The role of proinflammatory cytokines in lung ischemia-reperfusion injury

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Objective: Proinflammatory cytokines are known to play roles in ischemia-reperfusion injury of the heart, kidney, small bowel, skin, and liver. Little is known about their roles in ischemia-reperfusion injury of the lung. This study was undertaken to define the role of 2 proinflammatory cytokines, tumor necrosis factor α and interleukin 1β, in ischemia-reperfusion injury of the lung.

Methods: Left lungs of male rats were rendered ischemic for 90 minutes and reperfused for up to 4 hours. Treated animals received anti-tumor necrosis factor α or anti-interleukin 1β antibody before reperfusion. Increased vascular permeability in the lung was measured by using iodine 125-labeled bovine serum albumin. Neutrophil sequestration in the lung parenchyma was determined on the basis of activity. Bronchoalveolar lavage was performed to measure cell counts. Separate tissue samples were processed for histology, cytokine protein, and messenger RNA content by using Western blotting and the ribonuclease protection assay.

Results: Animals receiving anti-tumor necrosis factor α and anti-interleukin 1β demonstrated reduced injury compared with that seen in positive control animals (vascular permeability of 48.7% and 29.4% lower, respectively; $P < .001$). Vascular injury was reduced by 71% when antibodies to tumor necrosis factor α and interleukin 1β were administered together. Lung neutrophil accumulation was markedly reduced among animals receiving anti-tumor necrosis factor α and anti-interleukin 1β (myeloperoxidase content of 30.9% and 38.5% lower, respectively; $P < .04$) and combination blockade afforded even greater protection (52.4% decrease, $P < .01$). Bronchoalveolar lavage leukocyte content was also reduced by treatment with anti-tumor necrosis factor α, anti-interleukin 1β, and combination treatment. Reductions in permeability, myeloperoxidase, and bronchoalveolar lavage leukocyte content also resulted in a decrease in a histologic injury. Finally, anti-tumor necrosis factor α and anti-interleukin 1β treatment resulted in decreased messenger RNA expression for a number of early response and regulatory cytokines.

Conclusion: Tumor necrosis factor α and interleukin 1β help regulate the development of lung ischemia-reperfusion injury. They appear to promote injury by altering expression of proinflammatory and anti-inflammatory cytokines and influencing tissue neutrophil recruitment.

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Two of the most frequently studied cytokines in models of acute inflammatory injury models are tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β). These 2 proteins have a variety of proinflammatory activities that have led to both scientific and clinical investigations of their functional roles. TNF-α is a 17-kd, 157-amino-acid cytokine that is secreted by a wide spectrum of cells, including macrophages, monocytes, T cells, natural killer cells, and neutrophils.

IL-1β is a 17-kd, 153-amino-acid protein that is predominantly produced by monocytes and macrophages, although secretion from keratinocytes, endothelial cells, neutrophils, fibroblasts, and smooth muscle cells has been demonstrated. Both of these cytokines have an assortment of proinflammatory activities, including leukocyte chemoattraction, phagocyte stimulation, enhancement of downstream cytokine and chemokine production, and variable effects on cell growth and death.

These cytokines have been studied in isolation and in combination in multiple models of ischemia-reperfusion injury in an variety of vascular beds. Functional roles for TNF-α and IL-1β have been identified in liver,[1,2] heart,[3,4] brain,[5] kidney,[6] hind limb,[7] and gut[8] ischemia and reperfusion. These studies have demonstrated that TNF-α and, to a lesser extent, IL-1β promote injury in these vascular beds and that blockade of these cytokines decreases injury in a neutrophil-dependent manner. In addition to the local effects of these cytokines, secondary lung injury has also been investigated in the liver,[9] intestine,[10] and hind limb.[7] TNF-α promotes the generation of acute lung injury in all 3 of these vascular beds. Investigations of hind limb ischemia and reperfusion have demonstrated protection afforded by IL-1 receptor blockade.[7]

Studies of the roles of these 2 cytokines in acute nonischemic lung injury have been predominantly focused on inflammatory injury induced by lipopolysaccharide,[11] IgG immune complex,[12] and antiglomerular basement membrane antibody.[13] These studies have defined functional roles for TNF-α and IL-1β in each of these neutrophil-dependent models. The degree of protection afforded by blockade of either TNF-α or IL-1β is between 40% and 60%. The ability of the recombinant preparations of these 2 cytokines to directly cause lung injury has also been demonstrated and appears to be dependent on neutrophil recruitment.[14]

Surprisingly little work has investigated the roles of TNF-α and IL-1β in direct lung ischemia-reperfusion injury. Khimenko and colleagues[15] examined the effects of ventilated and nonventilated ischemia in an isolated buffer-perfused rat lung. Interpretation of these studies is difficult because they involve the use of isolated lung preparations, nonphysiologic perfusate, and brief ischemia-reperfusion periods. Despite these limitations, the authors were able to correlate increased injury with higher levels of TNF-α protein in the lung effluent. Other investigators have demonstrated a functional role for TNF-α in injury, which develops after 30 minutes and 4 hours of reperfusion. However, there was significant variability in positive control animals and inconsistent effects on collateral cytokine expression.[16] Chang and coworkers[17] investigated the effects of IL-1β in an isolated perfused rat lung. These studies must also be interpreted carefully because although increased IL-1 protein and mRNA expression was seen with ischemia and reperfusion, a functional role was not identified. Ultimately, there has not been a precise delineation of the functional roles of TNF-α and IL-1β in an intact animal model of ischemia and reperfusion.

Despite the similar physiologic effects of TNF-α and IL-1β, the combined blockade of these 2 mediators might afford more protection than blockade of either cytokine individually. Studies done in immune complex-induced lung injury suggest that the effects of combined blockade of TNF-α and IL-1β are additive.[18] Studies of combined cytokine blockade have not been done in lung ischemia-reperfusion injury, and information on such effects would be novel.

Studies of patients with adult respiratory distress syndrome[19] and those that have recently undergone lung transplantation[20] demonstrate increased locoregional expression of TNF-α and IL-1β. This expression is thought to be functionally important, and therefore animal studies detailing blockade of these cytokines would be clinically relevant. These studies seek to delineate the functional role of TNF-α and IL-1β and to investigate the mechanism of their action individually and in combination during direct lung ischemia-reperfusion injury.

Materials and Methods

Reagents

Rabbit antibody to rat TNF-α, rat IL-1β, and nonspecific rabbit IgG were purchased from PeproTech Inc (Rocky Hill, NJ). All other reagents were purchased from Sigma Chemical Co (St Louis, Mo) unless otherwise specified.

Animal Model

Pathogen-free adult male Long-Evans rats (Simonsen Laboratories, Inc, Gilroy, Calif) weighing between 280 and 320 g were used for all experiments. The University of Washington Animal Care Committee approved all experimental protocols. Animals were initially anesthetized with 30 to 35 mg of intraperitoneal pentobarbital. Subsequently, they were 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 a standardized inspired oxygen content of 60%, a rate of 80 breaths/min, and a positive end-expiratory pressure of 2 cm H_2O. Maximal peak pressures were maintained at less than 10 cm H_20. All animals received 0.4 mg of atropine intramuscularly after being connected to the ventilator to maintain their heart rates during anesthesia. Dissection was conducted by using an operating microscope, and a warming blanket was placed underneath the animals throughout the experiment. The animals were placed on the right side, and a left posterior lateral thoracotomy in the fifth intercostal space was performed. The left lung was mobilized atraumatically, and the inferior pulmonary ligament was divided sharply. At this time, all animals received 50 units of heparin dissolved in saline solution (total volume, 500 μL). Five minutes after the heparin was administered, the pulmonary hilum

was occluded (inflated) with a noncrushing microvascular clamp. During the experiment, the lungs were kept moist with periodic application of warm normal saline solution, and covering the incision with a plastic wrap minimized evaporative losses. The period of ischemia was constant at 90 minutes. At the end of the ischemic period, the clamp was removed from the hilum, and the lung was allowed to ventilate and reperfuse for periods of up to 4 hours. Animals were administered 0.5 mL of warm subcutaneous saline solution per hour to maintain hydration during the experiment. At the end of the reperfusion period, a midline incision from the neck to the pubis was created to allow access to the chest and abdominal cavities. Blood samples were obtained from the inferior vena cava just before animals were killed. 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 then separated from mediastinal tissues and analyzed as outlined below. Time-matched control animals underwent the same procedure, except the microvascular clamp was not applied to the hilum (sham thoracotomy alone).

Treated animals received polyclonal rabbit anti-rat TNF-α antibody, anti-rat IL-1β antibody (PeproTech), or both at either a low dose of 0.5 mg or a higher dose of 1 mg to determine the effects of TNF-α and IL-1β on ischemia-reperfusion injury. An additional group of animals received both anti-rat TNF-α and anti-rat IL-1β antibody at the lower dose. Antibody was dissolved in 500 μL of sterile phosphate-buffered saline solution (PBS) just before administration. It was then injected through the penile vein 5 minutes before removal of the vascular clamp from the hilum. Nonspecific IgG was administered in the same manner.

Lung Permeability Index

To quantitate lung injury caused by ischemia and reperfusion, a lung permeability index was measured in the following manner. Iodine 125-radiolabeled bovine serum albumin (BSA) was obtained from NEN Life Sciences (PerkinElmer Life Sciences, Wellesley, Mass). Before use of the iodine 125-radiolabeled BSA in vivo, serial dilutions were performed to obtain an activity of approximately 800,000 cpm (counts per minute). This volume of iodine 125-radiolabeled BSA, approximately 2 μL of the stock solution, was then brought to a final volume of 500 μL in a 1% BSA-PBS solution. Five minutes before removal of the hilar clamp or at an equivalent time in sham animals, the iodine 125-radiolabeled BSA mix was intravenously injected. Immediately before the death of the animals, 1 mL of blood was drawn from the inferior vena cava. The heart-lung block was then excised and flushed, as described previously. The counts were then quantitated for the left and right lungs, as well as the inferior vena caval blood with a gamma counter. The permeability index was then expressed as the ratio of the counts per minute in the left lung to the counts in 1 mL of inferior vena caval blood as follows:

Permeability index = Left lung (cpm)/1.0 mL of IVC blood (cpm)

This ratio was corrected for any variation in systemic distribution of radioactivity and provided a reproducible measure of lung microvascular permeability.

Myeloperoxidase Assay

The myeloperoxidase (MPO) assay was used to quantitate tissue neutrophil accumulation in the lung. Lungs for MPO analysis were harvested in a manner similar to that described above. The lungs were homogenized for 60 seconds in a solution of 0.5% hexadecyltrimethylammonium bromide and 5 mmol/L ethelynediamine tetraacetic acid in 50 mmol/L potassium phosphate buffer (pH 6.0). Samples were then ultrasonicated for 40 seconds in four 10-second bursts. The homogenized tissue was maintained on ice between all tissue processing periods. Samples were then centrifuged at 2300 rpm for 30 minutes at 4°C. The assay buffer was composed of 0.0005% H_2O_2 and 0.167 mol/L *o*-dianisidine dihydrochloride in 100 mmol/L potassium phosphate buffer (pH 6.0). Fifty microliters 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. Results of the MPO assay are recorded as the change in absorbance over 1 minute at 460 nm.

Bronchoalveolar Lavage

Additional animals underwent bronchoalveolar lavage (BAL) at the time of death; the heart-lung block was not flushed. With the use of the 14-gauge angiocatheter placed for ventilation, the lungs underwent lavage individually with 3 mL of cold sterile saline solution. The contralateral hilum was clamped to facilitate individual lung BAL analysis. At least 80% of the instilled fluid was recovered from each lung BAL fluid sample. This fluid was centrifuged (1500 rpm $\times$ 8 minutes at 4°C) to pellet the cells in the lavage fluid. The pellet was resuspended in 10 mL of sterile water to lyse red blood cells. This fluid was again centrifuged (1500 rpm $\times$ 8 minutes at 4°C). The supernatant was discarded, and the cells were counted with a hemacytometer (Hausser Scientific Co, Horsham, Pa).

Western Blot Analysis

Lung tissue was homogenized in 10 mL of solution containing 10 mmol/L *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (pH 7.9), 150 mmol/L NaCl, 1 mmol/L ethylenediamine tetraacetic acid, 0.5 mol/L phenylmethylsulfonyl fluoride, 0.6% NP-40, and a protease inhibitor cocktail (50 μL of a 1000$\times$ mixture). The homogenate was incubated on ice for 5 minutes, and 1-mL aliquots were placed into microfuge tubes for analysis. The remaining sample was stored as whole-cell lysates at 4°C. Samples were centrifuged at 14,000 rpm for 10 minutes at 4°C. The pellet was discarded, and the supernatant protein concentration was determined by using the bicinchoninic assay (Pierce Biotechnology, Inc, Rockford, Ill). Approximately 40 μg of protein were loaded on sodium dodecylsulfate-polyacrylamide gel electrophoresis gels (12%) and run at 100 V for 2 hours. After transfer to a polyvinylidene difluoride membrane, the membrane was stained with Coomasie blue to determine equal protein transfer. The membranes were then incubated with either anti-IL-1β or anti-TNF-α polyclonal antibody (PeproTech) at 1:1000 dilution overnight. A horseradish peroxidase-conjugated secondary antibody was applied for 1 hour, and the proteins were visualized with Pierce SuperSignal reagents (Pierce Chemical Company, Rockford, Ill) and autoradiography.

Ribonuclease Protection Assay

Lung RNA was isolated in guanidine thiocyanate, with 2 rounds of acid phenol-chloroform extraction and alcohol precipitation. RNA integrity was confirmed by means of agarose gel electrophoresis

and quantitated by means of optical density measurements (260 nm). RNA from each rat was evaluated with the Riboquant system (BD Biosciences Pharmingen, San Diego, Calif); rat template rCK1 was used for detection of cytokines. In vitro transcription was carried out in transcription buffer supplemented with (α-^{32}P) uridine triphosphate (3000 Ci/mmol, Amersham Biosciences, Piscataway, NJ) and T7 RNA polymerase. After DNAase I treatment, the riboprobe was isolated by means of phenol-chloroform extraction and ammonium acetate-ethanol precipitation, and labeling efficiency was determined by measuring Chernokov activity in a scintillation counter. Each riboprobe was diluted to the optimal activity defined by the manufacturer, added to 20 μg of kidney RNA, heated to 90°C, allowed to cool to 56°C, and annealed overnight. After RNase and proteinase K treatment, protected RNA hybrids were purified by means of phenol-chloroform extraction and ammonium acetate-ethanol precipitation and separated by means of electrophoresis on 5% polyacrylamide/8M urea gels. Gels were dried and subjected to autoradiography by using Kodak Biomax MS2 film (Eastman Kodak Company, Rochester, NY).

GTS

Immunohistochemistry

Whole-lung tissue specimens were immediately fixed in 10% neutral-buffered formalin. Tissue samples were dehydrated through a series of graded alcohol baths, cleared, infiltrated, and embedded in paraffin. Specimens were cut in 5-μm serial sections, baked overnight at 50°C, and archived. In preparation for immunocytochemistry, sections were dewaxed and rehydrated through exposure to graded baths to a final distilled water wash. Specimens were then blocked with 5% normal serum for 30 minutes at 37°C. After the blocking step, excess serum was removed, and the primary antibody was applied at the concentration predetermined by means of titration experiments. The primary antibody (8 μg/mL, PeproTech) was incubated for 1 hour at 37°C. After incubation, sections were rinsed 3 times in 1× PBS (pH 7.4). Manufacturer stock secondary antibody (Vector Laboratories, Burlingame, Calif) was then applied at a dilution of 1:250 and incubated for 30 minutes at 37°C. At this time, the Avidin-Biotin complex, ABC conjugate (Elite kit, Vector Laboratories), was made a minimum of 30 minutes before use. After the secondary antibody incubation, sections were rinsed 3 times, and the ABC conjugate (Vector Laboratories) was then applied to the sections. Staining was performed with diaminobenzidine tetrahydrochloride. Sections were then rinsed in running tap water for 10 minutes, dehydrated, cleared, and mounted with permanent mounting media. Stained sections were examined by using the image analysis software Image Pro Plus (Media Cybernetics, Silver Spring, Md). Ten random fields were acquired by using digital photography. Analysis was automated by calibrating and establishing a threshold for positive staining on the basis of intensity and a value acquired by the software representing a total area per field. Values for total areas were then averaged, and therefore comparisons among control values and experimental samples were performed.

Statistical Analysis

All data were presented as mean values ± SEM unless otherwise designated. Comparisons between multiple groups were made by using analysis of variance and within groups by using the Bonferroni or Dunnett modified 2-tailed t test.

Results

Changes in Lung Vascular Permeability

Four different groups of control animals were generated. Negative control animals were those that did not undergo any surgical manipulation. Sham thoracotomy-only control animals were connected to the ventilator and had a left thoracotomy and up to 5.5 hours of mechanical ventilation. A third group underwent thoracotomy and occlusion of the left hilum for 90 minutes. These animals were killed before any reperfusion. The final group was subjected to the full protocol, including 90 minutes of ischemia, followed by 4 hours of reperfusion (Figure 1). The permeability index in animals undergoing thoracotomy alone (0.18 ± 0.02) was double that seen in unmanipulated lungs (0.09 ± 0.006). The increase in permeability seen with thoracotomy and ventilation was statistically significant ($P < .001$). The difference in permeability between animals that underwent thoracotomy alone and those that were subjected to 90 minutes of ischemia without reperfusion was not statistically significant (0.18 ± 0.02 vs 0.22 ± 0.005, $P = .20$). A 7-fold increase in permeability was seen in animals that underwent 4 hours of reperfusion after 90 minutes of ischemia (0.75 ± 0.01). The difference in lung permeability index between unmanipulated control animals or those undergoing ischemia alone and those undergoing ischemia and reperfusion were highly statistically significant ($P < .001$).

There was no statistically significant difference in permeability values for positive control animals receiving PBS (0.75 ± 0.01) or nonspecific IgG (0.77 ± 0.02, Figure 2). Animals receiving antibody to TNF-α or IL-1β had a significant decrease in permeability at 4 hours of reperfusion when compared with that of positive control animals (permeability index of 0.43 ± 0.05 and 0.45 ± 0.02, respectively). This represented a 48.7% and 47.4% decrease in injury among those animals receiving antibody (0.5 mg) to TNF-α or IL-1β, respectively ($P < .001$). The higher dose of blockade of IL-1β correlated with a 61.6% reduction in vascular injury (0.34 ± 0.02, $P < .001$). However, administration of the higher dose of TNF-α (1 mg) did not significantly alter the protection seen at the lower dose (45%). The combined blockade of both cytokines decreased lung vascular injury by 71%, which was more protection than that afforded by blockade of either TNF-α or IL-1β in isolation ($P < .001$).

MPO Activity

MPO activity was measured in lungs from unmanipulated control animals and from animals subjected to 90 minutes of ischemia and 0, 1, 2, 3, and 4 hours of reperfusion (Figure 3). The change in absorbance at 460 nm in unmanipulated control animals was 0.061 ± 0.002. With ischemia only,

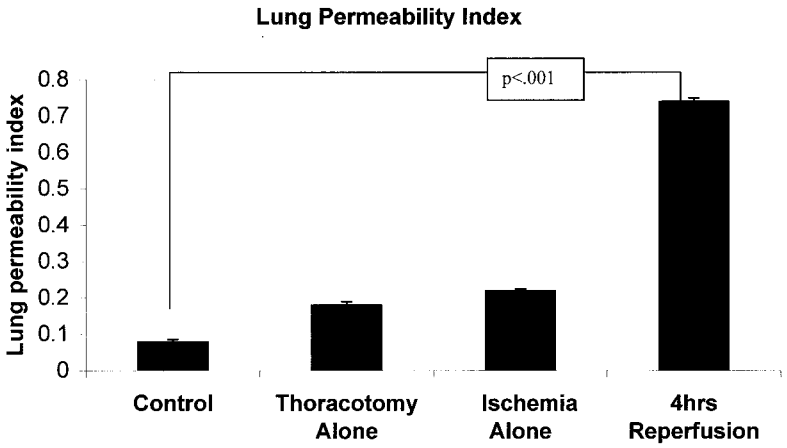

Figure 1. Left lung permeability in control animals. The lung permeability index is defined as counts per minute in the left lung divided by counts per minute in 1 mL of inferior vena caval blood. There is a 7-fold increase in lung vascular permeability in animals that undergo ischemia and reperfusion when compared with unmanipulated animals ($P < .001$). Animals that underwent thoracotomy alone and ischemia alone also had a statistically significant increase in permeability compared with that in unmanipulated control animals ($P < .04$; n = 4 in all experimental groups).

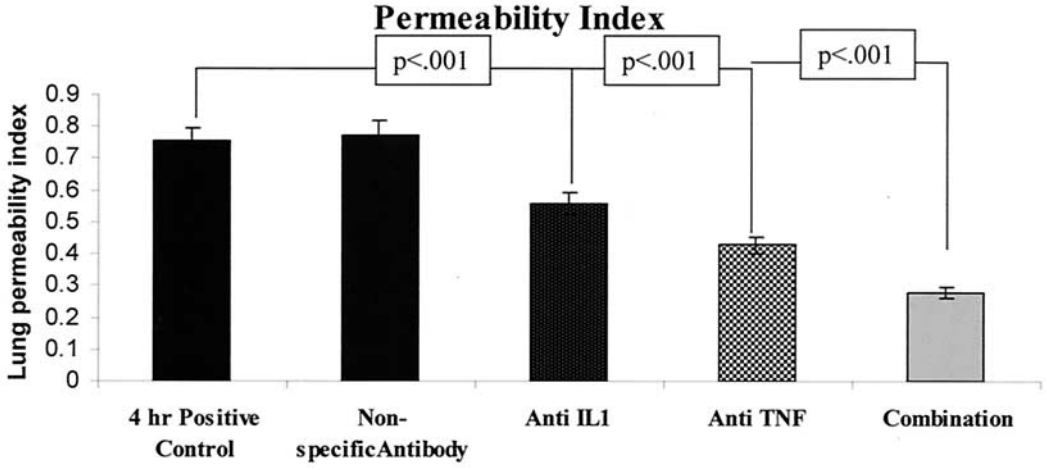

Figure 2. Left lung permeability in treated animals. Animals receiving anti-TNF-α, anti-IL-1β (0.5 mg), and combined blockade demonstrated a statistically significant decrease in lung vascular injury. Two groups of positive control animals are shown, one receiving saline solution and one receiving nonspecific rabbit IgG. There is no difference in permeability between these 2 groups. Animals that were administered anti-TNF-α and anti-IL-1β antibody (0.5 mg) demonstrated a 48.7% and 47.4% decrease, respectively, in lung vascular injury compared with that seen in control animals. Animals that received combined blockade demonstrated a 71% decrease in injury, which was statistically greater protection than that afforded by blockade of either cytokine alone (n > 3 in all experimental groups).

MPO activity increased only slightly when compared with that in unmanipulated control animals ($P = .03$). Reperfusion was associated with a steady increase in MPO activity that was detectable at 2 hours and markedly increased by 3 hours (0.26 ± 0.01) and 4 hours (0.41 ± 0.04) after reperfusion ($P < .001$, Figure 3). MPO content appeared to peak after 4 hours of reperfusion.

Animals receiving antibody to TNF-α and IL-1β had statistically significant decreases in MPO activity at 4 hours of reperfusion when compared with that in positive control animals (0.33 ± 0.03 for TNF-α and 0.30 ± 0.02 for IL-1β vs 0.46 ± 0.01, $P < .01$, Figure 4). The decrease in MPO content associated with TNF-α and IL-1β blockade correlated with a 30.9% and 38.5% decrease in tissue neutrophil

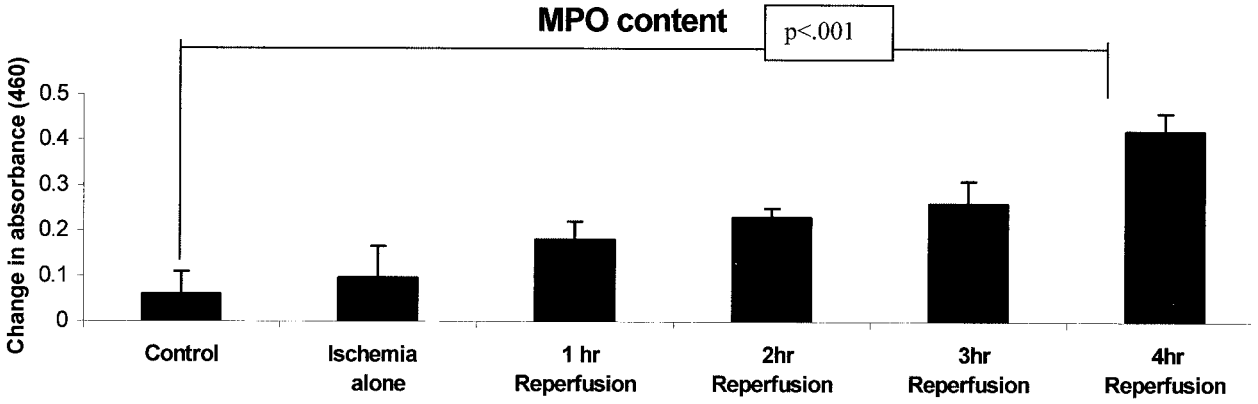

Figure 3. MPO content in control animals. MPO is measured as the change in absorbance at 460 nm over 1 minute. There is a steady increase in MPO content in the left lungs with increasing time of reperfusion. The increase in MPO content at 4 hours was statistically different from that of unmanipulated control animals ($P < .001$). Each experimental group includes 4 animals.

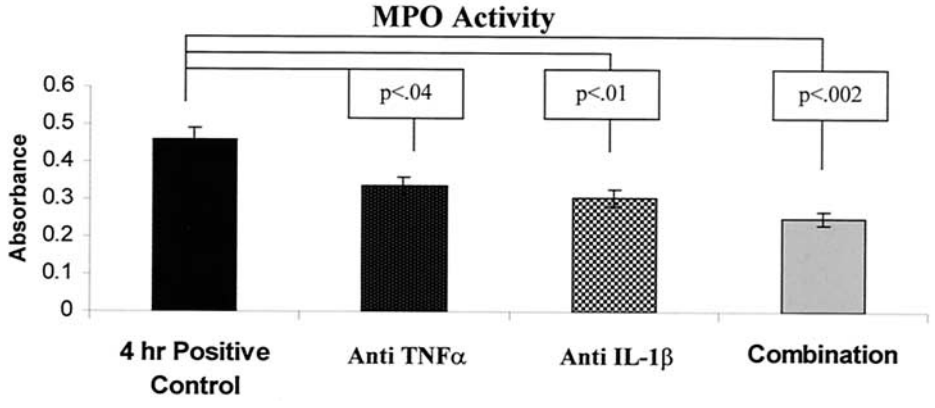

Figure 4. MPO in treated animals. Tissue neutrophil accumulation was decreased in animals receiving anti-TNF-α and anti-IL-1β antibody ($P < .04$). Animals that were administered antibody to both TNF-α and IL-1β demonstrated a 52.4% decrease in MPO content ($P < .002$, n > 3 in all experimental groups).

accumulation, respectively. Combined blockade with antibody to both cytokines decreased MPO content by 52.4%, a decrease in tissue neutrophil accumulation that was greater than that seen with individual cytokine blockade ($P < .002$).

BAL Neutrophil Content

BAL analysis was undertaken to evaluate neutrophil content in the distal airways and alveolar compartment. Baseline BAL neutrophil counts were recorded in 4 groups: unmanipulated control, sham thoracotomy alone, 90 minutes of ischemia alone, and 90 minutes of ischemia followed by 4 hours of reperfusion (Figure 5). Animals undergoing sham thoracotomy or ischemia alone did not demonstrate a significant increase in BAL neutrophil content; however, a statistically significant increase in BAL neutrophil content was seen with 4 hours of reperfusion ($P < .005$). The predominant cell type after 4 hours of reperfusion was the neutrophil, whereas all the other groups demonstrated mainly alveolar macrophages (>95%).

In animals treated with antibody to TNF-α and IL-1β, there was a statistically significant decrease in cell count in comparison with the 4-hour positive control animals ($P < .011$, Figure 6). This decrease in alveolar compartment cellular accumulation represented a 46% and 35.8% decrease in TNF-α and IL-1β antibody-treated animals, respectively.

Western Blot Analysis of TNF-α and IL-1β

TNF-α protein was not detected in the left lung homogenates of unmanipulated negative control animals, but protein was detected within 1 hour of reperfusion (Figure 7). The expression of TNF-α appeared to peak at 2 hours, as seen in the densitometric analysis shown below and then decrease to a baseline expression at 3 and 4 hours. IL-1β

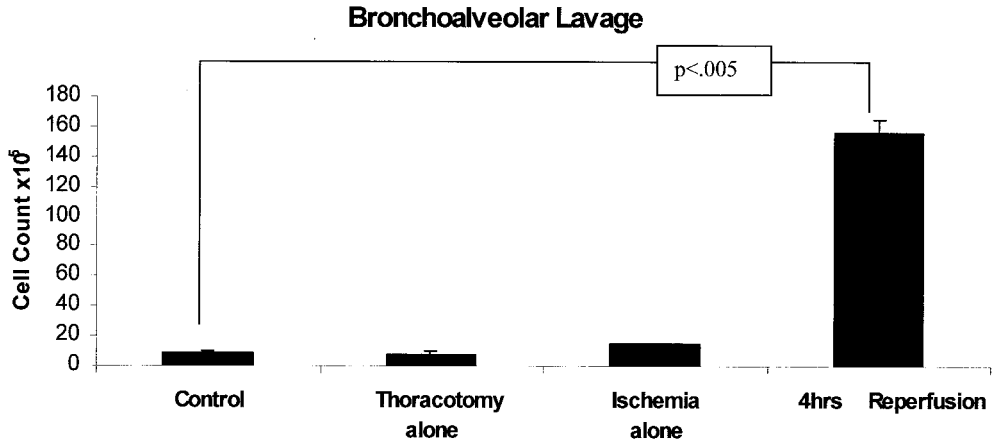

Figure 5. BAL cell count increased significantly in those animals undergoing ischemia followed by reperfusion compared with that in unmanipulated control animals ($P < .005$). A small increase in the cell count was seen with thoracotomy alone and ischemia alone (P = not significant). The predominant cell type in the unmanipulated animals was the alveolar macrophage, and the majority of the cells in the reperfused lungs were neutrophils (n = 4 in all experimental groups).

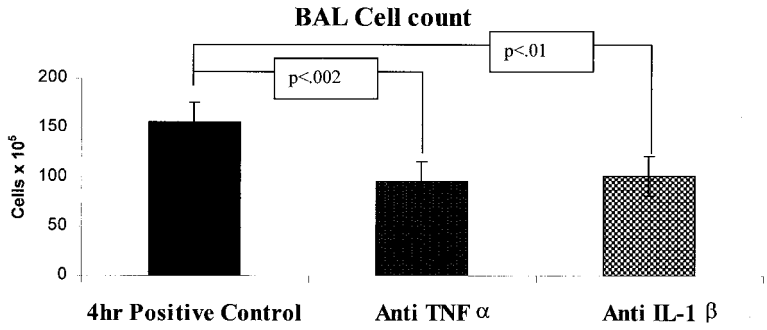

Figure 6. BAL cell count in treated animals. A significant decrease in alveolar leukocyte sequestration was also noted in animals receiving anti-TNF-α or anti-IL-1β antibody ($P < .02$, n = 4 in all experimental groups).

protein was not detectable in the negative control left lung homogenates (Figure 8). There was a slight amount of IL-1β detected at 1 and 2 hours of reperfusion that increased significantly at 3 hours and peaked at 4 hours. The densitometric analysis, quantitating the increased expression of IL-1β late during reperfusion, is shown below. The later expression of IL-1β contrasts with the earlier expression of TNF-α. This early appearance of TNF-α supports the implication that it regulates expression of other proinflammatory cytokines and effects neutrophil recruitment as early as 1 to 2 hours after reperfusion.

Ribonuclease Protection Assay

Ribonuclease protection assays were performed on lung extracts from 3 experimental groups: animals that underwent 90 minutes of ischemia and 3 hours of reperfusion and animals similarly treated but also receiving either anti-TNF-α antibody or anti-IL-1β antibody. Both left and right lungs were evaluated in all experimental groups (Figure 9). Animals that underwent ischemia and reperfusion of the left lung demonstrated significantly increased mRNA expression for a variety of mediators (IL-3, IL-5, IL-1, TNF-α, IL-2, and interferon γ). In reperfused lungs from animals treated with anti-TNF-α antibody, there was decreased expression of all inflammatory and regulatory cytokines compared with that seen in positive control lungs. This effect was most pronounced for IL-2, IL-4, IL-10, and TNF-α mRNA. Lung tissue treated with antibody to IL-1β also demonstrated a decreased expression of cytokine mRNA compared with that seen in tissue from positive control animals. Densitometric analysis for positive control and treated animals is shown in Figure 10. In a separate experiment, inhibition of the transcription factor nuclear factor κB with calcineurin inhibitors resulted in diminished ap-

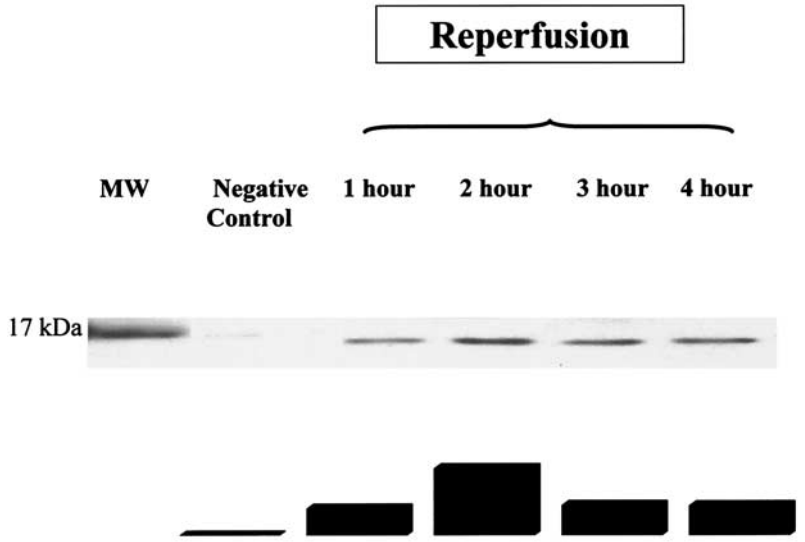

Figure 7. Western blot analysis of left lung homogenates for TNF-α. The molecular weight *(MW)* marker is shown in the far left lane. There is no detectable TNF-α protein in the negative control group. Protein is detected at 1 hour, peaks at 2 hours, and returns to a baseline level at 3 and 4 hours of reperfusion. Densitometric analysis of these differences is shown below (Western blots done in triplicate).

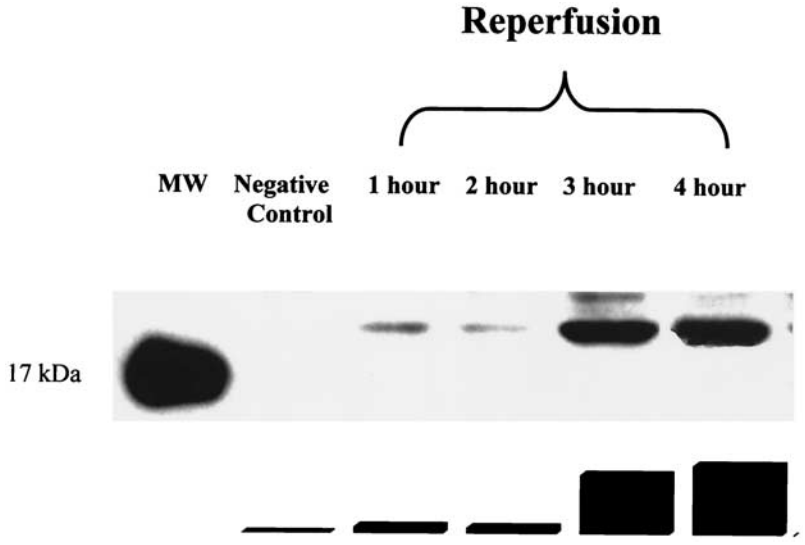

Figure 8. Western blot analysis of left lung homogenates for IL-1β. The molecular weight *(MW)* marker is shown in the far left lane. There is no detectable IL-1β protein in the negative control group. A small amount of protein is detected at 1 and 2 hours of reperfusion. At 3 hours, there is increased IL-1β detected, which peaks at 4 hours. Densitometric analysis is shown below (Western blots done in triplicate).

pearance of mRNA for TNF-α, but not IL-1β, in injured lungs. This suggests a potential divergence of mechanism of transcriptional regulation for these 2 cytokines (submitted manuscript).

Immunocytochemistry Analysis

Immunohistochemistry analysis data are shown in Figure 11. Surgical control and injured left lungs were processed for immunohistochemical analysis. A time course of injured lungs, including those that underwent ischemia alone and reperfusion of 15, 30, 60, 120, and 360 minutes, were stained for either TNF-α or IL-1β. In animals with negative assay results and surgical control animals, minimal staining was detected. Positive staining for TNF-α and IL-1β was present at the end of ischemia but became much more

intense within 15 minutes of reperfusion. In both TNF-α- and IL-1β-stained sections, staining was initially localized to lung macrophages. At later time points, TNF-α was detected in a more diffuse parenchymal pattern, but IL-1β remained localized to the macrophage throughout. However, the distribution of positively stained macrophages for IL-1β changed from those predominantly localized around the vasculature early in reperfusion to both interstitial and alveolar macrophages late in reperfusion.

Discussion

A precise delineation of the functional roles of TNF-α and IL-1β in an intact animal model of lung ischemia-reperfusion injury has not been previously elucidated. Vascular permeability studies demonstrate that blockade of both cytokines individually provided significant protective effects at 4 hours. The protection afforded by TNF-α blockade decreased lung vascular injury by 49%. Other studies have indicated slightly different degrees of protection associated with TNF-α antagonism. These differences in protection might be explained by the variability in the reported permeability of positive control animals in previously published experiments.[16,21,22] Studies of nonischemic inflammatory injury indicate that the protection afforded by TNF blockade is between 40% and 67%. These results are consistent with the degree of protection seen in our model of reperfusion injury.[13,18] IL-1β blockade has not been studied in an intact model of lung ischemia-reperfusion injury. Isolated perfused rabbit lungs demonstrated increased IL-1β mRNA and protein with reperfusion.[17] In this study the effects of IL-1β blockade on lung vascular permeability were less dramatic at the lower dose than those seen with TNF-α blockade; the protection afforded was 30%. However, with the higher dose, IL-1β blockade produce a more profound effect than TNF-α antagonism. Other studies have demonstrated that the effects of IL-1β antagonism are less significant than those seen with TNF-α antagonism.[7,13,18] This apparent difference might be explained in part by the dose used in this study.

Combined blockade with antibody to both of these cytokines was protective in an additive fashion. This might not seem intuitive because both of these cytokines have similar physiologic effects, but other investigators have also demonstrated an additive effect related to combined blockade in neutrophil-dependent models.[23] The effects of combined blockade were statistically more protective than either TNF-α or IL-1β blockade in isolation, which suggests that although these 2 cytokines have similar proinflammatory effects, the mechanism by which they effect injury might be different. TNF-α mRNA appears to be under a different transcriptional regulation than IL-1β, and therefore its functions might not be so redundant.

Neutrophil recruitment and subsequent neutrophil-mediated damage is well characterized in lung ischemia-reperfusion injury.[21] Both TNF-α and IL-1β increase the expression of selectins and cellular adhesion molecules and direct neutrophil chemotaxis through direct effects and through the production of chemokines.[24] As expected, blockade of these 2 cytokines was associated with a decrease in tissue (MPO content) and alveolar (BAL cell count) cell content. Decreases in MPO content of 36% and 40% were demonstrated with anti-TNF-α and anti-IL-1β antibody. Others have also correlated a decrease in MPO content with a blockade of these 2 cytokines.[7,18] Combined blockade of both cytokines decreased MPO content by 49%, and this is the first report of combined blockade of TNF-α and IL-1β resulting in an additive effect on neutrophil recruitment. These assays demonstrate that the proinflammatory effects of TNF-α and IL-1β are mediated at least partially through neutrophil recruitment and activation. Combined blockade of these 2 mediators is additive, suggesting that the mechanisms by which these 2 cytokines effect neutrophil recruitment might be distinct.

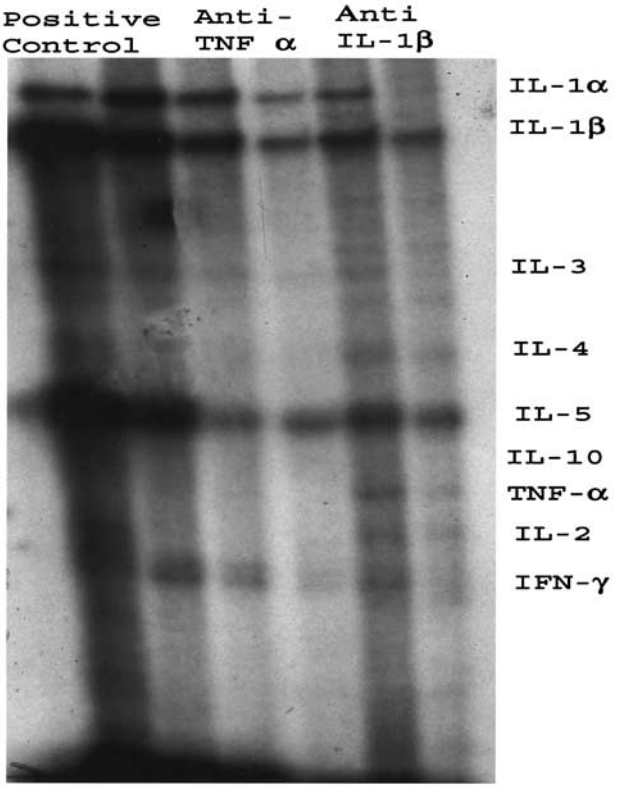

Figure 9. Ribonuclease protection assay. Three experimental groups are shown. The left and right lungs of the positive control are shown in the far left lanes. The adjacent lanes represent the left and right lungs of animals that underwent ischemia, 4 hours of reperfusion, and treatment with anti- TNF-α or anti-IL-1β antibody. There is a generalized decrease in expression of cytokines receiving antibody to TNF-α and IL-1β (ribonuclease protection assay done in triplicate).

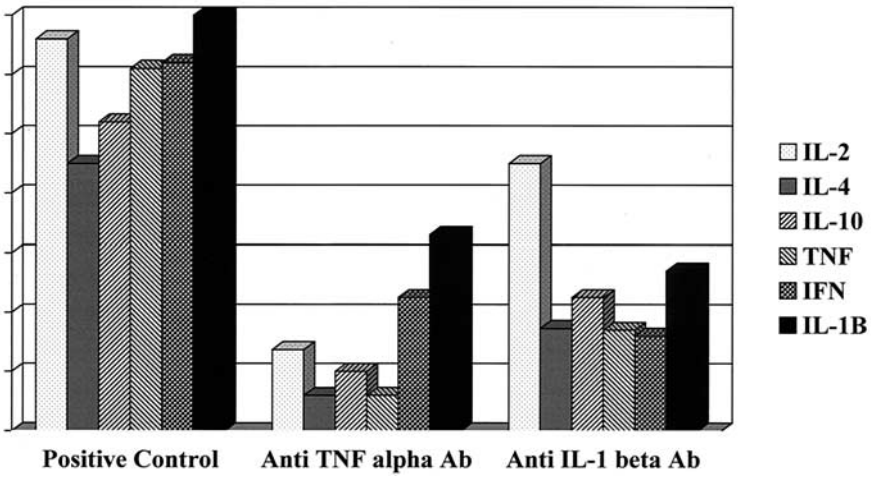

Figure 10. Densitometric analysis for a select group of cytokine mRNAs. By using Image J software and calibrating appropriately for gray scale, a decreased expression of message for TNF-α, IL-2, IL-1β, interferon γ, IL-4, and IL-10 are shown in response to TNF-α and IL-1β blockade. *Ab,* Antibody.

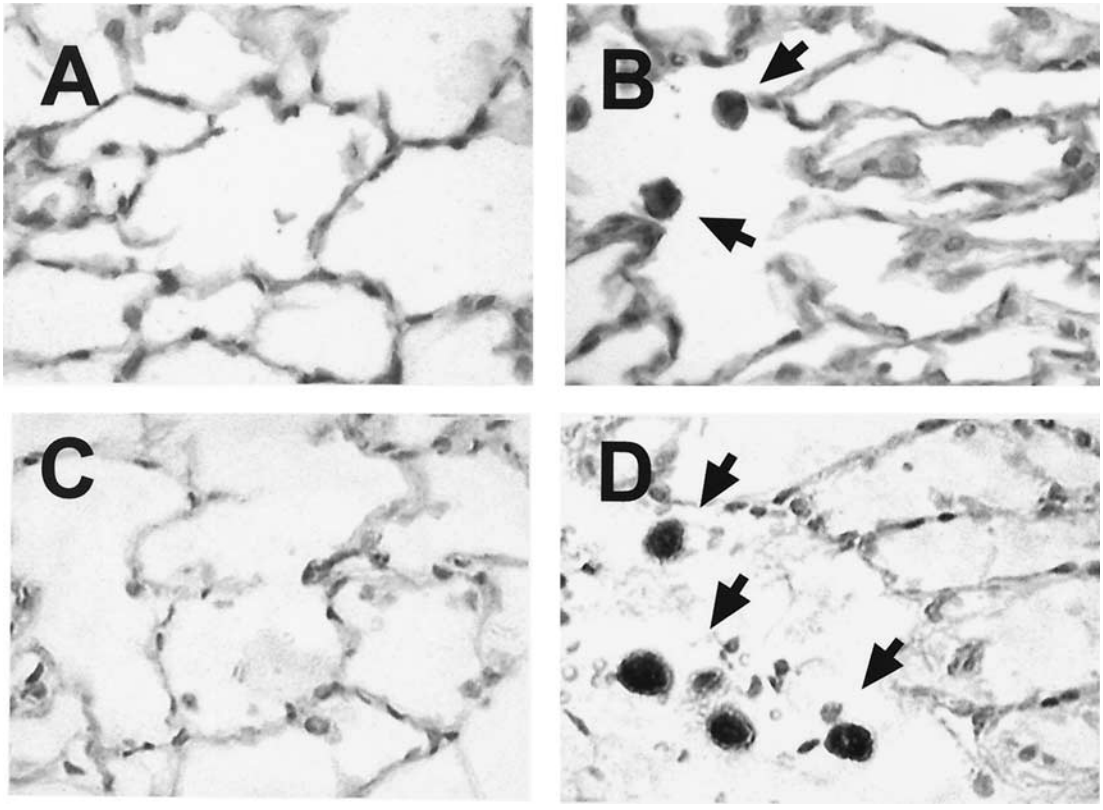

Figure 11. Immunohistochemical sections of lungs stained for TNF-α and IL-1β (representative samples, immunohistochemistry done on 3 different specimens per group). A and C, Negative surgical control demonstrating minimal staining for TNF-α and IL-1β, respectively; C and D, sections from injured lungs after 90 minutes of ischemia and 15 minutes of reperfusion showing intense positive brown staining for TNF-α and IL-1β, respectively, in mononuclear cells *(arrows).*

Western blots of left lung homogenates probing for TNF-α activity detects the production of TNF-α protein after 1 hour of reperfusion. Detectable protein levels was not evident in the negative control lungs. TNF-α protein appears at 1 hour, peaks at 2 hours, and then returns to baseline production at 3 and 4 hours of reperfusion. A precise delineation of TNF-α protein expression has not been elucidated in lung inflammation or lung ischemia-reperfusion injury. Early expression of TNF-α protein has been demonstrated in other models of reperfusion,[2,4,6] as well as in clinical situations involving lung reperfusion.[20] The mechanism affecting the early expression of TNF-α protein is still unclear, but some investigators have suggested that the early expression might represent the translation of constitutively expressed TNF-α mRNA.[25] Confirmation of this hypothesis would require the demonstration of new TNF-α protein in the presence of transcriptional blockade during ischemia and reperfusion.

Immunohistochemical analysis detects TNF-α protein within 15 minutes of reperfusion. Immunohistochemistry is a more sensitive modality for detecting small quantities of protein. The early expression of TNF-α from alveolar macrophages suggests that this cell is the key effector cell driving injury early after reperfusion. Later, during reperfusion, there is more diffuse expression of this cytokine throughout the parenchyma.

Expression of IL-1β protein is not detected in negative control left lungs (Western blot). Significant expression is detected in the injured lungs, which peaks after 3 hours of reperfusion; however, expression of IL-1β is seen soon after reperfusion in the immunohistochemical analysis. This again reveals the higher sensitivity of the histochemical analysis when compared with Western blotting. The expression of IL-1β appears to be less diffuse than TNF-α. IL-1β expression appears to be localized to macrophages. It does appear that the expression of IL-1β increases with reperfusion, thus explaining the detection of the protein in the Western blot later during injury.

Blockade of TNF-α demonstrates a blunting of the expression of the majority of cytokine mRNAs investigated on the ribonuclease protection assay shown. Not only are the expression of proinflammatory cytokines, such as IL-1, IL-2, and interferon γ, decreased, but the expression of regulatory cytokines IL-4 and IL-10 are also blunted. The same pattern of reduced message expression is also evident in the left lungs that received anti IL-1β antibody, although the effects are not as pronounced. The expression of collateral cytokine mRNA has not been investigated in lung ischemia-reperfusion injury, and these effects suggest that the protective effects of TNF-α and IL-1β blockade are also mediated in part through a diminution in expression of phlogistic cytokine mRNA.

TNF-α and IL-1β appear to play functional roles in warm lung ischemia and reperfusion. This is demonstrated through a decrease in lung injury (permeability, MPO, and BAL), as well as a modulation of cytokine expression (ribonuclease protection assay). Both TNF-α and IL-1β are expressed early during reperfusion (Western blot), and combined blockade of both of these cytokines appears to be additive.

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