Interleukin-6 production in hemorrhagic shock is accompanied by neutrophil recruitment and lung injury

CHRISTIAN HIERHOLZER, JÖRG C. KALFF, LAUREL OMERT, KATSUHIKO TSUKADA, J. ERIC LOEFFERT, SIMON C. WATKINS, TIMOTHY R. BILLIAR, AND DAVID J. TWEARDY

1Department of Surgery, 2Cell Biology, 3Medicine, and 4Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine and 5University of Pittsburgh Cancer Institute, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania 15213

Hemorrhagic shock (HS) initiates a cascade of inflammatory events after successful resuscitation that may result in organ impairment, including acute respiratory distress syndrome (ARDS) and increased mortality. We have previously shown that resuscitated HS in rats results in acute lung injury manifested by infiltration of polymorphonuclear neutrophils (PMN) into the interstitium and alveoli, pulmonary edema, and hypoxia (15). Chemokines of the C-X-C class, especially interleukin-8 (IL-8), have been demonstrated to be key mediators of PMN infiltration in HS (20, 28). Recent studies have demonstrated that IL-6 participates in the recruitment of PMN into tissue sites by induction of IL-8 (26).

The systemic response to inflammation includes the production of IL-6, which signals through activation of proteins that serve the dual function of signal transducers and activators of transcription (STAT) (18). We previously demonstrated activation of STAT proteins, particularly Stat3, in the lungs of animals subjected to HS and that levels of Stat3 activity increased with increasing severity of shock (13).

The current study was designed to determine if IL-6 production occurs in our rat model of HS where it may contribute to PMN infiltration and Stat3 activation. We report here that levels of IL-6 mRNA increased with increased duration of the ischemic phase of resuscitated shock, similar to our findings regarding Stat3 activity. In addition, levels of IL-6 mRNA and Stat3 activity were increased above control animals only in resuscitated HS animals. Furthermore, induction of IL-6 mRNA as well as the activation of Stat3 occurred simultaneously after resuscitation, with levels of both peaking 1 h after the onset of resuscitation. To test the hypothesis that IL-6 protein in the distal airways of the lung will cause PMN recruitment and lung injury, we administered IL-6 by intratracheal instillation into the lungs of anesthetized rats. IL-6 instillation resulted in increased bronchoalveolar lavage (BAL) fluid cellularity, accumulation of PMN into widened interstitial and alveolar spaces, and increased wet-to-dry ratios to levels observed previously in animals subjected to HS. These results suggest that IL-6 is produced in the lung in HS where it contributes to Stat3 activation, PMN infiltration, and lung injury.

MATERIALS AND METHODS

Animals. These studies were approved by the University of Pittsburgh Institutional Review Board for animal experimentation and conform to National Institutes of Health guidelines for the care and use of laboratory animals. Fasted male Sprague-Dawley rats (Charles River Breeding Laboratory, Cambridge, MA) were used for all phases of these studies.

HS protocols. For initial anesthesia, penthrane inhalation was used. The animals were intubated orally with a 14-gauge cannula. The right carotid artery and left jugular vein were cannulated with 21-gauge tubing after surgical preparation and isolation. The cannulas, syringes, and tubing were flushed with heparin sodium (1,000 U/ml) before all procedures. Arterial blood pressure was continuously monitored with a Spacelab 514 multimonitor (Spacelabs, Hillsboro, OR). A Harvard small-animal ventilator (Braintree Scientific, Braintree, MA) was used to administer a 2.5-ml tidal volume of room air at 72 strokes/min. After vascular cannulation, the animals received intravenous anesthesia (50 mg/kg pentobarbital sodium).

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Two HS protocols were performed: HS without resuscitation and HS with resuscitation as previously described (13). Briefly, the unresuscitated HS protocol was carried out to the predefined end points of compensated, decompensated, and irreversible shock. After the initial bleed of 2.25 ml/100 g body wt, mean arterial pressure (MAP) dropped sharply and then slowly stabilized. In compensated shock (1 h of ischemia), additional blood was withdrawn to maintain MAP at 40 mmHg for 1 h, and no shed blood was returned (0% shed blood return, SBR). In decompensated shock (2.5 h of ischemia), the MAP was maintained at 40 mmHg for 2.5 h and required 35% of the shed blood to be returned (35% SBR). In irreversible shock (3.5 h of ischemia), the MAP was maintained at 40 mmHg for 3.5 h and required 70% of the shed blood to be returned (70% SBR). Animals in the unresuscitated HS protocol were killed at the end of the ischemic phase. Time-matched sham control animals underwent all preparations and monitoring procedures but were not bled. Animals were randomly subjected to either the shock or sham protocol. There were three to five animals in each shock and sham group.

In the resuscitated HS protocol, animal preparation and hemorrhage were carried out as in the unresuscitated protocol to the defined end points: for the compensated shock (1 h of ischemia) groups to compensation end point with 0% SBR and for the decompensated shock (2.5 h of ischemia) groups to midcompensation phase with 35% SBR. Once the animals had attained these points in the protocol, they were resuscitated, and the MAP was normalized using shed blood plus two times the shed blood volume in lactated Ringer solution. Once the animal had recovered, all cannulas were removed, and the incision was closed. Rats were killed from 1 to 8 h after the initiation of resuscitation. Time-matched sham animals again served as controls, and there were four rats in each group at each time point. The irreversible shock group (70% SBR) was excluded from the resuscitation protocol because animals subjected to 70% SBR could not be resuscitated.

IL-6 instillation into the lungs of normal animals. Recombinant human IL-6 was obtained from Genzyme (Cambridge, MA). The specific activity was 8.1 × 10^7 U/mg as determined by the manufacturer. Endotoxin levels were 0.102 ng/µg as determined using a kinetic-chromogenic method. IL-6 was administered by intratracheal injection as previously described (14). In brief, male Sprague-Dawley rats were subjected to instillation of IL-6 or saline fluid by intratracheal injection. Animals were intubated orally. To prepare a surfactant-containing vehicle for IL-6 instillation, 20 ml of saline was used to lavage the lung of a normal rat. The lavage fluid was centrifuged (2,500 g, 5 min) to remove cells and debris. IL-6 doses (0, 10, 30, 100, or 300 ng) were diluted in 1 ml of cell-free lavage fluid and injected into the trachea of anesthetized animals followed by three strokes of air with a 3-ml syringe. After instillation and recovery from the anesthesia, all rats were active and alert in their cages. Animals were killed at 2, 4, 8, 12, 24, and 48 h after IL-6 instillation. Isolation of lungs and cells and measurement of lung injury. The rats subjected to the HS protocols were killed at the completion of each experiment. After the carcasses were flushed with cold (4°C) isotonic saline solution, the left lung was removed and used for wet-to-dry determination and PMN counts. The right lung was immediately frozen in liquid nitrogen and stored at −80°C. The right lung then was used for total cellular RNA extraction using the method of Chomczynski and Sacchi (7) and for protein extraction from frozen sections using high-salt buffer for use in electrophoretic mobility shift assay (EMSA) as described (12). In rats subjected to the instillation of IL-6, median sternotomy and preparation of the trachea were performed, and the left pulmonary hilus was isolated and ligated. The left lung was excised and removed for wet-to-dry ratio. The right lung was fixed by inflation with formaldehyde solution (4%). BAL was performed by injecting and retracting 3 ml of sterile saline 10 times into the airways. Cells were counted using a hemocytometer. A differential count was performed on Wright-stained cytocins.

For histopathological examination, the lungs of animals were sectioned and stained for myeloperoxidase (MPO; see Ref. 11) and with hematoxylin and eosin (H+E) using standard procedures. The stained slides were examined at ×400 magnification. Ten randomly chosen fields of each lung specimen were blindly scored for number of intensely staining MPO-positive PMN as described (14).

RT-PCR amplification. Total RNA (2.5 µg) was subjected to first-strand cDNA synthesis using oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (15). Primers for amplification of rat IL-6 cDNA were based on rat cDNA and were designed with the assistance of a PCR primer design program, PCR Plan (Intelligenetics, Mountain View, CA). The 5′ primer sequence was ACAAGGATGTGACGCTGAG. The 3′ primer sequence was ATGTTCTGGTCTTATTGGCAG. The primers amplified a product of ~339 bp in length. Restriction enzyme digestion with HindIII and EcoRI confirmed the identity of the fragment. Semi-quantitative RT-PCR was performed as described (12). Briefly, γ-32P end-labeled 5′ primer was used, and PCR conditions were as follows: denaturation at 94°C for 1 min; annealing at 57°C for 1 min; and polymerization at 72°C for 2 min in a Perkin-Elmer 480 thermocycler. The optimized cycle number was identified at 30 cycles. Rat peritoneal macrophages elicited with thioglycolate and RAW cells stimulated in vitro with lipopolysaccharide (LPS) served as a positive control for rat IL-6 mRNA. The negative control for each set of PCR reactions contained water instead of DNA template. Fifteen microliters of the PCR reaction were separated on a 10% polyacrylamide gel. After gel drying and exposure to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA), the relative radioactivity of the bands was determined by volume integration using laser scanning densitometry. Each gel contained the same positive control, which permitted normalization of samples and comparison between gels.

EMSA. EMSA was performed using whole tissue extracts of lung sections from the experimental groups as described (12). Binding reactions were performed using 20 µg of extracted protein and radiolabeled DNA-binding element. The activation of Stat3 was assessed using the high-affinity serum- and cytokine-induced Stat3-specific antibody (hSIE) duplex oligonucleotide that preferentially binds Stat3 and Stat1 (30). EMSA was performed on a 4% polyacrylamide gel as described (6). The level of Stat3 activation was quantitated using PhosphorImager analysis of gel shift band intensities. Where indicated, EMSA binding reactions were incubated with antibodies specific for Stat3a or Stat3b. The Stat3a-specific antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and was generated in rabbits against the COOH-terminal 20 of 21 amino acid residues of murine Stat3a. Stat3b-specific antibody was generated at Charles River Pharmaservices (Southbridge, MA) by immunizing chickens with the COOH-terminal 10 amino acid residues of human Stat3b conjugated to thyroglobulin (12).

In situ hSIE binding assay. Five-micrometer sections were cut on a cryostat microtome and mounted on positively charged “Superfrost” slides (Superfrost, Fisher, PA). Specimens were washed three times in 1× PBS and incubated in 1× PBS and DNase (1 µl/50 ml; Ambion, Austin, TX) for 30 min. After 30 min of incubation in 2% paraformaldehyde at

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room temperature, specimens were washed three times with 1× PBS and then incubated with the 35S-labeled hSIE duplex oligonucleotide for 60 min at 30°C and washed again three times in 1× PBS. Specimens were then dehydrated and submerged in Kodak photographic emulsion (1:1 dilution with deionized H2O). Specimens were exposed to Kodak film for 5 days, developed in D-19 (Kodak) for 20 min, and visualized with a Nikon FXA photomicroscope.

Immunohistochemistry. Frozen lung sections (5 µm) mounted on positively charged Superfrost slides were washed with 0.05 M PBS and PBS containing 1% bovine serum albumin (BSA) each three times, incubated in goat serum for 15 min, blotted, and incubated for 2 h at room temperature with mouse anti-human IL-6 monoclonal antibody, which cross-reacts with rat IL-6 (1:50 dilution; Endogen, Cambridge, MA) or with a nonspecific isotype-matched control. Specimens were washed three times with BSA, incubated in the fluorescent-labeled secondary indocarbocyanine-conjugated goat anti-mouse IgG antibody (1:250 dilution; Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h at room temperature, washed three times in BSA, incubated in Hertz stain for 30 s, and washed three times in BSA. After air drying, specimens were covered with coverslips and Gelmount (Biomeda) and inspected by fluorescent microscopy (Nikon FXA photomicroscope).

Statistics. Unless otherwise indicated, data are presented as means ± SE. Comparisons of means were performed using ANOVA followed by comparison of individual pairs of means using the Scheffé's test. Both tests are contained within the StatView 4.1 program (Abacus Concepts, Berkeley, CA).

RESULTS

IL-6 mRNA and protein are increased in the lungs of rats subjected to HS. We have previously reported that rats subjected to our HS protocol demonstrated evidence of acute lung damage with increased PMN infiltration and elevated wet-to-dry ratio (15). To determine if the proinflammatory cytokine IL-6 was expressed locally in the lung in HS where it may contribute to the PMN recruitment and lung damage, we determined IL-6 mRNA levels and IL-6 protein expression in the lungs of rats subjected to HS. Using RT-PCR with end-labeled 5´ primer, we performed semiquantitative RT-PCR to measure levels of IL-6 mRNA in the lungs of normal rats and rats subjected to the HS or sham protocol (Fig. 1). Lungs of normal rats demonstrated a low level of an amplified IL-6 product. Sham animals demonstrated a 5.1- to 11.2-fold increase in amplified IL-6 product over normal animals (NL; open bar), sham control animals (open bars), and shock animals (filled bars) was plotted. Differences between each shock and sham group were significant (P < 0.01 for each group). Levels of amplified IL-6 were increased 43% at 4 h after resuscitation in animals subjected to decompensated vs. compensated (* P = 0.01) and 69% at 8 h after resuscitation in animals subjected to decompensated vs. compensated HS (** P = 0.03).
examined (compensated/4 h, compensated/8 h, decompensated/4 h, decompensated/8 h). Comparison of compensated shock groups (1 h of ischemia) with decompen­sated shock groups (2.5 h of ischemia) at identical time points of death after resuscitation to determine the influence of the duration of the ischemic phase of shock on IL-6 mRNA levels revealed a 43% increase in IL-6 mRNA levels after 2.5 h of ischemia and resuscitation (decompensated shock) and death after 4 h versus 1 h of ischemia and resuscitation (compensated shock) and death after 4 h (P < 0.01). Similarly, we observed a 69% increase in IL-6 mRNA levels in animals subjected to 2.5 h of ischemia and resuscitation (decompensated shock) and death after 8 h versus animals subjected to 1 h of ischemia and resuscitation (compensated shock) and death after 8 h (P = 0.03). Comparisons made to determine the influence of the duration of the resuscitation phase on IL-6 mRNA levels revealed no difference in IL-6 mRNA levels 4 h after resuscitation compared with 8 h after resuscitation in either group.

To establish that increased IL-6 mRNA production in the lungs in HS is accompanied by increased IL-6 protein, we performed immunohistochemistry (Fig. 2). IL-6 protein was increased in shock animals compared with sham animals, with the most prominent staining occurring in cells lining the bronchioles. IL-6 protein was also observed in alveolar macrophages, but staining was less intense. The lungs of normal animals did not demonstrate specific staining for IL-6 protein (data not shown).

Increased expression of lung IL-6 in HS requires both the ischemic and resuscitation phases and occurs rapidly after resuscitation. To determine whether or not resuscitation from HS was required for the induction of IL-6 mRNA and, if so, to examine the kinetics of IL-6 mRNA production during the resuscitation phase, we measured levels of IL-6 mRNA at the end of the ischemic phase and at 1, 2, 3, and 4 h after resuscitation (Fig. 3, A and B). IL-6 mRNA was not increased in shock animals at the end of the ischemic phase compared with sham animals; however, levels of IL-6 mRNA were increased over sham animals after resuscitation at all time points examined, with the peak occurring 1 h after resuscitation and demonstrating a 5.9-fold increase over sham levels (P = 0.04). To confirm the failure of ischemia alone to induce IL-6 production above sham levels, we examined the lungs of animals for IL-6 mRNA production in two additional HS protocols in which animals were subjected to unresuscitated compensated and unresuscitated irreversible HS. With semiquantitative IL-6 RT-PCR, there was no difference in IL-6 mRNA levels between shock and sham animals regardless of the severity of shock (Fig. 3C). Even in animals subjected to irreversible HS, an ischemic injury that would have had a 100% lethality, levels of IL-6 mRNA were not elevated over levels found in sham animals.

Stat3 activation in HS requires the resuscitation phase and exhibits similar kinetics to IL-6 induction. IL-6 signals through activation of Stat3. We previously demonstrated activation of STAT proteins, particularly Stat3, in the lungs of animals subjected to resuscitated HS (13). It is not known if the resuscitation phase is necessary for the activation of Stat3 or the time course of the activation of Stat3 after resuscitation. Protein extracts of lungs from shock animals killed at the end of the 2.5-h ischemic phase (decompensated shock) did not demonstrate increased Stat3 activity compared with sham animals with the use of EMSA gel shift (Fig. 4, A and B). In contrast, we found that Stat3 activity
Fig. 3. Semiquantitative RT-PCR of IL-6 mRNA from lungs of rats subjected to unresuscitated and resuscitated HS. RT-PCR reactions were performed using total RNA (2.5 µg) from the lungs of animals subjected to the decompensated (2.5 h of ischemia) HS or sham protocol without resuscitation (0 h) or 1 (R1), 2 (R2), 3 (R3), or 4 (R4) h after resuscitation. Reaction products were separated on polyacrylamide gels. Gels were dried and exposed to a PhosphorImaging screen and developed using a PhosphorImager. The location of the amplified IL-6 fragment (339 bp) is indicated on right. In B, the radioactive signal within the amplified fragment was quantitated using scanner laser densitometry and ImageQuant software, and the mean ± SE of the sham groups (open bars) and shock groups (filled bars) was plotted. The differences between each resuscitated shock and sham group were significant (P < 0.01 for each comparison). There was no difference between the mean of the shock and sham groups without resuscitation (0 h; P = 0.6). The mean of the shock groups at 1, 2, 3, and 4 h was significantly increased over the unresuscitated (0 h) group (P ≤ 0.04). In C, RT-PCR reactions were performed using total RNA (2.5 µg) from the lungs of animals subjected to compensated (C; 1 h of ischemia) or irreversible (I; 3.5 h of ischemia) shock protocol without resuscitation (filled bars) or to the corresponding sham procedure (open bars). Reaction products were separated on polyacrylamide gels and quantitated as above. Values shown are means ± SE of each group (n = 4). There were no differences between shock and sham groups within either set (P = 0.7).
was increased after resuscitation at all time points examined, peaking 1 h after resuscitation with a level of activity 9.2-fold greater than sham controls (P < 0.02). Similar to IL-6 mRNA levels, there was no difference in Stat3 activity between shock and sham animals in unresuscitated compensated (1 h of ischemia) and irreversible (3.5 h of ischemia) HS (Fig. 4C).

Three distinct isoforms of Stat3 have been identified, Stat3α, Stat3β, and Stat3γ. Stat3α (92 kDa) is the predominant isoform expressed in most cells (2). Stat3β (83 kDa) arises from alternative splicing of the Stat3 gene transcript, resulting in a 50-nucleotide deletion at the 3' end of the open reading frame of Stat3α (6, 27). The third isoform, Stat3γ, is a 72-kDa protein identified in granulocyte colony-stimulating factor-activated mature neutrophils and is derived from Stat3α by posttranslational modification involving proteolysis (A. Chakraborty and D. J. Tweardy, unpublished observation). Stat3α and Stat3β each are supershifted by specific antibodies; no antibody is available that can supershift only Stat3γ. We added antibody specific to either Stat3α or Stat3β or both antibodies to binding reactions containing radiolabeled hSIE duplex oligonucleotide to determine which of these Stat3 isoforms are activated in the lungs of shock animals. Addition of both antibodies together supershifted a portion of the serum-inducible factor (SIF)-A complex. Addition of both antibodies together supershifted a greater portion than either alone but did not supershift all of the SIF-A complex (Fig. 4D). The remaining SIF-A complex has the mobility of Stat3γ (Fig. 4D). Thus all isoforms of Stat3 were activated in the lung in HS. The activation of Stat3γ corresponds to our finding of PMN recruitment into the lung after resuscitated HS.

To determine the cellular site of Stat3 activation in the lungs after HS, we performed an in situ binding assay using frozen lung sections and 35S-radiolabeled hSIE duplex oligonucleotide as a probe. The positions of the SIF-A, -B, and -C complexes, the supershifted complex, and the residual SIF-A complex after supershift of Stat3α and Stat3β (Stat3γ) are indicated.

![Fig. 4. Stat3 activation in the lung after unresuscitated and resuscitated HS.](image-url)

In A, protein extracts were obtained from frozen sections of lungs from animals subjected to uncompensated (2.5 h of ischemia) HS without resuscitation (R0) or uncompensated HS followed by 1 (R1), 2 (R2), 3 (R3), or 4 (R4) h of resuscitation or to the corresponding sham procedure. Extracts (20 µg) were used in electrophoretic mobility shift assays (EMSA) with the radiolabeled high-affinity serum-inducible element (hSIE) duplex oligonucleotide. The positions of the serum-inducible factor (SIF)-A (Stat3 homodimer), SIF-B (Stat1/Stat3 heterodimer), and SIF-C (Stat1 homodimer) complexes are indicated on right. In B, the SIF-A band was quantitated by PhosphorImager analysis, and the mean ± SE for shock animals (filled bars) and sham control animals (open bars) was plotted. Differences between each resuscitated shock and sham group were significant (P < 0.01 for each comparison). There was no difference between the mean of the shock and sham groups without resuscitation (0 h; P = 0.4). The mean of the shock groups at 1, 2, 3, and 4 h was significantly increased over the unresuscitated (0 h) group (P < 0.02). In C, EMSA was performed using extracts from the lungs of rats subjected to compensated (C; 1 h of ischemia) or irreversible (I; 3.5 h of ischemia) HS (filled bars) without resuscitation or to the corresponding sham procedure (open bars). The SIF-A band was quantitated by PhosphorImager analysis as above, and the mean ± SE for each group was plotted. There was no difference between the mean of the shock and sham groups in any of these unresuscitated animals (P = 0.5, 0.7, and 0.8). In D, extracts of lungs from a representative shock animal were incubated with antibodies specific for Stat3α or Stat3β or with both antibodies. The positions of the SIF-A, -B, and -C complexes, the supershifted complex, and the residual SIF-A complex after supershift of Stat3α and Stat3β (Stat3γ) are indicated.
hSIE duplex oligonucleotide (Fig. 5). Examination of the shock lung specimens after incubation with the hSIE probe was most notable for binding of labeled probe within alveolar cells. Specimens from sham animals did not demonstrate binding of the probe.

Intratracheal instillation of IL-6 into the lungs resulted in PMN infiltration and lung damage. IL-6 has been recently shown to cause tissue accumulation of PMN through the induction of chemokines such as IL-8 (26). To determine whether or not IL-6 protein alone is sufficient to cause PMN accumulation in the lungs of rats, we instilled IL-6 protein (300 ng) by syringe injection through an intratracheal tube. BAL was performed on IL-6-treated animals, and BAL cellularity was determined at 2, 4, 8, 12, 24, and 48 h (Fig. 6). The BAL cellularity was increased at all time points examined and peaked at 4 h, achieving a 5.6-fold increase compared with control saline-treated animals (P = 0.03). Examination of Wright-Giemsa stain of BAL cells at 4 h after IL-6 instillation revealed that 45 ± 8% of the isolated cells were PMN.

To examine IL-6-induced PMN recruitment into the lung in greater detail, lungs of rats were sectioned and stained for MPO and with H+E. Examination of MPO-stained sections of lungs of rats that received IL-6 revealed many MPO-positive cells compared with animals receiving the saline control (Fig. 7, A and C). To quantitatively assess the increase in PMN recruitment into the interstitium and alveoli of lungs of animals receiving IL-6 or saline control, MPO-stained slides from lung cross sections were examined at ×400 magnification at 2, 4, 8, 12, 24, and 48 h after IL-6 instillation. Ten random fields of each lung specimen were scored blindly for number of intensely staining MPO-positive PMN. The scores were pooled, and the means ± SE were calculated (Fig. 8A). Lungs of IL-6-treated animals demonstrated an increase in MPO-positive PMN in both the interstitium and alveoli at 4, 8, 12, 24, and 48 h compared with saline-treated animals, with the peak of PMN accumulation occurring from 4 to 12 h. To determine the minimum amount of IL-6 necessary to result in lung PMN accumulation, animals were examined 4 h after receiving 10, 30, 100, and 300 ng of IL-6 by tracheal instillation (Fig. 8B). There was a linear increase in MPO-positive cells (r = 0.74; P < 0.03) with increasing doses of IL-6. Instillation of as little as 30 ng
of IL-6 increased PMN infiltration by 7.1-fold over animals receiving saline control (P = 0.01).

PMN migration into alveolar spaces is not always accompanied by injury of the alveolar capillary wall in vivo and by pulmonary edema (8, 10, 19). To determine if IL-6-mediated PMN infiltration caused lung damage, we examined the lungs histologically and measured lung wet-to-dry ratio. The accumulation of PMN was accompanied by histological signs of lung injury with widened interstitium and interstitial and alveolar edema (Fig. 7). The wet-to-dry ratio increased in the IL-6-treated animals at 4, 8, 12, and 24 h compared with control animals (Fig. 9) and peaked at 4 h, achieving a 45% increase over normal lung (P = 0.03). To determine the minimum amount of IL-6 necessary to cause an increase in the wet-to-dry ratio, animals were examined 4 h after receiving 10, 30, 100, and 300 ng of IL-6 by tracheal instillation (Fig. 8B). We observed a linear increase in the wet-to-dry ratio (r = 0.82, P = 0.001) with increasing doses of IL-6. Instillation of as little as 30 ng of IL-6 increased the wet-to-dry ratio by 11% compared with animals receiving the saline control (P = 0.04).

**DISCUSSION**

In this study, we demonstrated that IL-6 is locally produced in the lungs of rats subjected to resuscitated HS. Levels of IL-6 mRNA increased with increasing duration of the ischemic phase of resuscitated shock. IL-6 protein was located in bronchoepithelial cells and in alveolar macrophages. Both the ischemic and resuscitation phases were required to increase IL-6 mRNA levels above those of sham control animals. We found increased activation of Stat3 compared with sham animals only in the lungs of rats subjected to resuscitated HS. IL-6 mRNA expression and Stat3 activation demonstrated similarly rapid kinetics, with levels of both peaking 1 h after the onset of resuscitation. Intratracheal instillation of IL-6 into the lungs of normal rats caused PMN infiltration and lung damage characterized by pulmonary edema. Our results indicate that IL-6 alone can induce lung injury and suggest
that the production of IL-6 may contribute to ARDS after HS.

We previously observed that the lungs of animals subjected to 2.5 h of ischemia followed by resuscitation and death at 8 h demonstrated the greatest increase in PMN infiltration and wet-to-dry ratio compared with sham animals (15). PMN accumulation (48 ± 4.9 MPO-positive PMN/×400 field) and wet-to-dry ratio (6.2 ± 0.3) in these animals were similar to PMN accumulation (61 ± 12 and 73 ± 11) and wet-to-dry ratio (6.05 ± 0.25 and 5.69 ± 0.28) observed in animals 4 and 12 h, respectively, after IL-6 instillation (300 ng).

The pathogenesis of ARDS is thought to involve a diffuse alveolar capillary injury. Activated neutrophils are the major cellular elements that mediate acute inflammation and have been implicated in the pathogenesis of the microvascular injury that occurs in ARDS (29). The results from our study indicate that the presence of IL-6 alone can lead to pulmonary recruitment of PMN and pulmonary edema. Thus increased IL-6 production in the lung of patients suffering from HS may be an additional mechanism that contributes to PMN-induced lung injury and possibly ARDS.

IL-6 is a multifunctional cytokine that is part of the acute inflammatory response. IL-6 stimulates neutrophilia and thrombopoiesis and induces the synthesis of acute-phase proteins (16, 17, 25). Sustained elevations of IL-6 in the plasma and BAL of patients suffering from ARDS have been demonstrated and negatively correlated with disease outcome and patient survival (5, 21). Although the acute phase of ARDS involves PMN recruitment and PMN-mediated tissue injury, there has been little evidence to suggest that IL-6 contributes directly to these processes. In a previous study, overexpression of IL-6 in the pancreas of transgenic mice promoted local inflammation (4). However, our study is the first to demonstrate that the presence of IL-6 in the lung alone is sufficient to promote PMN infiltration and pulmonary edema.

![Fig. 8. Effect of IL-6 instillation on accumulation of MPO-positive polymorphonuclear neutrophils (PMN) in interstitium and alveolar space. In A, IL-6 (300 ng) was instilled into the lungs of animals (n = 4), and the animals were killed at the time points indicated. Lungs were inflated, fixed in formaldehyde, sectioned, and stained for MPO. MPO-stained slides were examined at ×400 magnification. Ten random fields of each lung specimen were blindly scored for number of intensely MPO-positive PMN. Scores were pooled, and the means ± SE were plotted. Means were increased compared with normal lung at 4, 8, 12, and 24 h (*P ≤ 0.03). In B, IL-6 was instilled into the lungs of animals (n = 4) at the doses indicated. Lungs were harvested at 4 h, sectioned, and stained for MPO. MPO-positive PMN were quantitated and plotted as above. There was a linear increase in MPO-positive cells with increasing dose (r = 0.74; P < 0.03) 4 h after IL-6 instillation. There was a significant increase in MPO-positive PMN in the interstitial and alveolar spaces after administration of 30, 100, and 300 ng IL-6 (P = 0.01).

![Fig. 9. Effect of IL-6 instillation on lung wet-to-dry ratio. In A, IL-6 (300 ng) was intratracheally instilled into the lung of rats (n = 5; filled bars) by syringe injection. Control rats (n = 6; open bars) received saline control alone. Animals were killed at the times indicated, and the left lung was removed for determination of wet-to-dry ratio. Data presented are means ± SE. The wet-to-dry ratio was significantly increased in the IL-6-treated animals compared with control animals at 4, 8, 12, and 24 h (*P = 0.03, P = 0.04, P = 0.02, and P = 0.03, respectively). In B, IL-6 at the doses indicated was instilled into the lungs of animals (n = 5) as described above. Animals were killed at 4 h, and the left lung was removed for determination of wet-to-dry ratio. The wet-to-dry ratio increased with increasing IL-6 dose (r = 0.82; P = 0.001). A significant increase in lung wet-to-dry ratio was detected after administration of an IL-6 dose at 30, 100, and 300 ng (*P ≤ 0.03).]
In contrast to our findings of acute inflammation after IL-6 instillation, several studies have shown that IL-6 has anti-inflammatory effects. Exposure of cells to IL-6 or intraperitoneal injection of IL-6 was demonstrated to inhibit tumor necrosis factor-α and IL-1β production and to protect against LPS toxicity in vitro (1) and in vivo, respectively (3). IL-6 also decreased neutrophil infiltration into the lung in a mouse model of chronic pneumonia elicited by fungal exposure (9). In addition, intraperitoneal injection of IL-6 antibody and intratracheal administration of IL-6 (5,000 units) decreased BAL cellularity and peripheral blood neutrophilia. However, these models of LPS toxicity and chronic inflammation differ substantially in either the nature or duration of the insult from the acute inflammation occurring in our models of HS and IL-6 instillation.

Recent studies have demonstrated that IL-6 contributes to tissue recruitment of PMN by induction of chemokines (26); however, little is known about the mechanisms of IL-6-mediated local chemokine production. The promoter region of IP-10, a member of the C-X-C subgroup of chemokines, contains an interferon-stimulated response element capable of binding STAT proteins (23). Our findings in HS that IL-6 is produced in the lung and Stat3 is locally activated raise the possibility that IL-6-activated Stat3 may contribute to local chemokine production through transcriptional activation of chemokine genes such as IL-8.

The amount of IL-6 instilled into the lung of rats in our studies ranged from 10 to 300 ng. We observed a significant increase in lung wet-to-dry ratio and PMN infiltration after instillation of 30, 100, and 300 ng. In patients with ARDS, levels of IL-6 in BAL fluid exceeded 20 ng/ml, and IL-6 levels in plasma exceeded 1.5 ng/ml (22). Levels of IL-6 in lung tissue in humans or animals have not been reported. Where simultaneous lung tissue and plasma levels of a cytokine have been determined, such as IL-1β levels in steroid-induced Pneumocystis carinii pneumonia, a lung-to-plasma gradient greater than 60 was found (24). If such a gradient occurred for IL-6, the lung tissue levels of IL-6 in patients with ARDS would exceed 90 ng/ml. Consequently, the doses used in our studies may very well reflect lung tissue levels of IL-6 achievable in acute inflammatory states such as early ARDS.

To determine if IL-6 administered into the lungs results in systemic PMN activation and PMN-mediated damage to other organs such as the liver, we measured serum levels of liver enzymes, including alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, and alkaline phosphatase. Liver enzymes were within normal limits in all IL-6 instillation groups (data not shown). In addition, histological examination of representative cross sections of liver did not demonstrate increased PMN infiltration (data not shown). Taken together, these results indicate that IL-6 delivered intratracheally leads to lung injury as a result of its local effects on PMN recruitment and activation and not as a consequence of a systemic effect of IL-6 on PMN.