

# Inflammation and ischemia-induced lung angiogenesis

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**Wagner EM, Sánchez J, McClintock JY, Jenkins J, Moldobaeva A.** Inflammation and ischemia-induced lung angiogenesis. *Am J Physiol Lung Cell Mol Physiol* 294: L351–L357, 2008. First published December 21, 2007; doi:10.1152/ajplung.00369.2007.—A role for inflammation in modulating the extent of angiogenesis has been shown for a number of organs. The present study was undertaken to evaluate the importance of leukocyte subpopulations for systemic angiogenesis of the lung after left pulmonary artery ligation (LPAL) in a mouse model of chronic pulmonary thromboembolism. Since we (24) previously showed that depletion of neutrophils did not alter the angiogenic outcome, we focused on the effects of dexamethasone pretreatment (general anti-inflammatory) and gadolinium chloride treatment (macrophage inactivator) and studied Rag-1<sup>-/-</sup> mice (T/B lymphocyte deficient). We measured inflammatory cells in bronchoalveolar lavage fluid and lung homogenate macrophage inflammatory protein-2 (MIP-2) and IL-6 protein levels within 24 h after LPAL and systemic blood flow to the lung 14 days after LPAL with labeled microspheres as a measure of angiogenesis. Blood flow to the left lung was significantly reduced after dexamethasone treatment compared with untreated control LPAL mice (66% decrease;  $P < 0.05$ ) and significantly increased in T/B lymphocyte-deficient mice (88% increase;  $P < 0.05$ ). Adoptive transfer of splenocytes (T/B lymphocytes) significantly reversed the degree of angiogenesis observed in the Rag-1<sup>-/-</sup> mice back to the level of control LPAL. Average number of lavaged macrophages for each group significantly correlated with average blood flow in the study groups ( $r^2 = 0.9181$ ;  $P = 0.01$  different from 0). Despite differences in angiogenesis, left lung homogenate MIP-2 and IL-6 did not differ among study groups. We conclude that inflammatory cells modulate the degree of angiogenesis in this lung model where lymphocytes appear to limit the degree of neovascularization, whereas monocytes/macrophages likely promote angiogenesis.

dexamethasone; gadolinium chloride; T lymphocytes; Rag-1<sup>-/-</sup>

ANGIOGENESIS IN THE LUNG REQUIRES remodeling and growth of the systemic vasculature and becomes prominent in several pathological states where chronic inflammation prevails such as cystic fibrosis (5), asthma (23), and chronic pulmonary thromboembolic disease (3, 11, 20, 26). Pulmonary ischemia resulting from chronic pulmonary thromboembolism or other forms of pulmonary artery obstruction leads to proliferation of the systemic circulation within and surrounding the lung. In experimental animals, left pulmonary artery ligation (LPAL) creates a unique model of chronic pulmonary thromboembolism and subsequent angiogenesis (6, 12, 25, 32), which allows for investigation into the mechanisms of lung neovascularization. In mice, intercostal artery proliferation and perfusion of the lung develop within 4–5 days after LPAL (25, 31). We (24, 27, 28) have shown previously an increase in the total number of inflammatory cells in bronchoalveolar lavage fluid after

LPAL. Additionally, inflammatory cytokines macrophage inflammatory protein-2 (MIP-2) and IL-6 contribute to neovascularization in this model (24, 27). However, the cellular source of these cytokines and the contribution of inflammatory cells to the overall growth of new vessels are not clear. Corticosteroids have been shown to limit both inflammation and angiogenesis in asthmatic airways (13, 16). Numerous studies in a variety of angiogenesis models have selectively eliminated a leukocyte subpopulation and demonstrated an altered angiogenic outcome. Aurora and colleagues (2) showed that airway angiogenesis in response to a respiratory pathogen was substantially reduced in mice lacking lymphocytes. In the peripheral systemic vasculature, reductions in the number of Mac-3-positive monocytes/macrophages and CD3-positive T cells were associated with decreased neovascularization in hindlimb ischemia (30). In the LPAL model, where hypoxia does not play a role in eliciting growth factor release, trapped inflammatory cells are likely to modulate and perhaps direct angiogenic outcome. In the present study, we sought to confirm that inflammation is required for angiogenesis to proceed. We (24) previously showed that despite a large increase in the number of neutrophils in bronchoalveolar lavage fluid early (4 h) after LPAL, their presence was not critical for new vessel growth to the lung. Therefore, we questioned specifically whether lung macrophages and lymphocytes are essential for the process of neovascularization. The results presented suggest that, overall, inflammation promotes neovascularization, however, lymphocyte subsets provide further modulation.

## METHODS

### LPAL

Our protocol was approved by the Johns Hopkins Animal Care and Use Committee. Five- to six-week-old male C57BL/6 mice (wild-type; Charles River, Wilmington, MA) and Rag-1<sup>-/-</sup> homozygote mice raised on C57BL/6J background (Rag-1 KO; The Jackson Laboratories, Bar Harbor, ME) were used in this study. Mice were anesthetized (2% isoflurane in oxygen), intubated, and ventilated at 120 breaths/min (0.2 ml/breath) with the anesthetic/gas mixture. As previously described, a left thoracotomy was performed at the third intercostal space, and the left pulmonary artery was located, separated from the airway, and ligated using 6-0 silk suture (25, 27). The thoracotomy was closed with a suture while the mouse was placed on positive end-expiratory pressure (1 cmH<sub>2</sub>O). Lidocaine (2%) was applied topically for analgesia, and the skin incision was closed using methylacrylamide adhesive. The mouse was removed from the ventilator, extubated, and allowed to recover. Surgical control mice were treated the same as experimental mice in all respects except for ligating the left pulmonary artery. For lung tissue acquisition and lung lavage, anesthetized mice were killed by cervical dislocation at specified times after LPAL.

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### Experimental Groups

Four experimental groups of mice were studied after LPAL: 1) untreated or vehicle control C57BL/6 mice ( $n = 42$ ); 2) corticosteroid-treated mice (general anti-inflammatory agent;  $n = 31$ ); 3) gadolinium chloride ( $\text{GdCl}_3$ )-treated mice (monocyte/macrophage inactivator;  $n = 34$ ); and 4) Rag-1 KO mice (T/B lymphocyte depleted;  $n = 40$ ). Bronchoalveolar lavage of the left lung was performed 4 and 24 h after LPAL, IL-6, and MIP-2 protein levels were determined in the left and right lungs 4 and 24 h after LPAL, and neovascularization was assessed by blood flow determination 14 days after LPAL. These time points were selected based on previous data acquired in this model demonstrating an increase in lavaged inflammatory cells between 4–24 h after LPAL (24), an increase in left lung proangiogenic cytokines between 4–24 h after LPAL (24, 28), and an established neovasculature by 14 days after LPAL (27).

### Bronchoalveolar Lavage

In separate groups of animals 4 or 24 h after LPAL, immediately after death, the right lung was ligated and the left lung was washed ( $3 \times 0.3$  ml of 0.2% BSA/PBS). Bronchoalveolar lavage fluid was gently aspirated, the total recovered volume recorded, and total cell count (Bright Line Hemacytometer; Hausser Scientific, Horsham, PA) and differential cell counts (Cytospin 4; Shandon, Pittsburgh, PA; and Diff-Quick staining; Dade Behring, Newark, DE) were determined where  $\sim 1,000$  cells/mouse were evaluated.

### IL-6 and MIP-2 Protein

In separate groups of mice, 4 or 24 h after LPAL, mice were euthanized, and the upper third of the left lung and the entire right lung were dissected and excised. Lung samples were weighed, homogenized (Tris·HCl buffer and FastPrep Bio 101 with green kit; Thermo Savant, Holbrook, NY), and aliquoted for ELISA and bicinchoninic acid (BCA) assays. The samples were processed according to instructions provided by the Quantikine Mouse IL-6 and MIP-2 microplate ELISA kits (R&D Systems, Minneapolis, MN). Total protein measurements were made according to the BCA protein microplate assay kit (Pierce, Rockford, IL).

### Blood Flow Determination

To determine the extent of neovascularization, systemic blood flow to the left lung was measured 14 days after LPAL using 10- $\mu\text{m}$  radiolabeled microspheres. Diethylenetriamine pentaacetic acid (DTPA)-coated polystyrene microspheres (range 9.6–10.4  $\mu\text{m}$  in diameter; Kisker, Steinfurt, Germany) were bound to Technetium-99m radioligand (Cardinal Health, Dublin, OH). Fourteen days after LPAL, mice were anesthetized and ventilated as described above. The carotid artery was cannulated (PE 10), and 150,000 microspheres (stock = 1.5 million spheres per milliliter) were infused (0.1 ml at 0.04 ml/min; Harvard Apparatus, Holliston, MA). Mice were killed by exsanguination, and the left lung was excised. Gamma emissions from lodged, radiolabeled microspheres were immediately counted in the Hidex Triathler (Bioscan, Washington, DC). Left lung activity was normalized to whole body activity counted in a Capintec counter (Capintec, Ramsey, NJ), which had been calibrated to the Bioscan instrument. Left lung blood flow was expressed as percent of total measured blood flow (carcass and all organs), i.e., percent cardiac output.

### Treatments

**Corticosteroid treatment.** A relatively high dose of steroid was selected based on doses that were effective in airway and lung injury inflammation models in mice (18, 19, 22). Dexamethasone (10 mg/kg ip in 400  $\mu\text{l}$  volume; Sigma, St. Louis, MO) was administered to mice 24 h before LPAL, at the time of surgery, and subsequently every 24 h

until blood flow determination at 14 days. Vehicle-treated mice received PBS in the same volume and at the same frequency as dexamethasone.

**GdCl<sub>3</sub> treatment.** The chemical agent,  $\text{GdCl}_3$ , is taken up exclusively by phagocytic mononuclear cells and causes them to apoptose (14). This chemical has been used in a wide range of experiments and at a variety of concentrations (7–100 mg/kg iv and ip). Therefore, we selected and validated a treatment paradigm in a small pilot series. Twenty-four hours before LPAL, mice were treated with  $\text{GdCl}_3$  to reduce the number of macrophages in the lung (45 mg/kg ip in 150  $\mu\text{l}$  volume; Sigma). Since there is no pulmonary circulation to the left lung after LPAL, mice were exposed daily in a closed chamber to aerosolized  $\text{GdCl}_3$  (10 min, 45 mg/kg) to ensure maximum and sustained inactivation of lung macrophages. In preliminary experiments ( $n = 10$  mice total) at 4 h, 1 day, and 6 days after LPAL, total lavaged cells did not increase as previously reported for this model (24). Average number of total lavaged cells was  $2.3 \times 10^4$  cells/ml, which was considerably reduced (56% reduction) compared with previously reported values over the same time period after LPAL (24).

### Splenocyte Adoptive Transfer

Spleens were isolated from naïve C57BL/6 mice and freshly minced through a 70- $\mu\text{m}$  strainer. Cells were collected, and red cells removed using a lysis buffer (ACK; Quality Biological, Gaithersburg, MD) before being washed and then resuspended in PBS. Ten million cells were suspended in PBS (100  $\mu\text{l}$ ) and injected through a tail vein of Rag-1 KO mice 24 h before LPAL. Successful transfer and proliferation of lymphocytes was confirmed after 14-day microsphere injections by FACS analysis of splenocytes (anti-CD3<sup>+</sup> phycoerythrin for T cell staining and anti-CD19<sup>+</sup> fluorescein isothiocyanate for B cell staining; BD Pharmingen, San Jose, CA).

### Statistics

All data are presented as means  $\pm$  SE. Changes in inflammatory cells, lung protein, and blood flow (log transform of fractional flow) were evaluated by one-way ANOVA. Relevant within-group comparisons were made using Fisher's protected least significant differences test for multiple comparisons. Student's *t*-test for unpaired data was used for Rag-1 KO two-group comparisons. A *P* value  $\leq 0.05$  was accepted as significant.

## RESULTS

### Bronchoalveolar Lavage

Early (4 and 24 h) after LPAL, we performed bronchoalveolar lavage of the left lung evaluating equal numbers of animals at each time point within treatment groups ( $n = 4$  mice at 4 h,  $n = 4$  mice at 24 h). Average recovered lavage volume for all mice studied was  $740 \pm 20$   $\mu\text{l}$ . Since total lavaged cells did not differ between the vehicle-treated LPAL group and untreated LPAL group ( $P = 0.55$ ), results were combined into one control LPAL group for all subsequent endpoints. Furthermore, since no differences were observed between 4-h samples compared with 24-h samples in any treatment group ( $P = 0.80$ ), we combined the data from 4- and 24-h time points for each group for an average estimate of lavage cell changes early (4–24 h) after LPAL. The average total number of lavaged inflammatory cells for the sham surgical group, LPAL, and two treatment groups are presented in Fig. 1A. The average number of total cells in the LPAL group was  $4.3 \times 10^4$  ( $\pm 0.6 \times 10^4$ ) cells/ml, and significant differences in total lavaged cells were observed across groups ( $P < 0.01$ ). Dexamethasone and  $\text{GdCl}_3$  treatment both significantly reduced the number of lavaged inflammatory cells relative to the LPAL control group ( $P \leq$

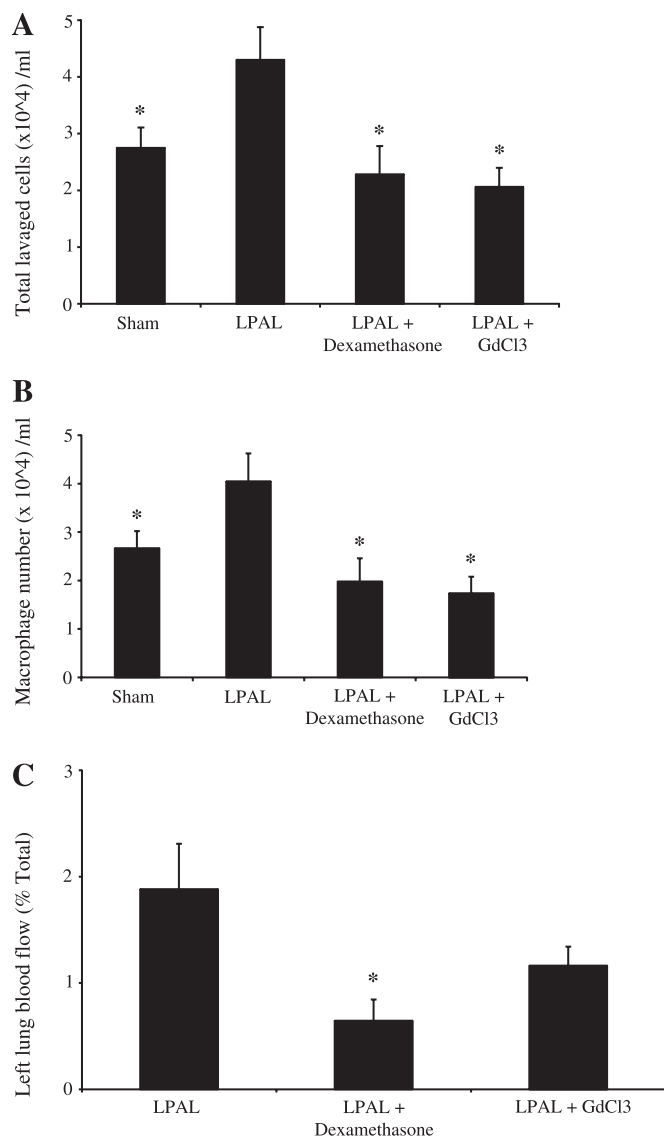


Fig. 1. A: total left lung lavaged inflammatory cells ( $\times 10^4$ /ml, 4–24 h after surgery in sham-operated mice ( $n = 8$  mice), after left pulmonary artery ligation (LPAL;  $n = 14$  mice), after LPAL and dexamethasone treatment ( $n = 8$  mice), and after LPAL and gadolinium chloride ( $\text{GdCl}_3$ ) treatment ( $n = 8$  mice). Both dexamethasone and  $\text{GdCl}_3$ -treated mice showed a significant reduction in total lavaged inflammatory cells that were not different from sham-operated mice ( $*P \leq 0.05$  from LPAL group). B: total left lung lavaged macrophages ( $\times 10^4$ /ml, 4–24 h after surgery in sham-operated mice, after LPAL, after LPAL and dexamethasone treatment, and after LPAL and  $\text{GdCl}_3$  treatment. Both dexamethasone and  $\text{GdCl}_3$ -treated mice showed a significant reduction in the number of lavaged macrophages that were not different from sham-operated mice ( $*P \leq 0.05$  from LPAL group). C: systemic blood flow to the left lung as a percent of total blood flow after LPAL. A significant decrease in blood flow to the ischemic left lung was observed after LPAL in dexamethasone-treated mice ( $n = 7$  mice) compared with LPAL ( $n = 8$  mice) alone. After LPAL and  $\text{GdCl}_3$  ( $n = 10$  mice) treatment, left lung blood flow showed a trend toward a reduction, which failed to reach statistical significance. ( $*P \leq 0.05$ ).

0.05). As expected, the sham surgical group had fewer total lavaged cells than the LPAL group ( $P \leq 0.05$ ). The average number of lavaged macrophages (Fig. 1B) in the LPAL group was  $4.05 \times 10^4$  ( $\pm 0.6 \times 10^4$ ) cells/ml, which comprised an average 93% of total inflammatory cells. The results for lavaged macrophages of the treatment groups exactly paral-

leled the results of the total number of cells and the statistical significance (Fig. 1B;  $P < 0.01$ ). Lavaged polymorphonuclear leukocytes averaged  $2.3 \times 10^3$  ( $\pm 0.6 \times 10^3$ ) after LPAL and did not vary across groups ( $P = 0.24$ ). So few lymphocytes were seen ( $<1\%$ ) that an accurate count could not be obtained with the applied methods.

Figure 2, A and B, shows the results from bronchoalveolar lavage after LPAL in the Rag-1 KO mice without ( $n = 4$  mice at 4 h,  $n = 4$  mice at 24 h) and with splenocyte transfer ( $n = 2$  mice at 4 h,  $n = 2$  mice at 24 h). Rag-1 KO mice demonstrated a significantly greater number of total lavaged inflammatory cells compared with the LPAL control group ( $5.8 \pm 0.4 \times 10^4$  vs.  $4.3 \pm 0.6 \times 10^4$  cells/ml;  $P \leq 0.05$ ). This increase was reversed in the group of Rag-1 KO mice with splenocyte transfer before LPAL ( $4.0 \pm 0.7 \times 10^4$  cells/ml;  $P \leq 0.05$ ). Rag-1 KO mice demonstrated a significantly greater number of macrophages than the control LPAL group ( $5.7 \pm 0.4 \times 10^4$  vs.  $4.0 \pm 0.6 \times 10^4$  cells/ml;  $P \leq 0.05$ ). Lavaged polymorphonuclear leukocytes did not differ between LPAL and Rag-1 KO groups ( $P = 0.28$ ). However, polymorphonuclear leukocytes increased in the adoptive transfer group ( $1.7 \pm 0.7 \times 10^2$  cells/ml;  $P = 0.004$ ), and too few lavaged lymphocytes were seen ( $<1\%$ ) in either KO group to obtain an accurate count with the applied methods.

#### MIP-2 and IL-6 Protein

Based on previous results demonstrating early changes in cytokine protein expression, we measured MIP-2 and IL-6 protein in ischemic left lungs and compared them to paired right lungs at 4 and 24 h after LPAL in the treatment groups. No significant differences were observed in left lungs between 4-h samples compared with 24-h samples in any treatment group for MIP-2 ( $P = 0.20$ ). Consequently, we combined the data from 4 and 24 h time points for each group for an average estimate of MIP-2 protein expression early after LPAL (Table 1). An increase in absolute MIP-2 protein normalized to total lung protein was observed in the ischemic left lung relative to the right lung in all treatment groups. This increase was statistically significant in all groups except for the Rag-1 KO mice where the right lung showed higher levels of MIP-2 protein. However, there were no significant differences among the four groups with regard to left lung MIP-2 protein ( $P = 0.85$ ). When comparing left lung 4- and 24-h IL-6, protein expression reached borderline significance ( $P = 0.06$ ). However, for consistency, simplicity, and an increase in sample size, we combined the two time points. IL-6 protein expression was significantly greater in the left lung compared with the right lung in all 4 groups ( $P \leq 0.05$ ). However, left lung IL-6 protein did not vary among treatment groups ( $P = 0.96$ ). On average, both MIP-2 and IL-6 protein showed a three- to fivefold greater level in the left lung relative to the right lung and did not differ among groups ( $P > 0.05$ ).

#### Blood Flow Determination

Neovascularization, as assessed functionally by blood flow determination 14 days after LPAL, is shown in Fig. 1C. Average blood flow for the control LPAL group ( $1.9\% \pm 0.4\%$ ;  $n = 8$ ) was consistent with that previously reported for this model and time point after LPAL (27). Blood flow was significantly decreased in the group of dexamethasone-treated



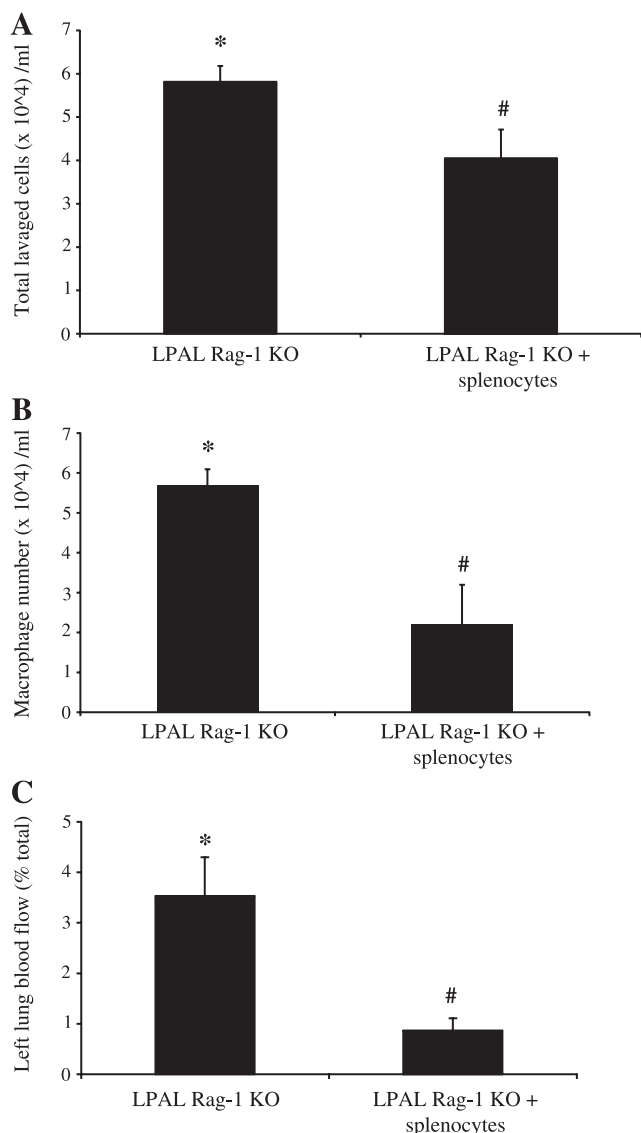


Fig. 2. A: total left lung lavaged inflammatory cells ( $\times 10^4$ )/ml, 4–24 h after LPAL in Rag-1<sup>-/-</sup> homozygote mice raised on C57BL/6J background (Rag-1 KO;  $n = 8$  mice) and Rag-1 KO mice after splenocyte adoptive transfer ( $n = 4$  mice). A significant increase in total lavaged cells was seen in Rag-1 KO mice compared with LPAL ( $*P \leq 0.05$ ). Splenocyte transfer caused a significant decrease in total lavaged cells in Rag-1 KO + splenocyte mice ( $\#P \leq 0.05$ ). B: total left lung lavaged macrophages ( $\times 10^4$ )/ml, 4–24 h after LPAL in Rag-1 KO mice and Rag-1 KO mice after splenocyte adoptive transfer. A significant increase in macrophages was seen in Rag-1 KO mice compared with LPAL ( $*P \leq 0.05$ ). Splenocyte transfer caused a significant decrease in macrophages in Rag-1 KO + splenocyte mice ( $\#P \leq 0.05$ ). C: systemic blood flow to the left lung as a percent of total blood flow after LPAL in Rag-1 KO mice ( $n = 7$ ) compared with LPAL wild-type mice ( $*P \leq 0.05$ ). However, splenocyte transfer caused a significant decrease in left lung blood flow in Rag-1 KO + splenocyte mice ( $n = 4$ ;  $\#P \leq 0.01$ ).

mice ( $n = 7$ ;  $P < 0.01$ ) compared with control LPAL. There were no significant differences in GdCl<sub>3</sub>-treated mice ( $n = 10$ ) compared with the control LPAL group.

In an additional series, lymphocyte-depleted mice were studied without and with splenocyte replacement of T/B lymphocytes. Left lung blood flow after LPAL was significantly

increased ( $3.5\% \pm 0.8\%$  in Rag-1 KO;  $n = 7$ ) relative to the control LPAL group ( $P \leq 0.05$ ). Successful transfer and proliferation of CD3<sup>+</sup> T cells and CD19<sup>+</sup> B cells in an additional group of Rag-1 KO mice were evaluated by FACS analysis of splenocytes at the time of blood flow determination. Relative to naïve C57BL/6 splenocytes, splenocytes from Rag-1 KO mice showed  $<1\%$  CD3<sup>+</sup> T cells and CD19<sup>+</sup> B cells. After adoptive transfer of splenocytes from naïve C57BL/6 mice to Rag-1 KO mice, average CD3<sup>+</sup> T cells in Rag-1 KO mice increased to 35% of the normal level observed in naïve C57BL/6 mice. CD19<sup>+</sup> B cells in Rag-1 KO mice after splenocyte transfer averaged 3% of the value observed in naïve mice spleens. As seen in Fig. 2C, blood flow in the group of Rag-1 KO with splenocyte transfer demonstrated a significantly lower blood flow than Rag-1 KO mice ( $0.9\% \pm 0.2\%$ ;  $n = 4$ ;  $P < 0.01$ ).

When examining all groups studied, overall, there was a significant positive correlation between average blood flow and the average number of lavaged macrophages for a given group (Fig. 3;  $r^2 = 0.9181$ ;  $P < 0.01$  different from 0).

## DISCUSSION

Inflammation is an essential component of angiogenesis in most organs. Inflammatory cells surrounding the microvasculature can have a profound effect on promoting new vessel growth (10, 17). Our laboratory showed previously that there is a sustained increase in inflammatory cells in bronchoalveolar lavage fluid after unilateral left pulmonary artery ischemia (24). Genes upregulated in the proangiogenic left lung included many proinflammatory cytokines (28). In the present study, we sought to confirm that lung inflammation is required for angiogenesis to proceed and whether specific trapped inflammatory cells may be the source of growth factors essential for neovascularization. Since we previously showed that despite a large increase in the number of neutrophils in bronchoalveolar lavage fluid early (4 h) after LPAL, their presence was not critical for new vessel growth to the lung (24), we focused in this study specifically on whether macrophage and lymphocyte depletion might alter the progression of neovascularization. The results presented suggest that overall neovascularization is closely associated with the number of lavaged macrophages in the ischemic lung early after LPAL. Furthermore, the availability of lymphocytes appears to regulate the number of lavaged macrophages and subsequent angiogenesis.

We initially sought to determine whether a general anti-inflammatory corticosteroid would alter angiogenic outcome in the mouse lung after LPAL. Corticosteroids have been shown

Table 1. MIP-2 and IL-6 protein

Treatment	MIP-2, pg/mg Protein		IL-6, pg/mg Protein	
	Left Lung	Right Lung	Left Lung	Right Lung
LPAL	101 ± 27*	34 ± 12	134 ± 34*	35 ± 8
LPAL + Dexamethasone	132 ± 48†	30 ± 12	132 ± 47†	31 ± 12
LPAL + GdCl <sub>3</sub>	149 ± 48†	25 ± 8	152 ± 49†	25 ± 8
LPAL Rag-1 KO	157 ± 74	100 ± 54	159 ± 47†	38 ± 11

For macrophage inflammatory protein-2 (MIP-2),  $n = 8$ /group; for IL-6,  $n = 8$ /group, except left pulmonary artery ligation (LPAL),  $n = 12$ .  $*P < 0.01$ ;  $\dagger P < 0.05$ . GdCl<sub>3</sub>, gadolinium chloride; Rag-1 KO, Rag-1<sup>-/-</sup> homozygote mice raised on C57BL/6J background.

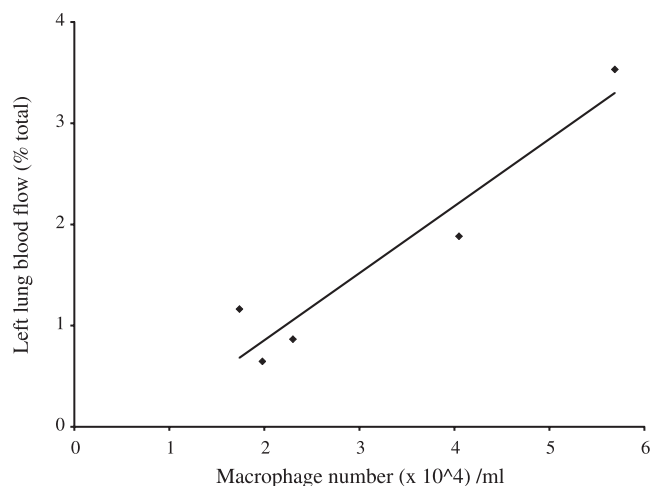


Fig. 3. Correlation between average number of macrophages and average systemic left lung blood flow after LPAL. A significant correlation exists between the group average number of macrophages obtained after bronchoalveolar lavage early after LPAL and the group average measurement of neovascularization 14 days after LPAL ( $P < 0.01$ ).

to limit both inflammation and angiogenesis in asthmatic airways (13, 16). In other organs where hypoxia and hypoxia-inducible growth factors are involved in the process of neovascularization, dexamethasone has been shown to limit new vessel growth in the cornea (7), brain (21), and skin (33). In the present study, where the ventilated left lung was ischemic but not hypoxic, we selected a high dose of dexamethasone, which was shown to limit lung inflammation in other injury models (18, 19, 22). Total lavaged cell numbers were significantly decreased early after LPAL (Fig. 1A), as was blood flow to the left lung (Fig. 1C), our functional estimate of angiogenesis 14 days after LPAL. Interestingly, however, the two cytokines previously shown to be important for angiogenesis in this model, IL-6 (24) and MIP-2 (27, 28), were not altered in left lung homogenates after corticosteroid treatment relative to control left lungs after LPAL alone (Table 1). These observations suggest that inflammatory cells within the lung per se are not major contributors to the available pool of these specific lung growth factors. In addition to alveolar macrophages, a variety of lung cells including epithelial cells, endothelial cells, and fibroblasts have been shown to secrete IL-6 and MIP-2 (4). The fact that the applied corticosteroid dosing regimen altered lung lavage cell number confirmed the effectiveness of treatment, at least at the early time points of 4 and 24 h after LPAL. Whether intraperitoneal dosing was effective at maintaining decreased lavaged inflammatory cells beyond 24 h and until a new functional vasculature was established by 5–7 days is not clear. However, since there was no pulmonary perfusion, corticosteroid treatment had to have its major effect before LPAL and then on the source of neovascularization, namely the intercostal vessels that subsequently invade the ischemic lung parenchyma. In a skin wound healing model, the preponderance of macrophages at the site of neovascularization was significantly decreased by corticosteroid treatment (33). Thus corticosteroid treatment in this model limited the number of inflammatory cells in the lung early after LPAL and the angiogenic outcome days later but did not alter two inflammatory cytokines known to participate in the growth of new

vessels. Despite a relatively high dose, corticosteroid treatment limited but did not completely inhibit inflammation and neovascularization as we (27) have shown previously a background value of 0.20% after 2 days of LPAL. This value, representing other systemic perfusion of the lung, potential lodging of microspheres from left heart back flow, and experimental error, is considerably less than the blood flow measured after 14 days of corticosteroid treatment.

The second approach to limiting inflammation involved treatment with  $GdCl_3$ , a particulate that is taken up by phagocytic mononuclear cells that causes their subsequent apoptosis (14). Numerous investigators have used this approach to eliminate the available alveolar macrophage population in experimental models. However, adequate and sustained delivery to the lung in this model without pulmonary circulation to the lung posed an experimental challenge. In a series of pilot experiments conducted for a 6-day trial period, the combination of intraperitoneal  $GdCl_3$  treatment before LPAL followed by daily aerosol challenge appeared to be effective at limiting the total number of lavaged inflammatory cells. Lavaged macrophages and total cell number were significantly decreased after LPAL (Fig. 1, A and B). However, angiogenesis evaluated by blood flow 14 days after LPAL, although trending downward, was not significantly different from control LPAL. At this time, it is not clear whether the treatment remained effective throughout the 14 day time period or whether the extent of the reduction in lung macrophages per se was insufficient to alter neovascularization. Since there was a significant reduction in lavaged cells early after LPAL, we fully expected to see a decrease in MIP-2 protein in lung homogenate relative to other treatment groups. Since we did not see this decrease despite a reduction in lavaged macrophages, we are forced to conclude that alveolar macrophages are not the major source of MIP-2 protein in this model. Furthermore, since we showed previously that both IL-6 removal (24) and CXCR<sub>2</sub> neutralization (27) decreased angiogenesis, we know that both proteins are important in this model. However, they appear to be necessary but not sufficient to elicit maximum neovascularization at the time point measured by blood flow determination (14 days). Since none of the treatments completely eliminated macrophages, small differences in protein among groups may have been difficult to discern.

The Rag-1<sup>-/-</sup> mouse was used to assess the importance of T/B lymphocytes in this angiogenesis model. These mice, with a homozygous mutation (Rag1<sup>tm1Mom</sup>), produce no mature T cells or B cells. Previous work by Aurora and colleagues (2) showed that the extent of tracheal angiogenesis after *Mycoplasma pulmonis* infection was significantly reduced in Rag-1<sup>-/-</sup> mice. In that model, when serum from infected wild-type mice was transferred into lymphocyte-deficient mice, the original angiogenic phenotype was restored. Thus our results demonstrating a substantial increase in the extent of neovascularization 14 days after LPAL in Rag-1<sup>-/-</sup> were quite surprising. Adoptive transfer of mature splenocytes from normal C57BL/6 mice into the Rag-1<sup>-/-</sup> mice reduced the level of angiogenesis. These results demonstrate that T/B cells regulate the inflammatory response, the number of lavaged macrophages, and neovascularization. Although contrary to what we predicted based on Aurora et al. (2), the observations are consistent with a growing body of evidence that T lymphocytes can have a major effect on the lung vasculature (29). T

lymphocyte depletion has been shown to enhance pulmonary vascular remodeling in a model of pulmonary hypertension (29) and depletion of regulatory T cells specifically has been shown to alter the progression of acute lung injury in an LPS model (8). Others have shown that regulatory T cells suppress immune responses (15) and decrease the number of macrophages at atherosclerotic sites (1). In our model, antibody detecting surface antigen for T cells (CD3<sup>+</sup>) was confirmed to be present 14 days after LPAL in the Rag-1<sup>-/-</sup> mice given splenocytes from naïve C57BL/6 mice. Thus the adoptive transfer and proliferation of this lymphocyte population was confirmed. Given the growing body of evidence suggesting that regulatory T cells have a major impact on lung inflammation and subsequent vascular remodeling, we suggest that these cells play a critical role in limiting the extent of lung inflammation and activated lung macrophages specifically. With regard to B cell transfer, proliferation and homing of CD19<sup>+</sup> B cells to the spleen appeared absent. Whether this was due to B cells homing to secondary lymphoid organs in this model or a complete lack of B cell proliferation is not clear. However, this result is also consistent with T cells being primarily responsible for the different angiogenic outcomes in the Rag-1<sup>-/-</sup> mice. Future studies are needed to identify lymphocyte populations in the lung and link these with angiogenic growth factors and subsequent neovascularization. In summary, T/B lymphocyte-depleted mice showed enhanced angiogenesis suggesting that normally T/B lymphocytes limit the extent of neovascularization.

When evaluating the angiogenic outcome in all experimental groups, blood flow was significantly correlated with the number of alveolar macrophages (Fig. 3). Although GdCl<sub>3</sub> treatment did not significantly decrease blood flow, the attenuated number of lavaged alveolar macrophages in the GdCl<sub>3</sub> group and the trend toward diminished blood flow in this group contributed to the overall positive correlation between average macrophage number and neovascularization. This correlation is consistent with the observations of Dahlqvist and colleagues (9) who showed an association of tissue macrophages and the development of new vessels in the airway mucosa after *Mycoplasma pulmonis* infection. In addition to the CXC chemokines, alveolar macrophages contain many growth factors/regulators of new vessel growth and may be the essential inflammatory component in this model. This speculation is based on the assumption that cells that are recovered from bronchoalveolar lavage are representative of resident cell populations. However, it is equally plausible that the lavaged macrophage is a marker of systemic inflammation and indicative of the inflammatory cells required for intercostal artery proliferation and invasion into the ischemic lung. Additionally, T/B lymphocytes appear to influence the number of macrophages and thereby affect the angiogenic outcome.

In summary, this study was designed to test whether inflammatory cells in the lung were the source or contributed to the release of proangiogenic growth factors early after pulmonary ischemia. With regard to IL-6 and MIP-2, two previously established growth factors in this model, results demonstrated that lung homogenate levels were not correlated with altered lavaged inflammatory cell profiles. However, the lavaged macrophage number appears to predict angiogenic outcome 14 days after LPAL. These results suggest that other products produced by macrophages are essential for the process of

angiogenesis to occur. Furthermore, lymphocytes modulate the availability of alveolar macrophages.

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