally believed to exacerbate tissue injury through the release of a variety of cytotoxic mediators, such as reactive oxygen species and proteases.^{23,24} The current study is entirely consistent with a critical role of neutrophils in causing lung IRI. More than 80% of neutrophils were successfully depleted with anti-Gr-1 antibody. However, in contrast to other reports,¹⁵ a significant number of circulating monocytes (80%) was also lost (Figure 1, A). The reduction in monocytes might have been due to the use of intravenous injection rather than intraperitoneal injection, as used previously.¹⁵ There was no significant reduction in lymphocytes. In the neutrophil-depleted mice lung IRI was significantly reduced, despite significant infiltration of CD3⁺ T cells. Furthermore, we found that protection from lung IRI correlated with a significant reduction in neutrophil infiltration but not other subtypes of leukocytes. These results demonstrate that activated neutrophils are end-effectors, which directly cause lung IRI. Although T lymphocytes might have some direct toxic effects on pulmonary tissues, they predominantly appear to

amplify an inflammatory response that is chemotactic to neutrophils.

CD4⁺ T Lymphocytes Mediate Inflammatory Responses During Reperfusion

An increasing body of evidence has shown that T cells contribute importantly to lung IRI. T cells are activated and infiltrate into the lung during reperfusion earlier than neutrophils.¹⁰ Inhibition of T cells before reperfusion has been shown to attenuate inflammation and decrease lung IRI.^{20,25} These data are consistent with the current results, which suggest that CD4⁺ T cells, or a subset of these cells, are activated during lung IRI.

To identify the role of CD4⁺ T cells in lung IRI, we used mice with antibody-induced depletion of either CD4⁺ or CD8⁺ T cells. After reperfusion, significantly less infiltration of CD3⁺ T cells was found in CD4- and CD8-depleted mice than in IgG control and neutrophil-depleted mice (Table 1). In sham mice there were higher numbers of peripheral lung T cells and macrophages than neutrophils. There was no significant change in the number of alveolar macrophages after 2 hours of reperfusion in all IRI groups; however, in the IgG control group there was a further increase in the number of infiltrating neutrophils and T cells, with the change of neutrophils being greater (Table 1). This increase in T-cell numbers might represent chemotaxis of circulating lymphocytes because circulating lymphocytes were significantly reduced during reperfusion. Although significant numbers of neutrophils are trapped (marginated or infiltrated) in the lungs, circulating neutrophil numbers were increased after reperfusion (statistical significance was reached only in IgG control mice). The reason for the increase in circulating neutrophil numbers is not clear but possibly reflects mobilization from bone marrow in response to granulocyte-macrophage colony-stimulating factor released by activated CD4⁺ T cells. Compared with CD8⁺ T cell-depleted mice, CD4⁺ T cell-depleted mice had significantly less infiltration of CD3⁺ T cells and neutrophils with correspondingly less lung IRI. However, neutrophil-depleted mice still exhibited increased numbers of CD3⁺ T cells, as seen in IgG control mice. Thus neutrophils have no effect on T-cell activation during lung IRI. Taken together, these results demonstrate that CD4⁺ T cells and not CD8⁺ T cells are activated during lung IRI and play a critical role in amplifying an inflammatory response, which culminates in the activation of neutrophils.

In lung IRI CD4⁺ T cells could become activated through either antigen-independent or antigen-dependent pathways. Antigen-independent mechanisms for T-cell activation have been described, which involve IL-12, TNF- α , CCL2, CCL3, CCL5, and IFN- γ -inducible protein 10.^{12,13,26} We found that CCL3, TNF- α , IL-17, and CXCL1 were significantly increased after IRI in all antibody-treated mice except CD4-depleted mice. In CD4-depleted mice production of

IL-17 and CXCL1 were significantly lower than seen in other antibody-treated mice after IRI (except for IL-17 in neutrophil-depleted mice), indicating that CD4⁺ T-cell activation precedes CXCL1 induction. These results are consistent with an antigen-independent pathway leading to CD4⁺ T-cell activation during lung IRI. IL-17 activates alveolar macrophages and epithelial type II cells to induce CXCL1, which is a strong chemotactic mediator for neutrophil infiltration.^{7,27} Another possibility is that CD4⁺ T cells are activated directly by factors produced during reperfusion to release cytokines/chemokines that transactivate other leukocytes and epithelial cells.

Clinical Effect

Identification of the initiatory signaling cascade through CD4⁺ T cells and macrophages will likely lead to specific pharmacologic interventional targets for the amelioration of lung IRI. For example, recent studies by our group have identified one such potential therapeutic agent to be the A2A adenosine receptor, which resides on leukocytes, including CD4⁺ T cells and neutrophils. Here we have shown that agonists that specifically activate A2A adenosine receptors significantly reduce neutrophil infiltration and attenuate lung IRI after transplantation.^{4,28} The possible protective role of A2A adenosine receptors on CD4⁺ T cells in the setting of lung IRI and the underlying molecular mechanisms are yet to be elucidated and are currently under investigation.

CONCLUSION

The current study suggests that sequential activation of CD4⁺ T lymphocytes and neutrophils occurs during lung IRI. CD4⁺ T cells accumulate in the lung during reperfusion but have little direct toxic effect. Instead, CD4⁺ T cells orchestrate the chemotaxis of circulating neutrophils to the lung. Activated neutrophils are end-effectors, which carry out damage-producing tasks, whereas CD4⁺ T cells play a central role in mediating this injury process. The results underscore the importance of CD4⁺ T lymphocytes as mediators of lung IRI.

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Discussion

Dr Craig Selzman (*Chapel Hill, NC*). That was a very nice presentation and a lot to take in in a short period of time. I am trying to make some relevance out of this because it is almost like you make the background for lung transplantation, but this is a hilar clamp model. I was wondering if you could speculate on the role of temperature and what was the effect on many of your markers in this model.

Dr Yang. Thank you for your insightful comments. It is important to maintain a lower temperature of the donor lung under clinical situations. In this particular study our aim was to characterize the inflammatory responses during lung IRI, and thus we purposely maintained normal body temperature during occlusion and perfusion. Thus we did not sort out the temperature factor in our model.

Dr Selzman. I guess what I was getting at is that normally the temperature for a lung transplantation is a little bit lower.

Dr Yang. You are right.

Dr Selzman. Therefore I guess if you put a tape around a pulmonary artery during a lobectomy and then you were going to reperfuse—therefore I would like you to try to speculate how you might be able to figure that out. Also, did you administer any kind of pulmonary protective agent before your experiment?

Dr Yang. It is feasible to lower the body temperature during reperfusion in our model, and it has been reported that hypothermia ameliorates lung IRI. Although we did not address the temperature factor in our model, I would speculate that a reduced temperature would lower the inflammatory response during reperfusion. As far as pretreatment is concerned, our laboratory has applied an adenosine A2A receptor agonist, ATL313, to this model and found that treatment before ischemia, as well as during reperfusion, is quite protective.

Dr Selzman. Have you ever done this in your organ perfusion model, your ex vivo model?

Dr Yang. You are talking about the isolated model previously?

Dr Selzman. It sounded like you had an isolated model.

Dr Yang. We do have an isolated, blood-perfused rabbit lung model in our laboratory. In studies using this model, the isolated lungs typically undergo cold ischemic storage followed by reperfusion with blood at normal body temperature (37°C) to simulate a clinical lung transplantation. All groups were treated similarly in terms of temperature. Therefore we could not determine from these studies whether the temperature had been a factor of protection against IRI. However, even with lower ischemic temperature in this model, reperfusion injury still existed, and intervention with anti-inflammatory agents, such as ATL313, before reperfusion, ischemia, or both exerted significant protective effects. We did not look into the effect of the temperature factor on lung IRI, but that is an excellent point. It would be most interesting to design a study to look into the role of lower temperature as part of the regimen to prevent lung IRI after transplantation.