Early activation of the alveolar macrophage is critical to the development of lung ischemia-reperfusion injury

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Objectives: Activation of the alveolar macrophage is critical to the development of nonischemic inflammatory lung injury. The present studies were undertaken to determine whether the alveolar macrophage plays a similarly important role in lung ischemia-reperfusion injury.

Methods: The left lungs of male rats were rendered ischemic for 90 minutes and reperfused for up to 4 hours. Treated animals received liposome-encapsulated clodronate, which depletes alveolar macrophages. Injury was quantitated in terms of vascular permeability, tissue neutrophil accumulation, and bronchoalveolar lavage fluid leukocyte, chemokine, and cytokine content. Lung homogenates were also analyzed for nuclear translocation of the transcription factors nuclear factor κ B and activator of protein 1.

Results: Depletion of alveolar macrophages reduced lung vascular permeability by 53% compared with that seen in control animals (permeability indices: 0.88 ± 0.07 to 0.46 ± 0.04 , $P < .001$). The protective effects of alveolar macrophage depletion correlated with a 50% reduction in tissue myeloperoxidase content (0.62 ± 0.07 to 0.33 ± 0.03 , $P < .006$) and marked reductions in bronchoalveolar lavage fluid leukocyte accumulation. Alveolar macrophage-depleted animals also demonstrated marked reductions of the elaboration of multiple proinflammatory chemokines and cytokines in the lavage effluent and nuclear transcription factors in lung homogenates.

Conclusion: It is likely that the alveolar macrophage is the key early source of multiple proinflammatory mediators that orchestrate lung ischemia-reperfusion injury. Depleting alveolar macrophages is protective against injury, supporting its central role in oxidant stress-induced cytokine and chemokine release and the subsequent development of lung injury.

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Lung ischemia-reperfusion injury (LIRI) occurs in up to 25% of human lung transplant recipients¹ and in 15% to 20% of patients undergoing pulmonary thromboendarterectomy.² Despite improvements in preservation techniques and limitation of ischemic times, reperfusion injury remains problematic after lung transplantation and might increase the occurrence of acute and chronic rejection.

Reperfusion injury manifests itself clinically as deteriorating respiratory function coupled with interstitial edema, resulting from an increase in endothelial permeability. In animal models this vascular injury has been defined as biphasic.³ Although the late phase is dependent on neutrophil sequestration,⁴ the early stage is neutrophil independent and occurs within 15 minutes of reperfusion. It has been proposed that this early injury incites the processes that ultimately lead to fully developed lung injury several hours later. Because this early injury occurs well before any significant neutrophil sequestration, it is most likely dependent on the activation of a resident cell. The alveolar macrophage (AM) is such a resident cell and is centrally involved in a variety of lung injury models.⁵⁻¹⁰ The AM, in response to oxidant stress *in vitro*, secretes a number of chemokines and cytokines known to promote neutrophil accumulation and therefore regulates the late phase of injury.^{11,12} Therefore, presuming that the early vascular injury is dependent on a resident cell and knowing that AMs are critical in a variety of injury models and secrete proinflammatory mediators in response to hypoxia and reoxygenation, we hypothesized that AM depletion would be protective against LIRI. Selective depletion of AMs by means of apoptosis¹³ was achieved by using the established technique of intratracheal instillation of liposome-encapsulated clodronate.^{5,9,10,14}

Materials and Methods

Reagents

Liposomal preparations of clodronate were prepared as described previously.¹³ Clodronate was a gift from Roche Diagnostics (Mannheim, Germany). All other reagents were purchased from Sigma Chemical (St Louis, Mo) unless otherwise specified.

Animal Model

Pathogen-free, adult male Long-Evans rats (Simonsen Labs, Gilroy, Calif) weighing 280 to 320 g were used for all experiments. The University of Washington Animal Care Committee approved all experimental protocols. Animals were anesthetized with 30 to 35 mg of intraperitoneal pentobarbital, shaved, and prepped. A 14-gauge angiocatheter was inserted into the trachea through a midline neck incision and secured with a 4-0 braided suture. Animals were then placed on a Harvard Rodent Ventilator (Harvard Apparatus Inc, Holliston, Mass) with an inspired oxygen content of 60%, a respiratory rate of 90 breaths/min, and 2 cm H₂O of positive end-expiratory pressure. Maximal peak pressures were maintained at less than 10 cm H₂O during the operation. All animals received 0.2 mg of atropine intramuscularly, and anesthesia was maintained with inhaled halothane. Dissection was conducted by using an operating microscope, and a warming blanket was placed underneath the animals throughout the experiment. A left anterolateral thoracotomy in the fifth intercostal space was performed. The left lung was mobilized atraumatically, and the inferior pulmonary ligament was divided. At this juncture, animals received 50 units of intravenous heparin in saline solution (total volume, 500 μ L). Five minutes after heparin was administered, the

left pulmonary artery, veins, and main-stem bronchus were occluded with a noncrushing microvascular clamp. During the experiment, the lungs were kept moist with periodic topical application of warm normal saline solution, and the incision was covered to minimize evaporative losses. At the end of the ischemic period, which was held constant at 90 minutes, the clamp was removed from the hilum, and the lung was allowed to ventilate and reperfuse for periods of up to 4 hours. Animals received 0.5 mL of warm subcutaneous saline solution per hour to maintain hydration during the experiment. A midline incision from the neck to the pubis was made at the end of the reperfusion period to afford access to the chest and abdomen. Blood samples were obtained from the inferior vena cava just before death. The heart-lung block was rapidly excised, and the pulmonary circulation was flushed through the main pulmonary artery with 20 mL of normal saline solution. The lungs were separated from the heart and mediastinal tissues and then analyzed as outlined below.

Treated animals, during a combination of ketamine- and xylazine-induced anesthesia, received 100 μ L of liposome-encapsulated clodronate in a total volume of 300 μ L of phosphate-buffered saline (PBS) solution by means of direct intratracheal instillation 24 hours before the beginning of the experiment. Control animals received the same volume of liposomes but containing saline solution rather than clodronate. Macrophage depletion was documented by means of bronchoalveolar lavage (BAL) of lung blocks with 6 mL of warm normal saline solution containing 0.5 mmol/L ethylenediamine tetraacetic acid (EDTA) followed by cell counts. In uninjured lungs the predominant cell type is the AM (>99%). The AM cell count was reduced by 74% at 24 hours after treatment, and of the remaining AMs, only 10% (2.3%) remained viable by means of trypan blue exclusion ($P < .04$).

Lung Permeability Index

As one quantification of lung injury caused by ischemia and reperfusion, a lung permeability index was determined in the following manner. Iodine 125–radiolabeled bovine serum albumin (¹²⁵I-BSA) was obtained from NEN Life Sciences (Boston, Mass). Before use of the ¹²⁵I-BSA *in vivo*, serial dilutions were performed to obtain an activity of 800,000 cpm per dose. This volume of ¹²⁵I-BSA, approximately 2 μ L of the stock solution, was then brought to a final volume of 500 μ L in a 1% BSA-PBS solution. Five minutes before removal of the hilar clamp or at an equivalent time in control animals, the ¹²⁵I-BSA preparation was injected intravenously. Immediately before the animals were killed, 1 mL of blood was drawn from the inferior vena cava. The heart-lung block was then excised and flushed as described previously. The radioactivity was quantitated in both the left and right lung, as well as in the inferior vena caval blood sample, by using a gamma counter. The permeability index was expressed as the ratio of counts per minute in the left lung to counts per minute in 1.0 mL of inferior vena caval blood, as shown in the following equation:

$$\text{Permeability index} = \frac{\text{Left lung (cpm)}}{1.0 \text{ mL of blood (cpm)}}$$

This ratio corrects for any variation in systemic blood levels of radioactivity and provides a reproducible measure of lung microvascular permeability.¹⁵⁻¹⁸

Myeloperoxidase Assay

Tissue myeloperoxidase (MPO) content was used to quantitate neutrophil accumulation in the rat lungs. After flushing the pulmonary circulation with 20 mL of saline solution, the lungs were homogenized for 60 seconds in a solution of 0.5% hexadecyltrimethylammonium bromide and 5 mmol/L EDTA in 50 mmol/L potassium phosphate buffer (pH 6.0). Samples were then sonicated for 40 seconds in four 10-second bursts. The homogenate was maintained on ice between all tissue processing. Samples were then centrifuged at 2300g for 30 minutes at 4°C, and the supernatants were recovered. The assay buffer was composed of 0.0005% H₂O₂ and 0.167 mol/L *o*-dianisidine dihydrochloride in 100 mmol/L potassium phosphate buffer (pH 6.0). Fifty milliliters of each sample was mixed with 1.45 mL of assay buffer, and the change in absorbance at 460 nm over 1 minute was recorded.

Bronchoalveolar Lavage

The same groups of animals underwent BAL at the time of death. Through an extended median sternotomy, a 14-gauge angiocatheter was placed in the trachea, and the lungs were lavaged individually with 3.0 mL of warm sterile saline solution. Individual lung BAL analysis was accomplished by clamping the contralateral hilum with a noncrushing microvascular clamp. At least 80% of the instilled fluid was recovered from each lung. This fluid was centrifuged (1500g for 8 minutes at 4°C) to pellet the cells. The supernatant was snap-frozen in liquid nitrogen for subsequent cytokine and chemokine analysis after the addition of a protease cocktail inhibitor consisting of leupeptin (1 µg/mL), aprotinin (1 µg/mL), trypsin inhibitor (5 µg/mL), and pepstatin A (1 µg/mL). The red blood cells were lysed, and the pellet was resuspended in normal saline solution. Cells were then counted using a hemacytometer (Hausser Scientific, Reading, Pa).

Enzyme-linked Immunoassay of BAL Fluid for Cytokine and Chemokine Content

Sandwich enzyme-linked immunoassays (ELISAs) for macrophage inflammatory protein (MIP) 2, cytokine-induced neutrophil chemoattractant (CINC), MIP-1 α , monocyte chemotactic protein 1, and regulated on activation normal T cell expressed and secreted were performed by adding 50 µL of a 10 µg/mL protein A-purified specific anti-chemokine antibody (Peprotech, Rocky Hills, NJ) to a carbonate-coating buffer solution (pH 9.6) in a 96-well (Dynex) immunoassay plate. The plate was incubated overnight at 4°C and subsequently washed with PBS containing 0.05% Tween. Nonspecific binding sites were blocked with 1% BSA in saline solution (30-minute incubation at 37°C). Samples and standards were diluted in saline solution, and 50 µL was added to each well (1-hour incubation at 37°C). A secondary affinity-purified biotinylated antibody (Peprotech) specific to the epitope being studied (0.5–2 µg/mL) was added to each well (1-hour incubation at 37°C). After a 30-minute incubation with a streptavidin–horseradish peroxidase conjugate (Pierce, Rockford, Ill), the assay was developed by adding *o*-phenylenediamine dihydrochloride substrate. The reaction was stopped by adding 50 µL of 3 mol/L H₂SO₄. The tumor necrosis factor (TNF) α ELISA was performed according to the manufacturer's guidelines (R&D Systems, Abingdon, Oxon, United Kingdom). Samples and standards were run in triplicate, and well-to-well variation did not exceed 5%.

Electrophoretic Mobility Shift Assay

At the end of the experimental protocols, the left lungs were snap-frozen in liquid nitrogen after flushing the pulmonary circulation with 20 mL of saline solution. The frozen tissue was ground to a fine powder and suspended in 4 mL of buffer containing 0.06% Nonidet P-40, 150 mmol/L NaCl, 10 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 1 mmol/L EDTA, and 0.5 mmol/L phenylmethylsulfonyl fluoride. The solution was homogenized and centrifuged for 15 seconds (12,000g). The pellet was discarded, and the supernatant was centrifuged again for 15 seconds (12,000g). The resultant pellet was suspended in 40 µL of buffer containing 40 mmol/L NaCl, 20 mmol/L HEPES, 0.2 mmol/L EDTA, 1.2 mmol/L MgCl₂, 0.5 mmol/L phenylmethylsulfonyl fluoride, 0.5 mmol/L dichlorodiphenyltrichloroethane, 25% glycerol, 5 µg/mL aprotinin, and 5 µg/mL leupeptin at 4°C for 20 minutes. This solution was centrifuged for 5 minutes, the pellet was discarded, and the supernatant containing the nuclear protein was stored at –70°C. Quantification of nuclear protein was performed by using the bicinchoninic acid assay.

Nuclear protein (10 µg) was incubated in a binding reaction with double-stranded phosphorous 32 end-labeled oligonucleotide containing either the nuclear factor (NF) κ B or activator protein (AP) 1 binding consensus sequence (Promega, Madison, Wis). Running unlabeled oligonucleotide probe in a cold competition binding reaction assessed the specificity of each probe. The binding reaction was carried out at room temperature for 60 minutes, and the proteins were resolved on a 6% nondenaturing polyacrylamide gel at 100 V for 1 to 2 hours. The gels were dried and autoradiographed. Duplicate samples for each specimen were analyzed.

Statistical Analysis

All groups contained 4 animals unless otherwise specified, and all data are presented as the mean $\pm$ SEM. Comparisons between multiple groups were performed by using analysis of variance, and those between individual groups were done by using the Bonferroni modification of the *t* test.

Results

Changes in Lung Vascular Permeability

Five different groups of animals were studied. The first group served as negative control animals and did not undergo any intratracheal instillation or surgical manipulation. A second group received intratracheal saline liposomes but no surgical manipulation. A third group, the positive control animals, underwent thoracotomy and occlusion of the left hilum for 90 minutes, followed by 4 hours of reperfusion (IR protocol). Additionally, both the fourth and fifth groups were subjected to the IR protocol. The fourth group received saline liposomes, and the fifth received liposomes containing clodronate (macrophage depleted) 24 hours before the operation. The permeability index in animals with unmanipulated lungs receiving saline liposomes (0.23 ± 0.01) was more than double that seen in unmanipulated lungs from animals receiving no intratracheal instillations (0.09 ± 0.006 , $P < .001$). A marked increase in permeability was

Figure 1. Left Lung permeabilities in control and treated animals. There is a 4-fold increase in lung vascular permeabilities in animals receiving saline liposomes that were subjected to ischemia and reperfusion when compared with noninjured animals that were administered saline liposomes ($P < .001$). There was no significant difference between saline liposome-treated or untreated animals, whether unmanipulated or subjected to the full IR protocol. However, in clodronate liposome-treated (macrophage-depleted) animals, there was a marked reduction in vascular permeability ($P < .025$; analysis of variance: $P < .001$, $n = 4$).

seen in IR protocol animals (0.82 ± 0.02 , $P < .001$) compared with that seen in the unmanipulated groups. IR protocol animals that received saline liposomes had a mean permeability index of 0.88 ± 0.07 , which was not significantly higher than that of the positive control animals. In contrast, macrophage-depleted IR protocol animals demonstrated a 53% (0.46 ± 0.09 , $P < .001$) reduction in vascular permeability compared with that seen in positive control animals (Figure 1).

MPO Activity

MPO activity was measured in lungs from negative control animals and animals that received saline liposomes. The changes in absorbance at 460 nm over 1 minute in negative control animals and saline liposome-treated animals were 0.04 ± 0.01 and 0.07 ± 0.03 , respectively. The MPO content from positive control animals (0.44 ± 0.05) increased significantly when compared with that from negative control animals ($P < .003$) and was further increased in saline liposome-treated IR protocol animals (0.62 ± 0.07). This finding is consistent with the demonstration of many investigators that intratracheal liposome administration results in significant basal neutrophil accumulation in a time-dependent fashion.¹⁴ The difference in MPO content between saline liposome-treated and positive control animals, however, was not significant. Despite this apparent inflammatory side effect of liposome instillation, macrophage-depleted IR protocol animals demonstrated a 50% (Figure 2) reduction in lung tissue neutrophil accumulation (0.33 ± 0.03) when compared with the saline liposome IR protocol group ($P < .02$).

Figure 2. MPO content. The increase in MPO content after ischemia and reperfusion was statistically significantly different from that seen in unmanipulated lungs ($P < .001$). Although positive control animals that received saline liposomes had higher tissue MPO contents than untreated positive control animals, this difference was not significant. Tissue neutrophil accumulation was markedly decreased in macrophage-depleted animals ($P = .018$; analysis of variance: $P < .001$, $n = 4$).

BAL Fluid Analysis

BAL fluid analysis was undertaken to evaluate leukocyte content in the distal airways and alveolar compartment. BAL leukocyte counts were recorded in the previously described 5 groups. In negative control animals and in those treated with saline liposomes, the cell counts were low and not significantly different. Cell counts increased significantly in both the positive control animals and in the saline liposome-treated IR protocol animals compared with those seen in their respective unmanipulated counterparts. The predominant cell type at the end of the 4-hour reperfusion period was the neutrophil ($>95\%$). Bronchoalveolar cell counts were reduced in macrophage-depleted IR protocol animals by 44% (12.8 ± 1.3 , $P < .001$) compared with those seen in saline liposome-treated IR protocol animals (Figure 3). These results are similar to the trends noted with MPO content in macrophage-depleted animals.

Macrophage Depletion Reduces Total Lung NF- κ B and AP-1 Activation After the IR protocol

To determine the mechanism of protection of macrophage depletion in our model of LIRI, we evaluated the nuclear translocation of 2 proinflammatory transcription factors. There was minimal translocation of NF- κ B and AP-1 detected in unmanipulated animals. Shown are representative electrophoretic mobility shift assays that detail the translocation of NF- κ B and AP-1 in IR protocol lungs with and without macrophage depletion (Figure 4). Significant activation of NF- κ B and AP-1 is apparent in the animals that underwent 90 minutes of ischemia, followed by 15 minutes or 4 hours of reperfusion. Macrophage-depleted animals

Figure 3. BAL cell count. The BAL cell count increased significantly in saline liposome-treated animals that underwent the IR protocol ($P < .001$) and in untreated groups that underwent the IR protocol ($P < .001$) when compared with that seen in their respective nonsurgical control animals. The predominant cell type in the unmanipulated animals was the AM, and the majority of the cells in the reperfused lungs were neutrophils. A significant decrease in alveolar leukocyte sequestration was clearly demonstrated in animals that received clodronate liposomes ($P < .001$; analysis of variance: $P < .001$, $n = 4$).

appear to have markedly attenuated NF- κ B nuclear translocation at 15 minutes of reperfusion but not at 4 hours. In contrast, AP-1 is reduced at both 15 minutes and 4 hours of reperfusion in macrophage-depleted animals. These differences are quantitated in the densitometric analysis depicted adjacent to the autoradiograph (Figure 4).

Macrophage Depletion Reduces the Elaboration of Chemokines and Cytokines in BAL Fluid

The expression of selected chemokines and cytokines were analyzed in the BAL fluid of injured lungs by using ELISAs developed in our laboratory to demonstrate whether a reduction in the nuclear translocation of the aforementioned transcription factors was associated with diminution in the elaboration of inflammatory mediators. MIP-2, CINC, MIP-1 α , and TNF- α were studied because we have previously shown that neutralizing antibodies directed against these mediators are protective against injury (unpublished data). Three groups were studied: unmanipulated saline liposome-treated animals and IR protocol groups that received either saline or clodronate liposomes. All 4 mediators studied were significantly increased with injury (Figure 5). MIP-1 α , MIP-2, and CINC increased significantly with ischemia followed by 4 hours of reperfusion. In macrophage-depleted animals MIP-1 α , MIP-2, and CINC secretion was reduced by 70%, 64%, and 83%, respectively (MIP-1 α , 52 ± 9 to 25 ± 3 pg/mL [$P < .007$]; MIP-2, 1712 ± 308 to 751 ± 227 [$P < .04$]; CINC, 109 ± 22 to 27 ± 11 [$P < .006$]). TNF- α secretion in the BAL effluent increased from 52 ± 6 pg/mL in unmanipulated animals to 426 ± 43 pg/mL in the IR

protocol group ($P < .05$). This was also significantly reduced with macrophage depletion by 39% to 296 ± 7 pg/mL ($P < .05$).

Discussion

Depleting AMs ameliorates lung reperfusion injury in rats, as demonstrated by a reduction in vascular permeability and tissue neutrophil accumulation. This reduction might represent either a delay or a genuine amelioration in injury. Experience with this and other models of ischemia and reperfusion would suggest that it is more likely a true reduction. Protection is still evident despite the basal increase in neutrophil sequestration that is a recognized side effect of intratracheal liposome instillation.¹⁴ Additionally, macrophage depletion reduces the nuclear translocation of 2 proinflammatory transcription factors, AP-1 and NF- κ B, early in reperfusion. This, in turn, is associated with a diminished expression of cytokines and chemokines, mediators known to play important roles in the development of lung injury.¹⁹ The reappearance of NF- κ B activation late in reperfusion might relate to possible transcriptional activation in pulmonary artery endothelial cells^{20,21} or to the stimulation of tissue neutrophils that accumulate late in reperfusion. Nonetheless, these data strongly support a central role for the AM in the oxidant stress-induced elaboration of cytokines and chemokines in the lung.

The AM that resides at the air-tissue interface is the early, rapid-response, immune effector cell to a wide variety of toxic, infectious, and allergenic stimuli.²² The macrophage has an incredible range of biologic activities by virtue of its ability to synthesize and secrete an array of growth factors, cytokines, chemokines, arachidonic acid metabolites, and oxygen radicals.²³ Suppression of AM function has been shown to be protective in a wide variety of models, including lipopolysaccharide instillation, immune-complex deposition, hyperoxia, and ozone and *Pseudomonas* species-induced lung injury.^{5-8,10}

Until recently, the role of the AM in LIRI has been nebulous. One study using an isolated perfused lung model showed that inhibition of macrophage function with gadolinium improved pulmonary hemodynamics early in reperfusion.²⁴ However, this effect was not associated with significant improvement in measurable parameters of tissue injury, such as MPO content and extravascular lung water. Furthermore, in that study the early improvement in injury associated with gadolinium treatment did not result in any protective effects later in reperfusion. These data would therefore suggest that the AM is not critical to the development of florid lung reperfusion injury. This contradiction might be explained in part by the model used. Although the isolated lung model is an established and useful tool for studying lung reperfusion injury, it unfortunately is not physiologic. In addition to the hypotheses concerning mac-

Figure 4. Electrophoretic mobility shift assay (EMSA) investigating nuclear protein expression of NF- κ B and AP-1 in injured left lung homogenates. Lanes were run in duplicate to verify reproducibility, and the electrophoretic mobility shift assay is depicted in B. *Lanes 1 to 4* represent animals that underwent ischemia and 15 minutes of reperfusion. *Lanes 1 and 2* are samples from positive control animals, and *lanes 3 and 4* represent macrophage-depleted animals. *Lanes 5 to 8* represent animals undergoing ischemia and 4 hours of reperfusion. *Lanes 5 and 6* are samples from positive control animals, whereas *lanes 7 and 8* represent macrophage-depleted animals. There is a marked reduction in nuclear translocation of NF- κ B and AP-1 in macrophage-depleted animals at 15 minutes of reperfusion. Although NF- κ B expression was increased in both groups at 4 hours of reperfusion, AP-1 expression remained decreased in macrophage-depleted animals. This was confirmed by means of densitometric analysis and is depicted graphically in A for NF- κ B and in C for AP-1.

rophage depletion using clodronate-encapsulated liposomes that are addressed in this study, we have also recently demonstrated marked protection against LIRI with gadolin-

ium in our warm ischemia model (unpublished data). Macrophage depletion with liposomes is an effective tool because liposomes are avidly engulfed on contact with the

Figure 5. ELISAs for the chemokines MIP-1 α , CINC, and MIP-1 and the cytokine TNF- α . This panel of graphs demonstrates the marked increases in chemokine and cytokine content in BAL fluid from injured left lungs. There are significant decreases in the elaboration of all 3 chemokines, as well as TNF- α in BAL fluid from macrophage-depleted animals (n = 4).

AM.¹⁴ The intracellular release of clodronate induces apoptosis,¹⁴ with minimal effect on surrounding cells. Gadolinium, however, has the advantage of inactivating both alveolar and pulmonary interstitial macrophages,^{6,7} possibly through apoptosis.²⁵ Although gadolinium effectively inhibits total body macrophage function, it is toxic when administered systemically.²⁶ Similarly, liposomes containing clodronate are not a practical means of suppressing AMs clinically. Intratracheal liposomes incite a modest inflammatory response, and the long-term effects of AM depletion are unknown. Additionally, this strategy would only target AMs¹⁴ and would still leave a substantial number of interstitial macrophages unaffected. The preferential sparing of interstitial macrophages might in part explain the incomplete protection seen in this study with liposome depletion.

Certain commonly prescribed immunosuppressive drugs might be clinically useful inhibitors of macrophage function. We have previously shown that pretreatment with cyclosporine A (CSA) is an effective means of reducing reperfusion injury¹⁵ and have further demonstrated that the AM chemokine and cytokine response to hypoxia and reoxygenation in vitro is severely blunted by CSA.¹¹ This suggests that in LIRI, the AM is functionally inhibited by CSA. Although in vitro and in vivo AMs have been shown to suppress T-cell function,²⁷ the fear that AM depletion or suppression might exaggerate the rejection process has been refuted.²⁸

Reperfusion injury has traditionally been conceptualized as an inflammatory response to a primary endothelial injury. However, in animal models of LIRI, cessation of blood flow while preserving ventilation of lungs with either air or nitrogen prevented reperfusion injury.²⁹ This suggests that the endothelium might not play the central role in initiating LIRI. The lung macrophage not only resides at the air-tissue interface but is also in close proximity to capillaries, facilitating its ability to influence inflammatory responses central to the development of oxidative lung injury. AMs are known to secrete early proinflammatory mediators, such as TNF- α and interleukin 1 β with ischemia and reperfusion, which, in turn, primes the endothelium.¹⁸ Additionally, AMs release oxygen- and nitrogen-centered free radicals, promote the chemotactic attraction of neutrophils, and, ultimately, appear more likely to be the centrally important effector cell in the development of LIRI.

References

1. Meyers BF, Lynch J, Trulock EP, Guthrie TJ, Cooper JD, Patterson GA. Lung transplantation: a decade of experience. *Ann Surg*. 1999; 230:362-70.
2. Jamieson SW, Auger WR, Fedullo PF, et al. Experience and results with 150 pulmonary thromboendarterectomy operations over a 29-month period. *J Thorac Cardiovasc Surg*. 1993;106:116-27.
3. Eppinger MJ, Jones ML, Deeb M, et al. Pattern of injury and the role of neutrophils in Reperfusion injury of Rat Lung. *J Surg Res*. 1995; 58:713-8.

4. Eppinger MJ, Deeb GM, Bolling SF, Ward PA. Mediators of ischemia-reperfusion injury of rat lung. *Am J Pathol.* 1997;150:1773-84.
5. Lentsch AB, Czermak BJ, Bless NM, Van Rooijen N, Ward PA. Essential role of alveolar macrophages in intrapulmonary activation of NF- κ B. *Am J Respir Cell Mol Biol.* 1999;20:692-8.
6. Fujii Y, Goldberg P, Hussain SN. Contribution of macrophages to pulmonary nitric oxide production in septic shock. *Am J Respir Crit Care Med.* 1998;157:1645-51.
7. Pendino KJ, Meidhof TM, Heck DE, Laskin JD, Laskin DL. Inhibition of macrophages with gadolinium chloride abrogates ozone-induced pulmonary injury and inflammatory mediator production. *Am J Respir Cell Mol Biol.* 1995;13:125-32.
8. Tullis MJ, Brown S, Gewertz BL. Hepatic influence on pulmonary neutrophil sequestration following intestinal ischemia-reperfusion. *J Surg Res.* 1996;66:143-6.
9. Berg JT, White JE, Tsan MF. Response of alveolar macrophage-depleted rats to hyperoxia. *Exp Lung Res.* 1995;21:175-85.
10. Limper AH, Hoyte JS, Standing JE. The role of alveolar macrophages in *Pneumocystis carini* degradation and clearance from the lung. *J Clin Invest.* 1997;99:2110-7.
11. Naidu BV, Krishnadasan B, Byrne K, et al. Regulation of chemokine expression by cyclosporine A in alveolar macrophages exposed to hypoxia and re-oxygenation. *Ann Thorac Surg.* 2002;74:899-905.
12. VanOtteren GM, Standiford TJ, Kunkel SL, Danforth JM, Strieter RM. Alterations of ambient oxygen tension modulate the expression of tumor necrosis factor and macrophage inflammatory protein-1 alpha from murine alveolar macrophages. *Am J Respir Cell Mol Biol.* 1995;13:399-409.
13. Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods.* 1994;174(1-2):83-93.
14. Berg JT, Lee ST, Thepen T, Lee CY, Tsan MF. Depletion of alveolar macrophages by liposome-encapsulated dichloromethylenediphosphate. *J Appl Physiol.* 1993;74:2812-9.
15. Krishnadasan B, Naidu B, Farr A, Verrier AD, Rosengart M, Mulligan MS. Preoperative cyclosporine and tacrolimus decrease lung ischemia-reperfusion injury in rats. *J Thorac Cardiovasc Surg.* 2002;123:756-67.
16. Krishnadasan B, Naidu B, Verrier E, Mulligan MS. The role of endogenous IL-4 and IL-10 in lung ischemia reperfusion. *Ann Thorac Surg.* In press.
17. Naidu B, Fraga C, Salzman A, Szabo C, Verrier E, Mulligan MS. Critical role of reactive nitrogen species in lung ischemia-reperfusion injury. *J Heart Lung Transplant.* In press.
18. Van Griensven M, Stalp M, Seekamp A. Ischemia-reperfusion directly increases pulmonary endothelial permeability in vitro. *Shock.* 1999;11:259-63.
19. Kunkel SL, Lukacs NW, Strieter RM, Chensue SW. The role of chemokines in the immunopathology of pulmonary disease. *Forum (Genova).* 1999;9:339-55.
20. Yan SF, Fujita T, Lu J, et al. Egr-1, a master switch coordinating upregulation of divergent gene families underlying ischemic stress. *Nat Med.* 2000;6:1355-61.
21. Faller DV. Endothelial cell responses to hypoxic stress. *Clin Exp Pharmacol Physiol.* 1999;26:74-84.
22. Brody AR. Whither goes the alveolar macrophage? Another small chapter is written on the localized response of this crucial cell. *J Lab Clin Med.* 1998;131:391-2.
23. Ward PA. Phagocytes and the lung. *Ann N Y Acad Sci.* 1997;832:304-10.
24. Fiser SM, Tribble CG, Long SM, et al. Lung transplant reperfusion injury involves pulmonary macrophages and circulating leukocytes in a biphasic response. *J Thorac Cardiovasc Surg.* 2001;121:1069-75.
25. Mizgerd JP, Molina RM, Stearns RC, Brain JD, Warner AE. Gadolinium induces macrophage apoptosis. *J Leukoc Biol.* 1996;59:189-95.
26. Spencer AJ, Wilson SA, Batchelor J, Reid A, Rees J, Harpur E. Gadolinium chloride toxicity in the rat. *Toxicol Pathol.* 1997;25:245-55.
27. Strickland DH, Thepen T, Kees UR, Kraal G, Holt PG. Regulation of T-cell function in lung tissue by pulmonary alveolar macrophages. *Immunology.* 1993;80:266-72.
28. Sekine Y, Bowen LK, Heidler KM, et al. Role of passenger leukocytes in allograft rejection: effect of depletion of donor alveolar macrophages on the local production of TNF-alpha, T helper 1/Thelper 2 cytokines, IgG subclasses, and pathology in a rat model of lung transplantation. *J Immunol.* 1997;159:4084-93.
29. Sakuma T, Takahashi K, Ohya N, et al. Ischemia-reperfusion lung injury in rabbits: mechanisms of injury and protection. *Am J Physiol.* 1999;276:L137-45.