Neutrophils as early immunologic effectors in hemorrhage- or endotoxemia-induced acute lung injury

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ACUTE LUNG INJURY is characterized by the accumulation of large numbers of neutrophils into the lungs and a pulmonary inflammatory response in which there is increased production of immunoregulatory cytokines (13, 18, 26, 27). Macrophages, neutrophils, endothelial cells, and other pulmonary cell populations have all been demonstrated to express proinflammatory cytokines, but the relative importance of each of these cell populations in contributing to the development of acute lung injury has not been well defined. Interleukin (IL)-1β appears to be the most important proinflammatory cytokine in bronchoalveolar lavage specimens from patients with acute respiratory distress syndrome (ARDS), the most severe form of acute lung injury (25). Tumor necrosis factor-α (TNF-α) and IL-8 also appear to have central roles in the initiation and potentiation of acute lung injury (8, 11, 19, 24).

Blood loss and sepsis are major risk factors for the development of acute lung injury (5, 15). Experimental models of hemorrhage or endotoxin administration demonstrate that both of these pathophysiological insults produce acute inflammatory lung injury (9, 17, 31). Neutrophils appear to be important in the genesis of acute lung injury since induced neutropenia followed by endotoxin challenge or complement activation attenuates increases in vascular permeability and other indexes of pulmonary damage (14, 32). However, the in vivo mechanisms by which neutrophils mediate lung injury after hemorrhage or endotoxemia remain incompletely understood.

Neutrophils can express proinflammatory cytokines, including IL-1α, IL-1β, IL-8, and TNF-α (2, 7, 23, 30, 34). After hemorrhage or endotoxemia, neutrophils are major contributors to lung IL-1β production (23). Expression of TNF-α and macrophage inflammatory protein-2 (MIP-2), a murine homologue of IL-8, is also increased in lung neutrophils after blood loss or endotoxin administration (2, 30, 33). Binding sites for the transcriptional regulatory factor nuclear factor-κB (NF-κB) are present in the promoters of IL-1β, TNF-α, and MIP-2, and activation of NF-κB is important in modulating the expression of these as well as other immunoregulatory mediators (3, 4, 28). After hemorrhage or endotoxemia, NF-κB activation is increased in lung but not in blood neutrophils, providing an explanation for the enhanced expression of proinflammatory cytokines in pulmonary neutrophil populations (30).

Although neutrophils are an important component of the inflammatory response that characterizes acute lung injury, limited information is available concerning their role as early immune effectors in this process. To examine this issue, we performed a series of experiments in which neutrophils were depleted and then pulmonary cytokine expression, NF-κB activation, and...
parameters of lung injury were examined after either hemorrhage or endotoxemia. These studies demonstrate that activated neutrophils have an important role in initiating the inflammatory processes involved in the development of hemorrhage- or endotoxemia-induced acute lung injury.

METHODS

Mice. Male BALB/c mice, 8–12 wk of age, were purchased from Harlan Sprague Dawley (Indianapolis, IN). The mice were kept on a 12:12-h light-dark cycle with free access to food and water. All experiments were conducted in accordance with institutional review board-approved protocols.

Models of hemorrhage and endotoxemia. The murine hemorrhage model used in these experiments was developed in our laboratory and reported on previously (1, 2, 23, 29–31). With this model, 30% of the calculated blood volume (laboratory and reported on previously (1, 2, 23, 29–31). With these experiments, the mice were anesthetized with methoxyflurane anesthesia and then killed by cervical dislocation. The thorax was opened with two lateral incisions along the rib cage. The right heart was injected with cold, sterile PBS (3–5 ml) until the lungs had been flushed thoroughly. The lungs were excised with care to avoid the peritracheal lymph nodes and rinsed in PBS. The lungs were briefly blotted. The lungs were homogenized for 30 s on ice in 300 μl of buffer RLT (QIAGEN, Valencia, CA) containing 3 μl of β-mercaptoethanol (Sigma, St. Louis, MO). RNA was isolated using the RNAeasy kit (QIAGEN) following the manufacturer’s protocol. Briefly, proteinase K was added to each sample, incubated at 55°C for 10 min, and then centrifuged at 12,000 rpm for 3 min. Ethanol (100%) was added to clear the lysate, and the samples were centrifuged at 12,000 rpm for 15 s. After washes, the samples were treated with DNase for 15 min at room temperature and the membrane was dried in buffer RPE. RNA was eluted from the membrane in 30 μl RNase-free water, and the quantity of RNA was determined at 260-nm absorbance.

Cytokine ELISA. After the lung vascular bed had been flushed by injecting 5 ml of chilled (4°C) PBS into the right ventricle, the lungs were homogenized for 30 s in lysis buffer containing 10 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.6% isopropyl, 5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, and 1 μg/ml pepstatin. The homogenates were centrifuged at 10,000 rpm at 4°C for 10 min, and the supernatants were collected. Protein content of the supernatants was determined using the bicinchoninic acid protein assay kit from Pierce Chemical (Pittsburgh, PA). Immunoanalytical assay for IL-1β, TNF-α, and MIP-2 were quantitated with commercially available ELISA kits (R&D Systems, Minneapolis, MN). With these assays, the threshold of sensitivity for IL-1β and MIP-2 is 3 pg/ml, and for TNF-α, it is 10 pg/ml.

Quantitative PCR. Groups of five mice, with results obtained from individual mice, were used for each experimental condition. qPCR was used in these studies because the amount of RNA obtained from each mouse was insufficient to prepare Northern blots for cytokine analysis. The animals were anesthetized with methoxyflurane and then killed by cervical dislocation. The thorax was opened with two lateral incisions along the rib cage. The right heart was injected with cold, sterile PBS (3–5 ml) until the lungs had been flushed thoroughly. The lungs were excised with care to avoid the peritracheal lymph nodes and rinsed in PBS. The lungs were briefly blotted. The lungs were homogenized for 30 s on ice in 300 μl of buffer RLT (QIAGEN, Valencia, CA) containing 3 μl of β-mercaptoethanol (Sigma, St. Louis, MO). RNA was isolated using the RNAeasy kit (QIAGEN) following the manufacturer’s protocol. Briefly, proteinase K was added to each sample, incubated at 55°C for 10 min, and then centrifuged at 12,000 rpm for 3 min. Ethanol (100%) was added to clear the lysate, and the samples were centrifuged at 12,000 rpm for 15 s. After washes, the samples were treated with DNase for 15 min at room temperature and the membrane was dried in buffer RPE. RNA was eluted from the membrane in 30 μl RNase-free water, and the quantity of RNA was determined at 260-nm absorbance.

Primers and probes for IL-1β, TNF-α, and MIP-2 were designed using Primer Express software supplied by Perkin-Elmer (Foster City, CA). The IL-1β primer and probe sequence consisted of forward primer, 5′-GCTGAAAG-CTTCAACCTCTCA-3′; reverse primer, 5′-TCGTTGTCTTG-GTTCTCCCTTGTA-3′; and probe, 5′-CAAGATATATCAACAA-CAGTGATATTTCTCCTAGAGC-3′. The TNF-α primer and probe consisted of forward primer, 5′-CTGTAGCC-CACCTGCAGTGATCTCAA-3′; reverse primer, 5′-CCCTGG-TATGAGATAGCAAATCG-3′; and probe, 5′-TGTGACGCCCTCGACAC-3′. The IL-1β primers and probes consisted of forward primer, 5′-CTGTAGCC-CACCTGCAGTGATCTCAA-3′; reverse primer, 5′-CTCTGG-TATGAGATAGCAAATCG-3′; and probe, 5′-TGCCCCGAG-TACGGTCTCCCTCAC-3′. The MIP-2 primers and probes consisted of forward primer, 5′-CTGAAAGCCCCCCAGGA-3′; reverse primer, 5′-AACGTITTTGACCAGCCCCTTGAG-3′; and probe, 5′-TGCCCCCAGACAGAAGTCTAGCAG-3′. To optimize the primer sets, a primer optimization experiment was performed as described in the manufacturer’s protocol. Based on the primer optimization, the concentration of primers and probe for IL-1β and TNF-α contained 200 nM for the probe, forward primer, and reverse primer. The primer and probe concentrations for MIP-2 consisted of 10 nM for the forward primer, 450 nM for the reverse primer, and 200 nM for the probe. In each experiment, ribosomal RNA control probe, forward primer, and reverse primer (Perkin-Elmer) at concentrations of 50 nM were used to normalize the amount of RNA in each sample.
All reagents used in the one-step RT-PCRs were purchased from Perkin-Elmer. Each one-step RT-PCR contained a total volume of 50 µl. The RT reaction was performed for 30 min at 48°C with MultiScribe reverse transcriptase with a final concentration of 0.25 U/µl. After the RT step, AmpliTaq Gold polymerase, at a final concentration of 0.025 U/µl, was activated by an increase in temperature to 95°C for 10 min followed by 40 cycles of amplification (95°C for 15 s and 60°C for 1 min) with a GeneAmp 5700 sequence detection system (ABI Prism, Foster City, CA). The quantity of cytokine mRNA was determined from a standard curve with 10-fold dilutions of known amounts of target RNA with each primer and probe set. RNA amounts were determined using software provided with the GeneAmp 5700 sequence detection system. Quantification was determined by dividing the amount of 18S ribosomal RNA by the target amount for each cytokine sample.

**Preparation of nuclear extracts.** Nuclear extracts were prepared as previously described (17, 18). In brief, lungs were snap-frozen in liquid nitrogen and then homogenized in buffer A. After cytoplasm was removed from the nuclei by 15 passages through a 25-gauge needle, nuclei were centrifuged at 4°C for 6 min at 600 g. After the nuclear pellet was incubated on ice for 15 min in buffer C, the extract was centrifuged at 4°C for 10 min at 12,000 g. The supernatant was collected, divided into aliquots, and stored at −86°C. Protein concentration was determined using Coomassie Plus protein assay reagent (Pierce Chemical) standardized to bovine serum albumin according to the manufacturer’s protocol.

** Electrophoretic mobility shift assay analysis.** Activation of the transcriptional factor NF-κB was determined by electrophoretic mobility shift assay (EMSA) analysis, as described previously in our laboratory (22, 29, 30). The κB DNA sequence, with enhancer motifs underlined, was annealed, forming double-strand DNA probes with single-strand ends consisting of sequences of four thymidines, allowing the ends to be labeled by base pairing with α [32P]dATP using Sequenase DNA polymerase: 5′-TTTTCGAGCTCGGGACCTT-TCCGAGC-3′ and 3′-GCTCGAGCCCTGAAGGCTCGTT- TT5′.

DNA binding reaction mixtures of 20 µl contained 1 µg of naked extract, 10 mM Tris-Cl, pH 7.5, 50 mM EDTA, 0.5 mM dithiothreitol, 1 mM MgCl2, 4% glycerol, 0.08 µg of poly(dI-dC):poly(dI-dC), and 0.7 fmol of 32P-labeled double-stranded oligonucleotide. After the samples were incubated at room temperature for 20 min, they were loaded onto a 4% polyacrylamide gel (acrylamide-bis-acrylamide, 80:1; 2.5% T, 1.5% C) and run at 200 V. The DNA was detected by autoradiography.

Supershift studies using anti-p50 or anti-p65 antisera (Santa Cruz Biotechnology, Santa Cruz, CA) as previously described (29, 30) were used to demonstrate specificity of κB oligonucleotide binding. Specificity of binding also was confirmed by ablation of the κB band through incubation with a 500-fold excess of unlabeled oligonucleotide.

**In situ immunolocalization of TNF-α.** Immunohistochemistry was performed as described previously (24). Briefly, control mice or endotoxin-treated mice were prepared as previously described. After the right ventricle was perfused with 5 ml of PBS (4°C), the lungs were gently infiltrated through the trachea with 1% low-melting-point agarose (Seakem, Rockland, ME) at 42°C. Lungs were then removed, en bloc and fixed in 4% paraformaldehyde-0.23 M sucrose solution overnight. Tissue was then embedded and treated with 0.2 M glucose and 1.5 U/ml glucose oxidase in PBS for 30 min followed by 10% hydrogen peroxide in PBS for 15 min, after which 5-µm sections were prepared. Immunohistochemistry was conducted using either rabbit polyclonal anti-mouse TNF-α antibodies or control rabbit serum (PharMingen, San Diego, CA) at a dilution of 1:1,000 using the Vectastain immunohistochemistry kit following the manufacturer’s protocol (Vector, Burlingame, CA).

**Statistical analysis.** For each experimental condition, the entire group of animals was prepared and studied at the same time. Mice in all groups had the same birth date and had been housed together. Separate groups of mice were used for myeloperoxidase assays, PCR, and EMSA. For PCR, each animal was analyzed individually before group data. Data are presented as means ± SD for each experimental group were calculated. One-way ANOVA and the Tukey-Kramer multiple comparisons test were used when more than two experimental groups were compared. Student’s t-test was used for comparisons between two data groups. P < 0.05 was considered significant.

**RESULTS**

**Elimination of neutrophils reduces hemorrhage- or endotoxemia-induced lung injury.** Hemorrhage or endotoxemia results in accumulation of neutrophils in the lungs (Fig. 1) and increased wet-to-dry weight ratios (Fig. 2). Endotoxemia- or hemorrhage-induced lung edema was significantly reduced in mice made neutropenic by treatment with cyclophosphamide (Fig. 2A). The ameliorative effects of cyclophosphamide treatment on hemorrhage- or endotoxemia-associated increases in lung wet-to-dry weight ratios appeared to be due to neutropenia, but not to other effects of cyclophosphamide, since there was no effect on lung edema in nonneutropenic cyclophosphamide-treated animals (i.e., given cyclophosphamide 1 day before endotoxin administration or hemorrhage).

To confirm the role of neutrophils in contributing to lung injury after hemorrhage or endotoxemia, mice were made neutropenic with anti-neutrophil antibodies. As was the case with cyclophosphamide-induced neutropenia, anti-neutrophil treatment significantly reduced lung edema produced by hemorrhage or endotoxemia (Fig. 2B).

![Fig. 1. Myeloperoxidase activity in lungs of mice (n = 6 in each group) that were either hemorrhaged, given endotoxin [lipopolysaccharide (LPS)] 1 h previously, or unmanipulated (control). **P < 0.01 vs. control.](image-url)
Effects of neutrophils on NF-κB activation in the lungs after hemorrhage or endotoxemia. The transcriptional regulatory factor NF-κB was activated in the lungs after hemorrhage or endotoxemia but to a greater extent by endotoxemia than by hemorrhage (Fig. 3). To determine the relative importance of neutrophils in affecting pulmonary NF-κB activation, we treated mice with either cyclophosphamide (CTX), A or polyclonal rabbit anti-neutrophil antibodies (B). The wet-to-dry weight ratio of each endotoxin-treated or hemorrhaged (Hem) animal was corrected by subtracting the mean value for normal animals, and the increased mean of the untreated and CTX and anti-neutrophil antibody-treated groups was plotted. The increase in wet-to-dry weight ratio in LPS-treated or hemorrhaged animals or nonneutropenic CTX-treated animals [i.e., given CTX 1 day before LPS administration or hemorrhage (1/4d CTX)] compared with sham animals was significant ($P < 0.001$). The decrease in wet-to-dry weight ratio in mice made neutropenic with anti-neutrophil antibodies or CTX [i.e., given CTX 1 and 4 days before LPS administration or hemorrhage (1/4d CTX)] was similar compared with nonneutropenic controls ($*P < 0.05$ and $**P < 0.001$). Effective neutropenia was >98% in mice treated with anti-neutrophil antibodies or with CTX on days 1 and 4 before hemorrhage or endotoxin administration. In contrast, administration of CTX 1 day before hemorrhage or endotoxemia caused a <10% change in neutrophil counts compared with untreated or PBS-treated animals.

Effects of neutrophils on NF-κB activation in the lungs after hemorrhage or endotoxemia. The transcriptional regulatory factor NF-κB was activated in the lungs after hemorrhage or endotoxemia but to a greater extent by endotoxemia than by hemorrhage (Fig. 3). To determine the relative importance of neutrophils in affecting pulmonary NF-κB activation, we treated mice with either cyclophosphamide or anti-neutrophil antibodies and then examined nuclear translocation of NF-κB in whole lung extracts obtained after endotoxin administration or hemorrhage (Fig. 3). The increases in lung NF-κB activation produced by hemorrhage or endotoxemia were reduced in mice made neutropenic by administration of either cyclophosphamide or anti-neutrophil antibodies. Mice treated with a single dose of cyclophosphamide 1 or 4 days before hemorrhage or endotoxin administration or with anti-neutrophil antibodies for 2 days before hemorrhage or endotoxin administration were not neutropenic and showed levels of NF-κB activation similar to those found in control mice not given either cyclophosphamide or anti-neutrophil antibodies.

Role of neutrophils in hemorrhage- or endotoxemia-induced increases in lung cytokine expression. Hemorrhage or endotoxemia resulted in increased pulmonary mRNA levels for IL-1β, MIP-2, and TNF-α (Fig. 4). Although the amounts of mRNA for these proinflammatory cytokines were significantly elevated compared with control levels after hemorrhage, the increases produced by endotoxin injection were ~10- to 100-fold greater than those occurring after hemorrhage.

No significant increases in lung IL-1β expression, compared with those in unmanipulated nonneutropenic control mice, were present after hemorrhage or
endotoxemia in neutropenic mice (Fig. 4). Neutropenia also significantly diminished levels of MIP-2 mRNA after hemorrhage or endotoxemia but not to control levels. The amounts of TNF-α mRNA in the lungs after hemorrhage, but not after endotoxemia, were also reduced in neutropenic mice.

IL-1β, MIP-2, and TNF-α proteins were significantly elevated in the lungs after hemorrhage or endotoxemia compared with unmanipulated, control conditions (Fig. 5). Mice treated with a single dose of cyclophosphamide 1 or 4 days before hemorrhage or endotoxin administration or with anti-neutrophil antibodies for 2 days before hemorrhage or endotoxin administration were not neutropenic and had increases in pulmonary MIP-2, IL-1β, and TNF-α proteins that were similar to those found in control mice not given either cyclophosphamide or anti-neutrophil antibodies. As was the case for mRNA levels, endotoxemia-induced increases in pulmonary IL-1β and MIP-2 proteins were more than 10-fold greater than those produced by hemorrhage.

The amounts of MIP-2 and IL-1β proteins in the lungs were significantly lower after hemorrhage or endotoxemia in neutropenic compared with nonneutropenic animals (Fig. 5). However, as with TNF-α mRNA, neutropenia reduced hemorrhage- but not endotoxemia-induced elevations in pulmonary TNF-α protein.

The present experiments are consistent with our previous study (23), where neutrophils were demonstrated to be a major cellular source of IL-1β in the lungs after endotoxemia. However, the above experiments, which showed that neutrophils had no effect on endotoxin-induced elevations in pulmonary TNF-α expression, suggested that cell sources other than neutrophils were responsible for endotoxin-associated increases in lung TNF-α. To examine this issue we performed immunohistochemical studies on lung sections obtained 1 h after endotoxin administration (Fig. 6). These experiments showed that alveolar macrophages and not neutrophils were the major pulmonary cell population expressing TNF-α after endotoxin treatment.

DISCUSSION

In these experiments, neutrophils were demonstrated to have a central role in initiating an acute inflammatory response and in causing injury to the lungs after hemorrhage or endotoxemia. Elimination of neutrophils using two techniques with different mechanisms for producing neutropenia, cyclophosphamide or anti-neutrophil antibody therapy, diminished hemorrhage- or endotoxin-induced lung edema, activation of NF-κB, and expression of IL-1β and MIP-2. TNF-α generation in the lungs after hemorrhage but not after endotoxin administration also appeared to be neutrophil dependent.

Previous experiments have shown that neutrophil depletion prevented endotoxin-induced lung edema (14). However, the role that neutrophils have in producing lung injury after hemorrhage has not been well explored previously. In the present studies, both hemorrhage and endotoxemia induced NF-κB activation and proinflammatory cytokine expression in the lungs. However, the magnitude of NF-κB activity and enhancement of cytokine expression was greater after endotoxemia than after hemorrhage. For example, in the case of pulmonary IL-1β mRNA or protein, the increase from baseline, control conditions produced by hemorrhage was less than fivefold, whereas levels rose more than 20-fold after endotoxemia. Similar differences between hemorrhage- and endotoxemia-induced effects were found for MIP-2 and TNF-α. These results indicate that the magnitude of induction of signaling pathways initiated by endotoxemia and leading to NF-κB activation and proinflammatory cytokine expression is substantially greater than that produced by hemorrhage.

In a previous study (30), we found that the enhanced expression of IL-1β, TNF-α, and MIP-2 in lung neutrophils is dependent on activation of xanthine oxidase after hemorrhage but not after endotoxemia. Additionally, activation of the transcription factor cAMP-responsive element binding protein was regulated by xanthine oxidase-dependent pathways after hemorrhage but not after endotoxemia. Our previous study (30), coupled with the present results in which both the magnitude and patterns of endotoxin-induced increases in pulmonary cytokine expression differed from those present after hemorrhage, indicated that the...
intracellular signaling pathways initiated by endotoxemia and leading to increased expression of proinflammatory cytokines in lung neutrophils are distinct from those associated with hemorrhage. Despite differences between endotoxemia or hemorrhage on NF-κB activation and cytokine expression, the magnitude of increase in lung edema was similar with the two conditions. Although these results are consistent with there being a threshold effect for pulmonary damage, with no additional injury occurring even if there are further increases in NF-κB activation and proinflammatory cytokine release, it is possible that histopathological alterations, other than the measured increase in lung edema, may differ between hemorrhage and endotoxemia, reflecting the more pronounced effect of endotoxemia on NF-κB activation and cytokine expression. An alternate explanation for the present findings is that additional potent neutrophil-derived proinflammatory mediators not measured in these experiments were released after hemorrhage in amounts similar to those after endotoxemia and that such mediators were more important than IL-1β or MIP-2 in causing lung injury.

Neutrophil depletion prevented lung edema but did not decrease pulmonary TNF-α mRNA or protein levels after endotoxemia. These findings, like those of our previous experiments (30), indicate that transcriptional regulatory pathways activated in the lungs by hemorrhage and leading to enhanced proinflammatory cytokine expression are distinct from those associated with endotoxemia. These results also suggest that TNF-α does not have a central role in producing acute lung injury after endotox-
emia. Such findings are consistent with those in humans with ARDS, where IL-1β was demonstrated to be the most potent proinflammatory mediator in bronchoalveolar lavage, with TNF-α being much less significant (25).

Activation of the transcriptional factor NF-κB is important in modulating acute inflammatory responses (3, 4, 28). Association of NF-κB heterodimers with κB binding sites in the promoters of cytokines such as MIP-2. Lung homogenates were obtained from mice (n = 6 in each group) that were unmanipulated (control), given LPS 1 h previously (LPS), hemorrhaged 1 h previously (Hem), or treated with CTX 1 and 4 days before LPS administration (LPS 1/4d CTX) or hemorrhage (Hem 1/4d CTX). *P < 0.05 and ***P < 0.001 vs. control. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. nonneutropenic Hem or LPS.

Fig. 5. Effects of neutrophil depletion on cytokine protein in the lung. A and B: IL-1β. C and D: TNF-α. E and F: MIP-2. Lung homogenates were obtained from mice (n = 6 in each group) that were unmanipulated (control), given LPS 1 h previously (LPS), hemorrhaged 1 h previously (Hem), or treated with CTX 1 and 4 days before LPS administration (LPS 1/4d CTX) or hemorrhage (Hem 1/4d CTX). *P < 0.05 and ***P < 0.001 vs. control. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. nonneutropenic Hem or LPS.

Fig. 6. TNF-α is localized to alveolar macrophages after endotoxemia. Mice were either given PBS with LPS (A) or PBS alone (B) intraperitoneally. One hour later, the lungs were infiltrated with 1% agarose, removed en bloc, sectioned, and stained using rabbit anti-TNF-α antiserum. Photographs represent 1,000 magnifications of the sections. Arrows identify alveolar macrophages, which are positively stained for TNF-α in LPS-treated mice.
IL-1β, TNF-α, and MIP-2, as well as of other proinflammatory mediators, including intercellular adhesion molecule-1 and tissue factor, enhances expression of these proteins. Increased activation of NF-κB occurs among lung cell populations in models of acute lung injury due to hemorrhage or endotoxemia (6, 20, 21, 29, 30). NF-κB activation is also enhanced in alveolar macrophages from patients with ARDS (22). Previous experiments showed that inhibition of NF-κB activation prevented hemorrhage- or endotoxin-induced proinflammatory cytokine expression and neutrophil accumulation in the lungs (6, 20, 30).

In these experiments, hemorrhage- or endotoxemia-induced NF-κB activation was diminished in the lungs of neutropenic mice. We previously demonstrated that NF-κB is activated in lung neutrophils after hemorrhage or endotoxemia (30). One explanation for the present results is that most of the activation of NF-κB occurring in the lungs during hemorrhage or endotoxemia is in infiltrating neutrophils. In that case, neutrophil depletion decreases NF-κB activation in whole lung extracts simply by eliminating the major cell population in which such activation of NF-κB occurs. However, an alternate explanation is that neutrophils, by generating reactive oxygen intermediates, cytokines, or other proinflammatory mediators, initiate an inflammatory response in the lungs through which NF-κB becomes activated in other pulmonary cell populations. In this scenario, neutrophils are the initiators of an inflammatory response but are not the major cell population in which NF-κB is activated. Further in situ studies will resolve this issue.

Inhibition of neutrophil accumulation in the lungs, such as through the use of anti-adhesion molecule therapies, decreases the severity of lung injury after endotoxin administration, ischemia-reperfusion, or other pathophysiological insults (10, 16). The benefit of such interventions generally has been ascribed to inhibiting the effector functions of neutrophils, primarily the release of reactive oxygen intermediates and proteolytic mediators by which the lungs are directly damaged. However, neutrophils may have an alternate role in which they initiate inflammatory responses. Neutrophils are transcriptionally active and can produce a range of immunoregulatory molecules, such as cytokines, that are capable of potentiating inflammatory responses (2, 7, 30, 33). The present experiments are consistent with this early immunomodulatory role for neutrophils because they show that neutrophils are responsible for the initial activation of NF-κB, as well as of the increases in proinflammatory cytokines that occur in the lungs after hemorrhage or endotoxemia.

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