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Alveolar macrophage activation is a key initiation signal for acute lung ischemia-reperfusion injury

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Zhao, Minqing, Lucas G. Fernandez, Allan Doctor, Ashish K. Sharma, Alexander Zarbock, Curtis G. Tribble, Irving L. Kron, and Victor E. Laubach. Alveolar macrophage activation is a key initiation signal for acute lung ischemia-reperfusion injury. *Am J Physiol Lung Cell Mol Physiol* 291: L1018–L1026, 2006. First published July 21, 2006; doi:10.1152/ajplung.00086.2006.—Lung ischemia-reperfusion (I/R) injury is a biphasic inflammatory process. Previous studies indicate that the later phase is neutrophil-dependent and that alveolar macrophages (AMs) likely contribute to the acute phase of lung I/R injury. However, the mechanism is unclear. AMs become activated and produce various cytokines and chemokines in many inflammatory responses, including transplantation. We hypothesize that AMs respond to I/R by producing key cytokines and chemokines and that depletion of AMs would reduce cytokine/chemokine expression and lung injury after I/R. To test this, using a buffer-perfused, isolated mouse lung model, we studied the impact of AM depletion by liposome-clodronate on I/R-induced lung dysfunction/injury and expression of cytokines/chemokines. I/R caused a significant increase in pulmonary artery pressure, wet-to-dry weight ratio, vascular permeability, tumor necrosis factor (TNF)- α , monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-2 expression, as well as decreased pulmonary compliance, when compared with sham lungs. After AM depletion, the changes in each of these parameters between I/R and sham groups were significantly attenuated. Thus AM depletion protects the lungs from I/R-induced dysfunction and injury and significantly reduces cytokine/chemokine production. Protein expression of TNF- α and MCP-1 are positively correlated to I/R-induced lung injury, and AMs are a major producer/initiator of TNF- α , MCP-1, and MIP-2. We conclude that AMs are an essential player in the initiation of acute lung I/R injury.

pulmonary transplantation; clodronate; inflammation; chemokines

ISCHEMIA-REPERFUSION (I/R) is an unavoidable consequence of transplantation, and I/R-induced lung injury is an inflammatory response. A major characteristic of this injury is leukocyte infiltration in the lungs, which play a critical role in the pathophysiology of I/R injury (15, 41). However, we and others (16, 18) have demonstrated that lung I/R injury occurs as a biphasic response in which the acute phase is neutrophil independent. This finding is important because it reinforces the concept that resident lung cells (i.e., cells residing in the donor lung rather than circulating leukocytes from the recipient) initiate I/R injury upon transplantation. Using a blood-perfused rabbit lung model, we have demonstrated that pulmonary macrophages within the donor lung contribute to acute I/R injury (18).

Alveolar macrophages (AMs) play an important function in the primary defense system (3, 28). Once activated, AMs produce a variety of bioactive products such as tumor necrosis factor (TNF)- α , monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-2 (4, 25). A recent study observed a significant increase in the number of AMs in bronchoalveolar lavage (BAL) fluid that occurred as early as 1 h after hypoxia treatment (29). Cytokines and chemokines contribute greatly to the inflammatory response, and their expression is essential for trafficking of leukocytes to sites of inflammation (15, 17, 28, 31). In human lung transplantation, TNF- α , interferon (IFN)- γ , interleukin (IL)-8, IL-10, IL-12, and other pro- and anti-inflammatory cytokines/chemokines are elevated in the lung (11). In response to oxidative stress, AMs release proinflammatory cytokines and chemokines such as TNF- α , IFN- γ , and MIP-2 (a rodent analog of IL-8) (9, 36). Anti-TNF- α antibody pretreatment results in marked reduction of leukocyte infiltration and injury after lung I/R (24, 26). Using a buffer-perfused mouse lung model, our laboratory recently demonstrated that TNF- α -deficient mice undergo significant improvement of lung function after I/R (32). These studies strongly imply that AM-produced cytokines/chemokines play a critical role in the induction of acute lung I/R injury.

Given the ability of cytokine/chemokine production by AMs and the critical roles of these chemokines in leukocyte recruitment and activation during inflammation, we investigated whether selective depletion of AMs would protect the lung from acute I/R injury. To assess the impact of AMs on lung injury in the absence of circulating leukocytes, we used an isolated, buffer-perfused mouse lung model of I/R injury that eliminates nonmarginated (circulating) leukocytes from the pulmonary circulation. Using this model, and utilization of a well-established macrophage depletion technique, we studied: 1) the kinetics of lung injury and function after I/R and macrophage depletion, 2) corresponding expression of key cytokines/chemokines, and 3) whether AMs are a major source of these cytokines/chemokines. We hypothesized that depletion of AMs would protect the lung from acute I/R injury and reduce the expression of specific proinflammatory cytokines/chemokines.

MATERIALS AND METHODS

Study design and animals. With the use of an isolated lung model, lungs from male C57BL6 mice (25–30 g body wt) from Jackson

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Laboratory (Bar Harbor, ME) were subjected to 60 min ischemia followed by 60 min reperfusion with Krebs-Henseleit buffer (Sigma, St. Louis, MO). As a control, sham lungs received 120 min of reperfusion without ischemia, and the final 60 min of sham perfusion were compared with the 60 min of reperfusion after ischemia. Four groups of animals were used. Two groups of mice were pretreated 24 h before experimentation with liposome-encapsulated clodronate (lip-clo) to deplete AMs. One of these two groups was subjected to I/R (I/R/Clo) whereas the other was the corresponding sham group (Sham/Clo). The remaining two groups were without AM depletion and were pretreated with liposome-encapsulated PBS (lip-PBS) 24 h before experimentation. One of these groups underwent I/R (I/R/PBS), whereas the other was the corresponding sham group (Sham/PBS). Animal acquisition was under the supervision of the Center for Comparative Medicine and a licensed veterinarian. All animals received humane care in compliance with the "Principles of Laboratory Animal Care," formulated by the National Society for Medical Research, and *The Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Science and published by the National Institutes of Health.

Isolated perfused lung model. For this study, we used an isolated, buffer-perfused mouse lung system (Hugo Sachs Elektronik, March-Huggstetten, Germany) previously described (32). Mice were anesthetized with ketamine and xylazine. A tracheostomy was performed, and animals were ventilated with room air at 100 breaths/min and a tidal volume of 7 μ l/g body wt with a positive end-expiratory pressure of 2 cmH₂O. A midline abdominal incision was made, and the inferior vena cava was cannulated with a 30-gauge needle and injected with 50 units of heparin. The animals were exsanguinated by inferior caval transection. The subdiaphragmatic portion of the animal was excised and discarded. The anterior chest plate was removed, exposing the lungs and heart. With the use of an operating microscope, the thymus was dissected cephalad exposing the great vessels. A 4–0 silk suture was passed behind the pulmonary artery (PA) and aortic root. A partial half-knot was created with the suture, leaving room for a cannula to be passed in the PA. A small curvilinear incision was made in the right ventricular outflow tract with the perfusate flowing at 0.2 ml/min, and the PA cannula was passed under direct vision through the pulmonary valve and in the main PA. Next, the partial half-knot was tightened. The left ventricle was immediately vented with a small incision at the apex of the heart. The mitral apparatus was carefully dilated, and the left atria cannula was passed through the mitral valve and in the left atrium. The placement of the PA and left atrial cannulas was further confirmed by pressure tracings generated by the PULMODYN data acquisition system (Hugo Sachs Elektronik). The lungs were then perfused at a constant flow of 60 μ l·g body wt⁻¹·min⁻¹ with Krebs-Henseleit buffer containing 2% albumin, 0.1% glucose, and 0.3% HEPES (335–340 mosmol/kgH₂O). The Krebs solution was prepared to mimic mixed venous blood using an oxygenator (Living Systems Instrumentation, Burlington, VT) with titrated gases generating a pH of 7.35–7.40, a P_O₂ of 60–70 mmHg, and a P_{CO}₂ of 50–60 mmHg. The buffered perfusate and isolated lungs were maintained at 37°C throughout the experiment by use of a circulating water bath.

All lungs were allowed to equilibrate during a 15-min stabilization period. After equilibration, the ventilation rate was decreased to 50 breaths/min, and the fraction of inspired oxygen was decreased to <1%. To initiate the ischemic period, hypoxic ventilation was maintained with 95% nitrogen and 5% carbon dioxide, and perfusion was arrested. After 60 min of ischemia and hypoxic ventilation, the perfusion and room air ventilation were then resumed, initiating the reperfusion period. Hemodynamic and pulmonary parameters were recorded throughout the reperfusion period by the PULMODYN data acquisition system (Hugo Sachs Elektronik). Ventilation with hypoxic gas, rather than stopping ventilation altogether, during ischemia was used to avoid atelectasis while still maintaining ischemia. Atelectasis

and reexpansion has been shown to induce injury involving edema, oxidant generation, and apoptosis (13, 42, 46). This reexpansion-induced injury could obscure the effects of I/R injury, an issue we wished to avoid.

AM depletion. The lip-clo and lip-PBS were prepared as described by Van Rooijen and Sanders (45). Briefly, 0.86 ml egg lecithin (100 mg/ml in chloroform) and 8 mg cholesterol (in 10 ml chloroform) were combined in a 500-ml round bottom flask. The chloroform phase was then removed by vacuum rotation evaporation, resulting in a thin milky phospholipid film on the inner wall of the flask. The phospholipid film was dispersed in 10 ml clodronate (0.25 g/ml in PBS for lip-clo preparation) or 10 ml PBS (for lip-PBS preparation). This suspension was incubated at room temperature for 2 h under nitrogen gas, sonicated for 3 min, and maintained at 4°C overnight under nitrogen gas. The nonencapsulated clodronate was then removed by centrifugation (10,000 g). The lip-clo and the lip-PBS were washed (with PBS) and collected (by centrifugation) three times. Finally, the pellets of lip-clo and lip-PBS were resuspended with 4 ml PBS and stored in nitrogen gas-filled bottles at 4°C. All of these reagents were purchased from Sigma.

The mice were anesthetized with halothane, and tracheas were exposed by surgical resection. The animals were placed supine, with the head elevated ~30°. To efficiently deliver the lip-clo (or lip-PBS) solution to the alveolus and avoid airway blockage by the solution, we improved the technique of intratracheal administration. A 100- μ l lip-clo solution (containing 0.25 g/ml clodronate) followed by 100 μ l air in a 1.0-ml syringe was administered intratracheally with one stroke injection in the lungs of the AM-depleted groups. In the same way, the non-AM-depleted groups were administered 100 μ l lip-PBS instead. Using this delivery method, we found that the 100 μ l air are sufficient to deliver the solution in the airspace of the lung with no observed mortality. With the use of this technique, efficient and even distribution throughout the lungs was documented by instillation of an Evans blue dye solution, which resulted in an even distribution of staining within both lungs. After instillation, the neck incision was closed with sterile sutures, and mice were allowed free access to food and water. Later (24 h), the animals were then used in the experiment.

BAL fluid collection and cell counts. After perfusion, the lungs were lavaged with 0.5 ml PBS. This procedure was performed three times, and the fluid was pooled together. An average of 1.3 ml total BAL fluid was collected from each mouse. The BAL fluid was immediately centrifuged at 4°C (1,500 g, 5 min). The supernatant was collected and stored at –80°C until further assay. To calculate the percent decrease in AM numbers by liposome-clodronate, 100 μ l BAL fluid from each mouse were fixed upon slides by cytocentrifugation (Shandon Cytospin centrifuge; Thermo Electron, Waltham, MA), and the slides were stained with Diff-Quick (HEMA 3 Stain Set; Biochemical Sciences). Macrophages were then identified and counted under microscopy (\times 20 magnification) in three random fields-of-view per slide. The counter was blinded as to group identities.

Lung wet-to-dry weight ratio. Lung wet-to-dry weight ratio was used as an indicator of pulmonary edema using separate groups of animals ($n = 6$ /group). The lower lobe of the right lung from each animal 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 calculated as the wet-to-dry weight ratio. The remaining lung tissue was frozen in liquid nitrogen and stored at –80°C for RNA extraction.

Total RNA isolation and semiquantitative RT-PCR. RNA from lung tissue was extracted by homogenization of tissue in Trizol solution (Roche, Indianapolis, IN) according to the manufacturer's instructions. The RNA was alcohol precipitated, and the pellet was dissolved in diethylpyrocarbonate-treated water. Total RNA was determined by spectrometric analysis at 260 nm, and purity was evaluated by the ratio of 260/280 nm. Total RNA (5 μ g) was

reverse transcribed into cDNA, and amplification was performed using an RT-PCR kit (Promega, Madison, WI). The PCR primer pair sequences for mouse TNF- α , MCP-1, and MIP-2 were as follows: 5'-CTT CCA GAC TCC AGG CGG-3' (sense) and 5'-GCT ACG ACG TGG GCT ACA G-3' (antisense) for TNF- α ; 5'-TTA AAA ACC TGG ATC GGA ACC AA-3' (sense) and 5'-GCA TTA GCT TCA GAT TTA CGG GT-3' (antisense) for MCP-1; 5'-GAA CTG CGC TGT CAA TGC CT-3' (sense) and 5'-GTT AGC CTT GCC TTT GTT CA-3' (antisense) for MIP-2. The specific primer sequences 5'-GAA ATC GTG CGT GAC ATC AAA G-3' (sense) and 5'-TGT AGT TTC ATG GAT GCC ACA G-3' (antisense) for the housekeeping gene, β -actin, were used as an internal control. The cDNA was amplified after determining optimal conditions. RT-PCR product (20 μ l) was separated on 2% agarose and stained with ethidium bromide. The gels were then photographed, and PCR products were quantified with FluorChem IS-8900 System software (Alpha Innotech, San Leandro, CA) and normalized to the housekeeping gene β -actin to represent the mRNA level of each cytokine/chemokine. The background optical density reading for each band was subtracted locally. Each assay was conducted three times to ensure reproducibility.

ELISA for TNF- α , MCP-1, and MIP-2. The protein levels of TNF- α , MCP-1, and MIP-2 in BAL fluid were measured in triplicate using ELISA kits for mice according to the manufacturer's instructions. The ELISA kits for TNF- α and MCP-1 were purchased from BD Biosciences (San Diego, CA), and the MIP-2 ELISA kit was purchased from R&D Systems (Minneapolis, MN).

Lung vascular permeability. Disruption of the vascular permeability barrier is a significant pathological consequence of acute lung injury. As another indicator of lung injury, lung vascular permeability was assessed using separate groups of animals ($n = 6$ /group). We took advantage of the isolated, buffer-perfused, lung I/R model since it allowed precise control of perfusion rate. At the completion of reperfusion, the perfusion solution (Krebs solution) was replaced with 30 mg/ml BSA solution (in PBS) and perfused through the lungs at the same perfusion flow rate as during reperfusion for 3 min. After this, the perfusion solution was changed back to Krebs solution, and perfusion was continued for an additional 5 min to wash out the extra BSA solution from the lung vasculature. Using a BSA ELISA kit (Bethyl Laboratories, Montgomery, TX), BSA concentration in BAL fluid was measured according to the manufacturer's instructions. The sensitivity of the kit is 6.25 ng/ml. Each sample was measured in triplicate.

Neutrophil quantification in the lung. To quantitate the number of neutrophils in the lung, lungs were removed, minced, and digested with 125 U/ml collagenase type XI, 60 U/ml hyaluronidase type I-S, and 60 U/ml DNase 1 (all Sigma) at 37°C for 60 min. A cell suspension was made by passing the digested lungs through a 70- μ m cell strainer (BD Falcon, Bedford, MA). Remaining erythrocytes were lysed, and remaining cells were resuspended and counted. The fraction of leukocytes in the suspension was determined by flow cytometry (FACS Calibur; Becton Dickinson, San Jose, CA). Neutrophils were identified by their typical appearance in the forward/sideward scatter and their expression of CD45 (clone 30-F11; BD Biosciences-Pharmingen), clone 7/4 (BD Biosciences-Pharmingen), and GR-1 (clone RB6-8C5). Isotype controls were used to compensate for nonspecific antibody binding.

Statistical analysis. Data are presented as means \pm SE. ANOVA was used to determine if significant differences existed between groups. Tukey's honest significant difference multiple-comparison test was used to determine which groups were significantly different when the ANOVA results were significant. Two-way repeated-measures ANOVA was used to compare pulmonary and hemodynamic data during reperfusion. Data were considered significant at $P < 0.05$.

RESULTS

AM depletion by lip-clo. Depletion of AMs by lip-clo was verified by differential counting of cells in BAL fluid 24 h after intratracheal treatment. Mice treated with PBS alone had an average of 364 ± 26 AMs/3 fields-of-view. Mice treated with lip-PBS had an average of 358 ± 11 AMs/3 fields-of-view, whereas mice treated with lip-clo had an average of 74 ± 17 AMs/3 fields-of-view. Thus lip-PBS treatment did not alter AM numbers, whereas lip-clo treatment depleted AMs by 79.4%.

AM depletion prevents I/R-elevated pulmonary artery pressure. In lungs without AM depletion, I/R significantly increased pulmonary artery pressure (PAP) throughout reperfusion compared with sham lungs (IR/PBS vs. Sham/PBS, $n = 8$ /group, $P < 0.001$; Fig. 1). However, the AM-depleted lungs (IR/Clo) displayed no significant increase in PAP above sham (Sham/Clo). The *time 0* point of reperfusion represents a few seconds after initiation of reperfusion. The ischemic lungs react to the reestablishment of perfusion by undergoing an immediate, but short-lived, rise in PAP compared with corresponding sham lungs. These data indicate that AM depletion prevents I/R-induced increases in PAP.

Pulmonary compliance is improved by AM depletion. I/R resulted in a significant decrease of pulmonary compliance in the IR/PBS lungs compared with Sham/PBS ($n = 8$ /group, $P < 0.01$; Fig. 2). However, the significant difference between I/R and sham groups was neutralized by AM depletion (IR/Clo vs. Sham/Clo; Fig. 2). Interestingly, pulmonary compliance in the AM-depleted lungs was lower than the corresponding non-AM-depleted lungs (Sham/Clo vs. Sham/PBS).

AM depletion prevents I/R-induced increase in wet-to-dry weight ratio. To evaluate I/R-induced lung edema, lung wet-to-dry weight ratio was measured after reperfusion (Fig. 3A). In the I/R group, without AM depletion, the ratio was significantly higher than the sham group (IR/PBS vs. Sham/PBS, $P = 0.0015$, $n = 6$ /group). However, the significant difference between I/R and sham lungs was abolished in AM-depleted lungs (IR/Clo vs. Sham/Clo, $P = 0.9936$). The wet-to-dry ratios did not differ between the two sham groups (Sham/PBS vs. Sham/Clo, $P = 0.9092$). However, a significant decrease was observed in the IR/Clo group vs. IR/PBS ($P = 0.0005$). These results indicate that AM depletion protects the lung from I/R-induced edema.

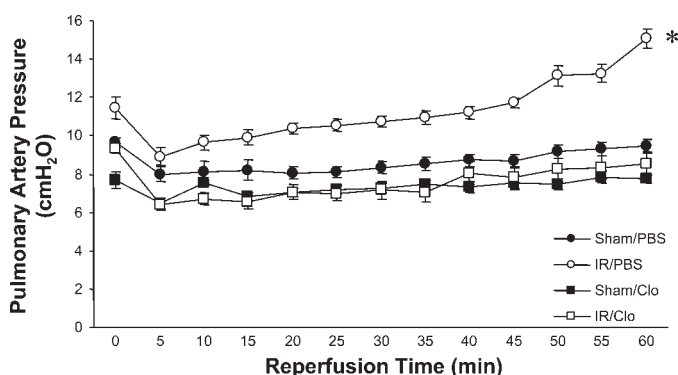


Fig. 1. Dynamic changes in pulmonary artery pressure (PAP) during 60 min reperfusion. Sham/PBS, sham lung without alveolar macrophage (AM) depletion; IR/PBS, ischemia-reperfusion (I/R) lung without AM depletion; Sham/Clo, sham lung, AM depleted with clodronate; IR/Clo, I/R lung, AM depleted with clodronate. The PAP in the IR/Clo group is reduced to the level similar to sham group (Sham/Clo). * $P < 0.001$, IR/PBS vs. Sham/PBS and IR/Clo.

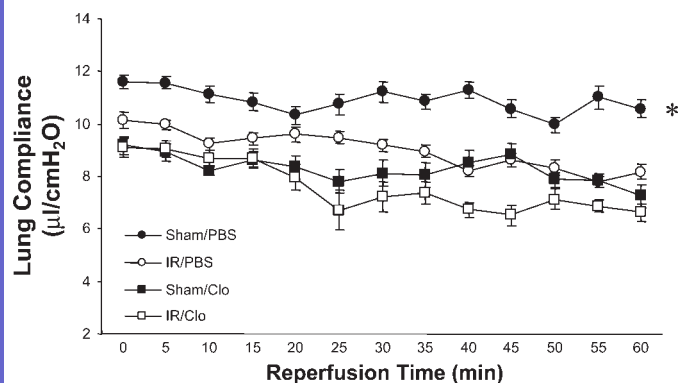


Fig. 2. Dynamic changes in pulmonary compliance during 60 min reperfusion. I/R induced a significant change in pulmonary compliance in non-AM-depleted lungs (* $P < 0.01$, IR/PBS vs. Sham/PBS). In AM-depleted lungs, there was no significant difference between sham and I/R (IR/Clo vs. Sham/Clo).

AM depletion prevents increased pulmonary vascular permeability after I/R. To additionally assess I/R-induced lung injury, we assessed vascular permeability by directly measuring BSA concentration in BAL fluid after reperfusion. In the lungs without AM depletion, I/R increased BSA concentration threefold vs. sham (IR/PBS vs. Sham/PBS, $P < 0.0001$; Fig. 3B). This increase of vascular permeability induced by I/R was attenuated after AM depletion (IR/Clo vs. Sham/Clo, $P = 0.0823$). In other words, the BSA concentration in the IR/Clo group was significantly decreased compared with the IR/PBS lungs ($P < 0.0001$) to levels similar to Sham/Clo. There was no difference between the two sham groups. These results suggest that AMs contribute to pulmonary vascular leakage during acute lung I/R injury, and AM depletion reduces pulmonary vascular permeability.

Effects of AM depletion on TNF- α , MCP-1, and MIP-2 protein expression. ELISA analysis of BAL fluid showed that I/R significantly elevated TNF- α protein by twofold compared with sham (IR/PBS vs. Sham/PBS, $P < 0.0001$; Fig. 4A). This high level of TNF- α expression was dramatically decreased in AM-depleted lungs after I/R to where there was no significant difference between Sham/Clo and IR/Clo groups ($P = 0.1810$, $n = 6$ /group). These data indicate that AM depletion reduced the capacity for TNF- α production after I/R.

Similar to TNF- α , MCP-1 in BAL fluid was significantly increased more than threefold after I/R (IR/PBS vs. Sham/PBS, $P < 0.0001$; Fig. 4B). This elevated MCP-1 expression was prevented after AM depletion to where the levels did not differ significantly from sham (IR/Clo vs. Sham/Clo, $P = 0.1960$). There was no significant difference observed between the sham groups (Sham/PBS vs. Sham/Clo, $P = 0.2674$), but MCP-1 was significantly reduced in the IR/Clo group vs. the IR/PBS group ($P < 0.0001$). These results demonstrate that I/R-induced MCP-1 expression in BAL requires the presence of AMs in the lung.

Unlike TNF- α and MCP-1, MIP-2 protein expression showed a significant difference between the two sham groups. Here, MIP-2 protein level was significantly lower in the AM-depleted sham lungs (Sham/Clo vs. Sham/PBS, $P = 0.0007$; Fig. 4C). A significant elevation of MIP-2 expression occurred in both I/R groups compared with their corresponding shams (IR/PBS vs. Sham/PBS, $P < 0.0001$; and IR/Clo vs. Sham/Clo,

$P < 0.0001$; Fig. 4C). Importantly, the level of MIP-2 was significantly reduced in the AM-depleted I/R lungs (IR/Clo vs. IR/PBS, $P < 0.0001$). These results show that AM depletion reduces MIP-2 protein expression regardless of I/R.

Expression of TNF- α , MCP-1, and MIP-2 mRNA after AM depletion. To further confirm the impact of AM depletion on TNF- α , MCP-1, and MIP-2 expression, mRNA levels were assessed by semiquantitative RT-PCR (Fig. 5). In the IR/PBS group, TNF- α mRNA was induced 2.5-fold vs. the Sham/PBS group (Fig. 5A). The AM-depleted lungs (IR/Clo) showed only a 1.7-fold increase in TNF- α mRNA vs. the Sham/Clo lungs. MCP-1 (Fig. 5B) and MIP-2 (Fig. 5C) mRNA showed similar changes to what was observed for TNF- α . After I/R, MCP-1 was elevated by 3.6-fold and MIP-2 by 4.1-fold, whereas AM depletion reduced these increases to 1.6-fold and 1.8-fold, respectively. These results indicate that TNF- α , MCP-1, and MIP-2 mRNA levels in lung tissue are positively correlated with their protein levels in BAL fluid. Depletion of AMs significantly downregulated the mRNA level, thus suggesting

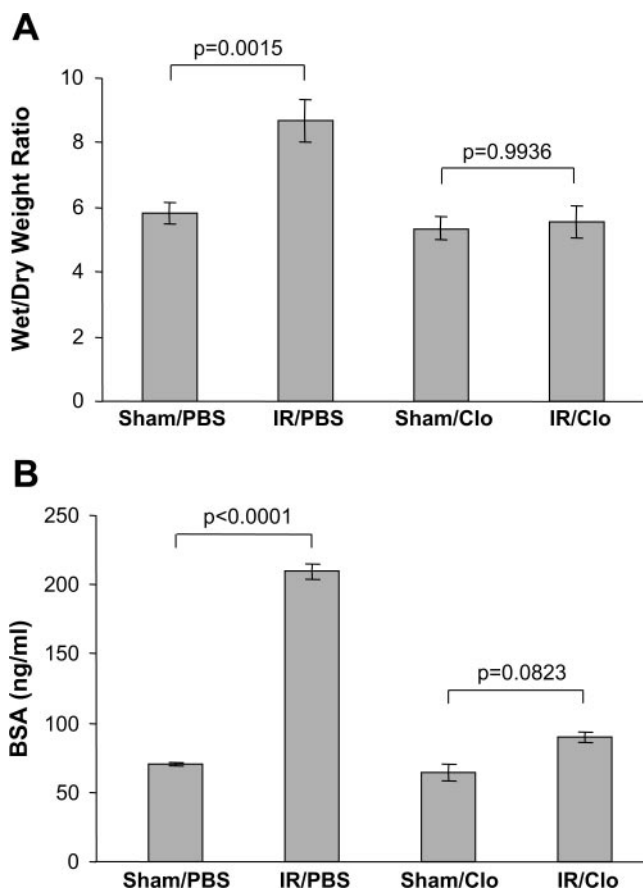


Fig. 3. Lung injury after I/R is attenuated after AM depletion. A: I/R significantly increased wet-to-dry wt ratio in non-AM-depleted lungs (IR/PBS vs. Sham/PBS, $P = 0.0015$). AM depletion abolished the I/R-induced increase in wet-to-dry wt ratio (Sham/Clo vs. IR/Clo, $P = 0.9936$). Wet-to-dry wt ratio was also significantly reduced in IR/Clo vs. IR/PBS ($P = 0.0005$). B: lung vascular permeability was assessed by measuring BSA concentration in bronchoalveolar lavage (BAL) fluid. In non-AM-depleted lungs, I/R significantly increased BSA concentration by 3-fold compared with sham (IR/PBS vs. Sham/PBS, $P < 0.0001$). AM depletion abolished the I/R-induced increase in BAL concentration (Sham/Clo vs. IR/Clo, $P = 0.0823$). BAL concentration was also significantly reduced in IR/Clo vs. IR/PBS ($P < 0.001$).



that the AM is likely a major cellular source of TNF- α , MCP-1, and MIP-2 in I/R-induced acute lung injury.

Neutrophil quantification in the lung. To determine if lung neutrophils are washed out by buffer perfusion in this model, nonperfused lungs were compared with lungs that were buffer-perfused for 20 min ($n = 4$ /group). The lungs were then digested, and total neutrophil counts were determined by flow cytometry as described in MATERIALS AND METHODS. The results

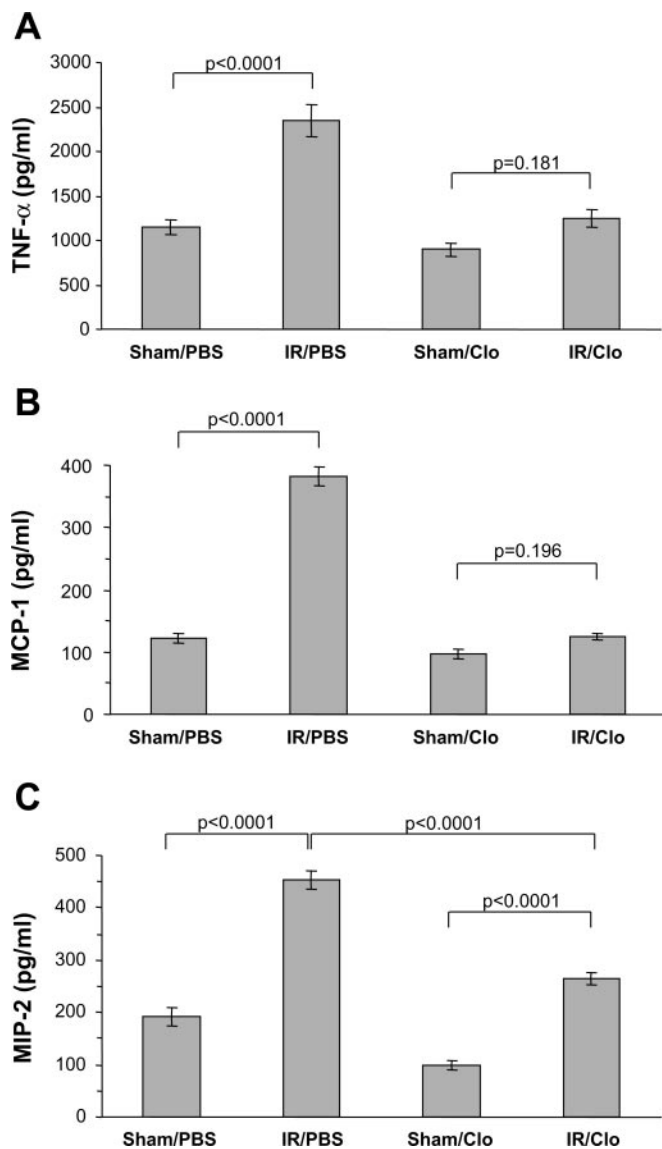


Fig. 4. ELISA analysis of tumor necrosis factor (TNF)- α , monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-2 protein expression in BAL fluid. **A:** TNF- α protein was significantly elevated over 2-fold after I/R in non-AM-depleted lungs (IR/PBS vs. Sham/PBS). In AM-depleted lungs, there was no significant increase in TNF- α protein after I/R compared with sham lungs (IR/Clo vs. Sham/Clo). **B:** MCP-1 protein was significantly elevated over 3-fold after I/R in non-AM-depleted lungs (Sham/PBS vs. IR/PBS). In AM-depleted lungs, there was no significant increase in MCP-1 protein after I/R compared with sham lungs (IR/Clo vs. Sham/Clo). **C:** MIP-2 protein was significantly elevated over 2-fold after I/R in non-AM-depleted lungs (Sham/PBS vs. IR/PBS). However, MIP-2 protein level was reduced in the Sham group treated with liposome-clodronate (Sham/Clo vs. Sham/PBS, $P = 0.0007$). I/R also significantly elevated MIP-2 expression in AM-depleted lungs (IR/Clo vs. Sham/Clo).

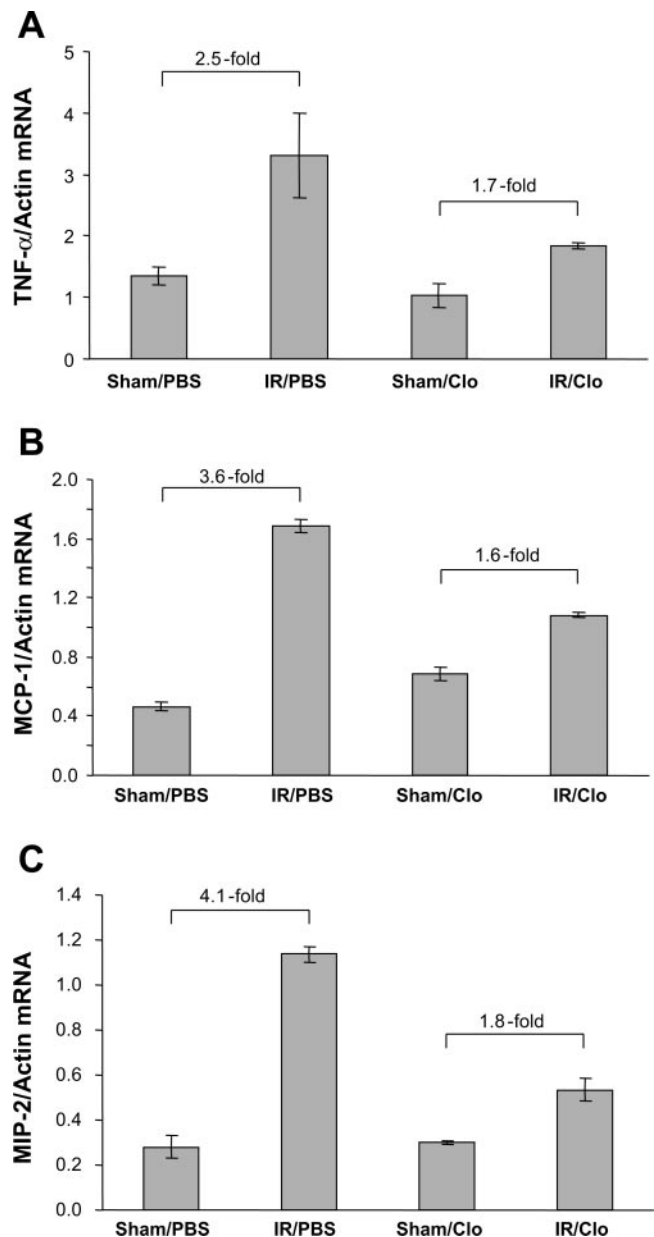


Fig. 5. Expression of mRNA for TNF- α (A), MCP-1 (B), and MIP-2 (C) in lung tissue. Without AM depletion, the mRNA of each of these three cytokine/chemokines was dramatically increased by I/R (IR-PBS vs. Sham-PBS). AM depletion severely reduced the increase in cytokine/chemokine mRNA expression after I/R (IR/Clo vs. Sham/Clo).

indicated that 20 min of buffer perfusion does not wash out a significant number of neutrophils from the lungs (Fig. 6).

DISCUSSION

An isolated, buffer-perfused mouse lung I/R model was employed in this study. By using this model and a well-established AM-depletion technique, we specifically investigated the role of AMs in acute lung I/R injury. The results of this study indicate that: 1) I/R-induced lung dysfunction and injury are attenuated after AM depletion, 2) I/R-induced TNF- α , MIP-2, and MCP-1 protein expression are downregulated after AM depletion, and 3) I/R-induced mRNA expres-

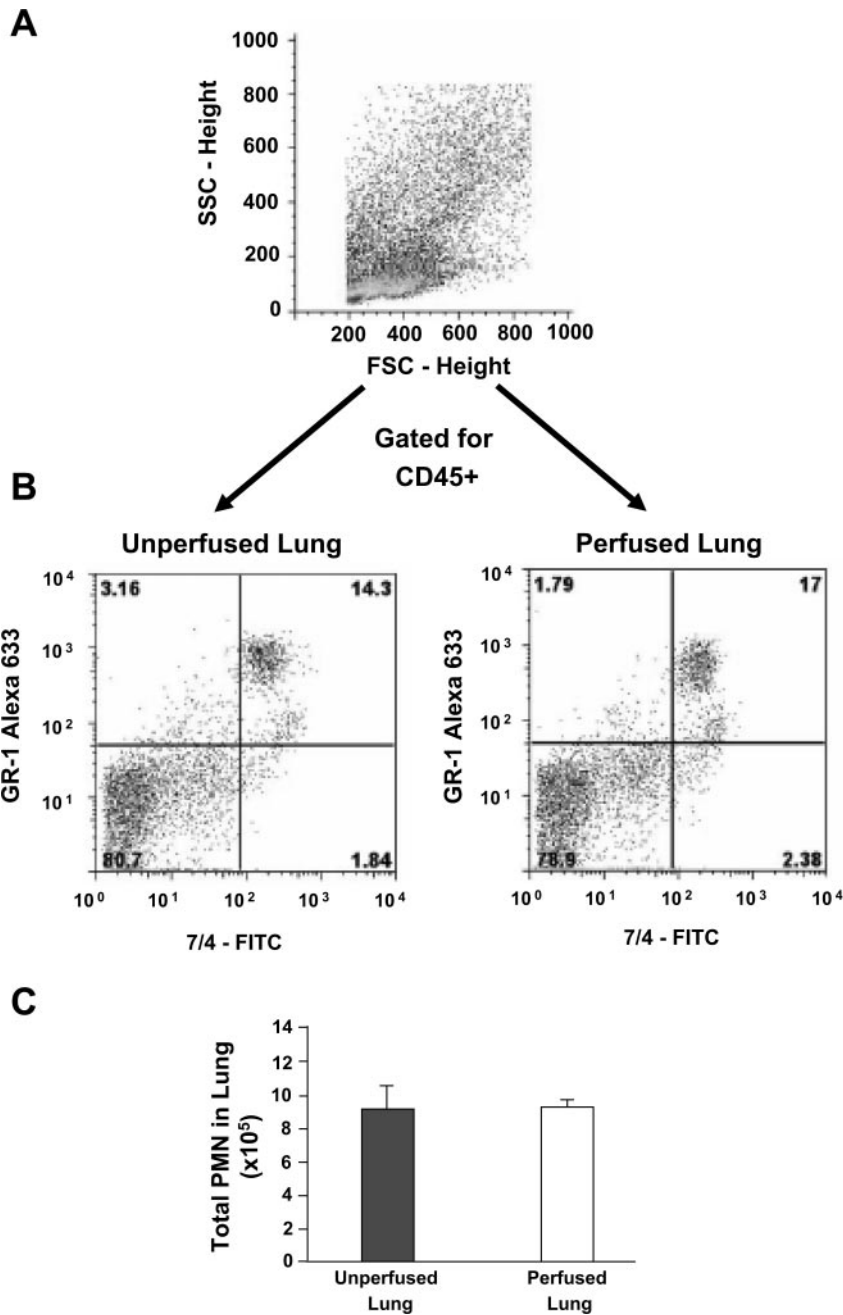


Fig. 6. Neutrophil quantitation in the lung. With the use of flow cytometry, total lung neutrophils were counted in nonperfused lungs vs. lungs that were buffer perfused for 20 min. The total cell population (A) was gated for expression of CD45 and further identified by the expression of clone 7/4 (x-axis in B) and GR-1 (y-axis in B). C: quantitation of total neutrophils in the lung indicating that 20 min of buffer perfusion does not significantly change the neutrophil population within the lung. SSC, sideward scatter; FSC, forward scatter; PMN, polymorphonuclear leukocytes (neutrophils).

sion of these cytokine/chemokines in lung tissue are also suppressed after AM depletion.

Neutrophil counts in the lungs indicated that 20 min of buffer perfusion does not wash out a significant number of neutrophils. This is not too surprising because many of these neutrophils are likely adherent to the pulmonary endothelium, and perhaps a longer perfusion time would wash out more cells. In addition, this is likely a result of the anatomical structure of the pulmonary vascular bed. It has been shown that neutrophils are dramatically slowed relative to the flow of red blood cells because of the need to contract and elongate to fit through narrower capillaries, leading to a high concentration of neutrophils in the pulmonary capillary bed (22). Thus, although there is little removal of neutrophils in this model, there

certainly is no contribution from nonmarginated leukocytes from the pulmonary circulation. It is possible that the marginated neutrophils, which remain even after buffer perfusion, can contribute to I/R injury. This does not affect our conclusion, however, that AMs are key initiators of lung I/R injury.

It is known that macrophages can be activated by many stimuli, including low O₂ tension (33, 43), resulting in expression of various inflammatory cytokines and chemokines (4, 9, 25, 36). The important role of macrophages in I/R injury has been demonstrated in the liver, gut, and heart (6, 23, 44). Our prior studies suggest that AMs play a pivotal function in acute lung I/R injury (18). Recently, the function of AMs in lung I/R injury was evaluated in two conflicting studies (37, 38), each of which utilized lip-clo depletion of

AMs. One study by Nakamura et al. (38) concluded that AM depletion is detrimental to the lungs, whereas a study by Naidu et al. (37) concluded that AM depletion is beneficial. The results of our current study support the results of Naidu et al. since we also show that AM depletion protects the lung from acute I/R lung injury.

There are two possible reasons that may explain these controversial conclusions. First, one source of conflict could be because of the different clodronate pretreatment times used. In Nakamura et al.'s report, the rats received clodronate 72 h before experimentation, and considerable numbers of neutrophils were found in the BAL (38). In our and Naidu et al.'s (37) studies, the animals were pretreated with clodronate for 24 h. It is well-known that neutrophils are critical components of the inflammatory cascade in lung I/R injury (2, 10). In preliminary experiments, we tested the effect of intratracheal administration of lip-clo on neutrophil influx in mouse lungs and observed, 72 h after administration, a large number of neutrophils in the BAL of the lungs that was not observed at 24 h (data not shown). Nakamura et al.'s (38) study described a significant increase in baseline neutrophils in BAL fluid of clodronate-treated animals, and other studies have also reported that lipo-clo treatment can lead to a recruitment of neutrophils in the lung (5, 14). Thus, in our current study, we optimized the lip-clo delivery time point (24 h before experiment) so that there was minimal neutrophil infiltration. In addition, we used a buffer-perfused model that eliminates contribution from non-marginated (circulating) neutrophils.

A second possible reason for the conflicting results is that different control groups were used. In Nakamura et al.'s study, the AM-depleted I/R group (clodronate-treated) was directly compared with a non-AM-depleted I/R group (Hanks' balanced salt solution-treated). However, because no clodronate-treated sham controls were included in this study, this may not be a valid comparison. This is important because clodronate-induced AM death is the result of apoptosis and necrosis, which can contribute to tissue injury and remodeling (40, 45). This effect would be amplified at 72 h after clodronate treatment vs. 24 h.

Of particular interest in our study was AM function during acute lung I/R injury. To help distinguish the effects of I/R stimulation from the effects of apoptotic AMs in the lung, we designed four groups (two groups with AM depletion and two groups without AM depletion) and thus each I/R group had its own sham group as a control. In this way, each of the I/R animals has the exact same treatment as the corresponding sham animals (IR/Clo vs. Sham/Clo and IR/PBS vs. Sham/PBS). This permitted a more accurate evaluation of the role of AM in lung I/R injury.

It is worth commenting on the observation that both lip-clo-treated groups (Sham/Clo and IR/Clo) had lower pulmonary compliance compared with their corresponding lip-PBS-treated groups (Sham/PBS and IR/PBS). The liposome itself has been demonstrated to impose no effect on pulmonary compliance, even when repeatedly administered (7). Our preliminary experiments showed that non-liposome-treated sham lungs (i.e., pretreatment with PBS alone) exhibited the same level of pulmonary compliance as lip-PBS-treated lungs (data not shown), supporting the view that the liposome itself does not have an effect. Therefore, reduced pulmonary compliance in the lip-clo-treated groups indicated that the clodronate itself

(or AM depletion) may have an effect on pulmonary compliance. It has been demonstrated that phospholipase A₂, a major surfactant degradation enzyme, is selectively expressed in AMs (1). We speculate that, during AM depletion, apoptotic or necrotic AMs may release phospholipase A₂ to adjacent alveolar epithelial cells and destroy the surfactant air-liquid interface, resulting in higher alveolar surface tension and, in turn, decreased pulmonary compliance in the clodronate-treated lungs. Moreover, clodronate could have other unknown pharmacological effects that can affect compliance through mechanisms not involving macrophages. However, the use of clodronate is an effective and well-accepted technique for macrophage depletion and has been used successfully in many models (19, 35, 39). Also, we cannot completely eliminate the possibility that the clodronate may have been contaminated with endotoxin and thus participate in a preconditioning effect that could be protective against I/R. We do not believe that this is the case, however, because the Sham/Clo group was included as a control with which to compare the IR/Clo experimental group.

TNF- α , a potent proinflammatory cytokine, is released in response to I/R in the context of lung transplantation. AMs are probably the initial source of TNF- α . Reducing TNF- α levels by inhibition of TNF- α -converting enzyme was shown to prevent rejection of transplanted lungs and lead to reduced chemokine production, such as MCP-1 (20). In lung allograft rejection models, utilizing anti-TNF- α neutralizing antibody, marked reduction of leukocyte infiltration and lung I/R injury was observed (24, 26). Our laboratory has recently demonstrated that TNF- α is a key initiating factor in acute lung I/R injury using TNF- α knockout mice (32). In the present study, we found that the expression of TNF- α positively correlates with I/R-induced lung dysfunction and is significantly down-regulated by AM depletion. Elevated chemokine expression is an essential event for leukocyte infiltration, and MCP-1 is an important chemokine for monocyte recruitment and activation (21, 30, 31, 47) as well as early accumulation of neutrophils at sites of inflammation (30, 31). Furthermore, macrophages from MCP-1 knockout mice showed a significant increase in the expression of RANTES, MIP-1 β , MIP-1 α , and MIP-2 mRNA (17). This suggests that MCP-1 has a critical regulatory function to many chemokines. Given these important functions of MCP-1, our results showed that MCP-1 expression is positively correlated with I/R-induced lung dysfunction and is significantly diminished by AM depletion. These observations strongly indicate that AMs are a critical producer/initiator of TNF- α and MCP-1.

MIP-2, a murine analog of IL-8 that is expressed by many cells in the lung, is also a potent chemoattractant for neutrophil recruitment and activation (16, 34, 43). In the current study, we observed that, unlike TNF- α and MCP-1, MIP-2 protein expression remained significantly elevated in IR/Clo lungs, although MIP-2 was also significantly reduced compared with IR/PBS. In other words, MIP-2 elevation after I/R was not completely prevented by macrophage depletion but was intermediately reduced. We and others (12, 47) have demonstrated that MIP-2 can be produced by nonimmune cells such as epithelial and endothelial cells in response to TNF- α , which may help explain why MIP-2 levels remained significantly increased in IR/Clo lungs. It is also interesting that the AM-depleted sham lungs (Sham/Clo) displayed reduced

MIP-2 protein level compared with the non-AM-depleted sham lungs (Sham/PBS). MIP-2 mRNA level, however, was found to be similar in these two groups, indicating that clodronate treatment directly inhibits the basal level of MIP-2 protein expression.

Although AMs can produce cytokines/chemokines upon activation, non-AM cells in the lung such as alveolar epithelial cells can also produce cytokines/chemokines in response to leukocyte-released proinflammatory cytokines (8, 27, 47). Our results show that AM depletion dramatically downregulates cytokine/chemokine protein and mRNA expression. Taken together, our results indicate that AM depletion is protective against acute I/R-induced lung dysfunction and injury. AMs are a major producer/initiator of TNF- α , MCP-1, and MIP-2. The protein expression levels of TNF- α and MCP-1 are positively correlated to I/R-induced lung dysfunction and injury. Therefore, this study indicates that AMs are essential contributors to the early phase of I/R-induced lung injury.

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