A low level of TNF-α mediates hemorrhage-induced acute lung injury via p55 TNF receptor

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Hemorrhagic shock induces acute lung injury manifested by neutrophil accumulation and increased microvascular permeability (1, 2). Neutrophil accumulation is a component of the pulmonary inflammatory response that contributes to increased microvascular permeability and respiratory dysfunction in a variety of lung injury models (1, 4, 9). Indeed, neutrophil depletion attenuates acute lung injury after hemorrhagic shock (1) or endotoxemia (35). Although neutrophil accumulation plays a central role in lung injury, the mechanism by which hemorrhagic shock provokes the inflammatory response remains obscure.

Hemorrhagic shock has been reported to promote pulmonary production of proinflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 (5, 6, 16, 20, 34). TNF-α induces chemokines (12, 37, 38) and adhesion molecules (11, 18, 25) in a variety of cell types and mediates tissue injury caused by proinflammatory stimuli such as lipopolysaccharide (LPS; see Refs. 21 and 26) and ischemia (13). It is likely that TNF-α, serving as a proximal mediator, provokes a pulmonary inflammatory cascade, resulting in recruitment of neutrophils and tissue injury. However, results of previous studies remain controversial regarding tissue TNF-α levels after resuscitation from hemorrhagic shock (5, 14, 24, 31, 34). A number of studies have observed increased levels of TNF-α after hemorrhagic shock (5, 24, 31, 34), whereas others have demonstrated no change in TNF-α (14). Moreover, the temporal profile of TNF-α production varies greatly among those studies reporting TNF-α overproduction (5, 31, 34). These discrepancies may be the result of different resuscitation protocols, i.e., timing, solution, and volume used in those studies. In addition, factors induced by reperfusion of underperfused tissues may influence tissue production and release of proinflammatory cytokines. We have observed that unresuscitated hemorrhagic shock induces lung neutrophil accumulation and lung injury in mice (1, 39) and that lung injury is associated with pulmonary expression of TNF-α (1, 2). Thus the relationship of TNF-α to lung neutrophil accumulation and lung injury could be determined in this model in the absence of potential confounding factors introduced by resuscitation.

Although hemorrhagic shock induces TNF-α expression in the lung, the level of pulmonary TNF-α expression is relatively low compared with that stimulated by bacterial LPS (1, 24). Several studies suggest that a physiologically relevant dose of recombinant TNF-α is insufficient to induce lung injury in naive rats or mice (30, 42). However, neutralization of TNF-α with a
monoclonal antibody attenuates lung injury in a mouse model of hemorrhagic shock (2), suggesting that endogenous TNF-α does contribute to lung injury in the setting of hemorrhagic shock. TNF-α signaling is mediated by two types of cell surface receptors (15). The p55 TNF-α receptor is the dominant effector in TNF-α biology and mediates most of the proinflammatory effects of TNF (25, 27–29, 38). The p75 TNF-α receptor has been found to play an important role in ligand passing and thus enhances p55 receptor signaling (41). The role of TNF-α in hemorrhagic shock-induced lung neutrophil recruitment and lung injury, however, remains to be defined, and it is unknown which TNF-α receptor is involved. Thus determination of the role of TNF-α and its receptors in lung neutrophil recruitment and lung injury will provide important information regarding therapeutic suppression of the pulmonary inflammatory response to hemorrhagic shock.

The purposes of this investigation were to examine 1) the temporal relationship of pulmonary TNF-α production to lung neutrophil accumulation after hemorrhagic shock, 2) whether lung neutrophil accumulation and lung injury after hemorrhagic shock are provoked by TNF-α and, if so, 3) which TNF-α receptor mediates these effects.

MATERIALS AND METHODS

Animals. Male mice of the C57BL/6, p55 TNF-α receptor knockout (p55 TNFR KO), and p75 TNF receptor knockout (p75 TNFR KO) genotypes between the ages of 8 and 10 wk were obtained from Jackson Laboratory (Bar Harbor, ME). TNF-α knockout (TNF KO) mice of the same age range were generous gifts from Dr. David Riches of the National Jewish Medical and Research Center (Denver, CO). Mice were kept on a 12:12-h light-dark cycle with free access to food and water. All animal experiments were approved by the University of Colorado Health Sciences Center Animal Care and Research Committee. During experiments, all animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (Department of Health, Education, and Welfare Publication No. [National Institutes of Health (NIH)] 85–23, revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20892).

Chemicals and reagents. Rat monoclonal antibody to mouse neutrophil p40 antigen (clone 7/4) was purchased from Serotec (Oxford, UK). Rat IgG and indocarbocyanine (Cy3)-conjugated donkey anti-rat IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Fluorescein-conjugated wheat germ agglutinin, and the Cy3-conjugated donkey anti-rat IgG (1:300 dilution with PBS containing 1% BSA). Control sections were incubated with nonimmune rat IgG (1 μg/mL). After being washed with PBS, sections were incubated with Cy3-conjugated donkey anti-rat IgG (1:300 dilution with PBS containing 1% BSA). The cell surface was counterstained with fluorescein-conjugated wheat germ agglutinin, and the nucleus was counterstained with bis-benzimide. The sections were mounted with aqueous media. Microscopy analysis was performed with a Leica DMRXA digital microscope equipped with Slidebook software (1.1.1., Denver, CO). Neutrophils in five random fields (800 × 800 pixels or 0.017 mm²/field) were counted by a blinded viewer. Neutrophil count is expressed as mean per square millimeter.

Myeloperoxidase assay. The method for myeloperoxidase (MPO) assay has been described previously (36) with minor modifications. Tissue was homogenized in 1.0 ml of 20 mM potassium phosphate (pH 7.4) for 30 s on ice. The homogenate was centrifuged at 24,000 g for 30 min at 4°C. The pellet was resuspended and sonicated on ice for 90 s in 10 vol of hexadecyltrimethylammonium bromide buffer (0.5% hexadecyltrimethylammonium bromide in 50 mM potassium phosphate, pH 6.0). Samples were incubated in a water bath (56°C) for 2 h and then centrifuged at 5,000 g for 10 min. The supernatant was collected for assay of MPO activity as determined by measuring the H2O2-dependent oxidation of 3′,3′,5′,5′-tetramethylbenzidine at 460 nm.

Assessment of lung leak. EBD was used to assess lung leak. EBD solution (2.5 mg/ml, 20 mg/kg body wt) was injected

The sham procedure involved cardiac puncture under methoxyflurane anesthesia, but no blood was removed. Animals were killed at 0.5, 1, 2 and 4 h after blood removal. After anesthesia and heparinization (40 mg/kg of pentobarbital sodium and 2,000 U/kg of heparin ip), the chest was opened, and lung tissue samples were prepared for the assessment of TNF-α levels and neutrophil accumulation. A portion of the lung tissue was embedded in tissue-freezing medium and frozen in dry ice-chilled isopentane. The remaining lung tissue was frozen in liquid nitrogen. All samples were stored at −70°C before use. A separate group of animals was killed 4 h after blood removal for the assessment of lung leak.

For comparison of hemorrhagic shock with LPS in the induction of lung TNF-α production and neutrophil accumulation, an endotoxemia model was used as reported previously (23). Wild-type mice received an injection of LPS (500 μg/kg in 200 μl of normal saline ip). Control animals were treated with 200 μl of normal saline (ip). Lung tissue was collected 1 and 4 h after treatment for measurement of TNF-α levels and neutrophil accumulation.

To examine whether hemorrhagic shock-induced lung neutrophil accumulation and lung injury could be restored in TNF KO mice, a group of TNF KO mice was injected with recombinant murine TNF-α (80 pg/g body wt) through a tail vein. Animals were subjected to sham treatment or hemorrhagic shock 5 min after injection of TNF-α. Lung neutrophil accumulation and lung leak were examined 4 h after treatment.
through a tail vein 3 h after hemorrhagic shock or sham treatment. Animals were anesthetized (40 mg/kg of pentobarbital sodium and 2,000 U/kg of heparin ip), and the chest was opened 1 h after injection of EBD. The pulmonary vasculature was flushed free of blood by gentle infusion of 10 ml of normal saline into the beating right ventricle. The lungs were then excised, weighed, and homogenized in formamide (0.5 ml/100 mg tissue). The homogenate was incubated for 18 h at 37°C and centrifuged at 10,000 g for 30 min. The supernatant was collected, and the optical density was determined spectrophotometrically at 620 nm. EBD concentration in lung homogenate was calculated against a standard curve and is expressed as micrograms of EBD per gram of tissue.

**TNF-α ELISA.** Lung tissue was homogenized with 4 volumes of PBS and centrifuged at 10,000 g for 20 min at 4°C. The resulting supernatant was collected for determination of TNF-α by ELISA as previously described (22). The detection limit for TNF-α was 5.1 pg/ml. Absorbance of standards and samples was determined spectrophotometrically at 450 nm using a microplate reader (Bio-Rad, Hercules, CA). Results were plotted against the linear portion of the standard curve. TNF-α level is expressed as picograms per gram of tissue.

**Statistical analysis.** Data are expressed as means ± SE. An ANOVA was performed with Statview 4.0 statistical analysis software (SAS Institute, Cary, NC), and a difference was accepted as significant if the P value was smaller than 0.05 as verified by the Bonferroni-Dunn post hoc test.

**RESULTS**

**Temporal recruitment of neutrophils to the lung after hemorrhagic shock.** Lung neutrophil accumulation in wild-type mice after hemorrhagic shock was assessed by immunofluorescent staining using a rat monoclonal antibody to mouse neutrophils. Neutrophils were present in lungs of sham-treated animals (Fig. 1A). Lung neutrophil count increased after hemorrhagic shock, and neutrophils were localized in the interstitial space adjacent to alveolar epithelial cells and in capillaries (Fig. 1B). No immunoreactivity was detected in negative control sections incubated with a nonimmune rat IgG (Fig. 1C). LPS induced similar neutrophil accumulation in the lung (Fig. 1D).

**Temporal neutrophil accumulation in lungs.** Temporal neutrophil accumulation in lungs is shown in Fig. 2. Hemorrhagic shock induced lung recruitment of neutrophils in a time-dependent fashion. Neutrophil count was similar at all time points after sham treatment, and it was 117.7 ± 46.2/mm² in the pooled sham control. Hemorrhagic shock increased lung neutrophil count by more than threefold to 385 ± 46.7/mm² (P < 0.05 vs. sham) 30 min after hemorrhage and continued to increase at later time points. By 4 h after blood removal, lung neutrophil count was 1,087.8 ± 95.7/mm² (P < 0.01 vs. sham). A sublethal dose of LPS induced a comparable level of neutrophil accumulation in lungs at 4 h (Fig. 2). The results of immunofluorescent staining were confirmed by MPO assay. Lung MPO activity increased fivefold (4.99 ± 0.31 vs. 0.91 ± 0.54 U/g in sham, P < 0.01) 4 h after hemorrhagic shock.

**Lung leak induced by hemorrhagic shock.** Lung leak was assessed in wild-type mice using the EBD assay. As shown in Fig. 3, lung EBD concentration was 16.8 ± 1.5 µg/g in sham-treated animals, and it increased 2.5-fold to 42.8 ± 7.1 µg/g 4 h after hemorrhagic shock (P < 0.01 vs. sham).

**Fig. 1.** Immunofluorescent detection and localization of neutrophils in lungs. At 4 h after hemorrhage or sham treatment, neutrophils in lungs of wild-type mice were visualized by immunofluorescent staining with a specific rat monoclonal antibody against mouse neutrophils followed by indocarbocyanine (Cy3)-conjugated anti-rat IgG (red). The cell surface was counterstained with fluorescein-conjugated wheat germ agglutinin (green), and the nucleus was counterstained with bis-benzimide (blue). A: neutrophils were present in lungs of sham-treated animals. B: hemorrhagic shock increased lung neutrophil accumulation. Neutrophils were localized in both the microvasculature and the interstitial space (arrow). C: neutrophil immunoreactivity was absent in lung sections incubated with nonimmune rat IgG in replacement of the primary antibody. D: lipopolysaccharide administration also increased lung neutrophil accumulation in both the microvasculature and the interstitial space (arrow). Bar = 10 µm.
Temporal changes in lung TNF-α level after hemorrhagic shock. Hemorrhagic shock induced a slight and transient increase in lung TNF-α in wild-type mice (Fig. 4). Lung TNF-α was similar at different time points after sham treatment and was 73.3 ± 17.45 pg/g wet tissue in pooled sham control. No increase in lung TNF-α was detected 30 min after blood removal. Lung TNF-α increased to 159.6 ± 32.9 pg/g wet tissue (P < 0.05 vs. sham) at 1 h and returned to the level of sham control 2 h after blood removal. In contrast, a sublethal dose of LPS induced a dramatic increase in lung TNF-α in wild-type mice (2,979 ± 992 pg/g wet tissue, P < 0.01 vs. saline control and hemorrhagic shock 1 h). Although LPS induced a dramatic increase in lung TNF-α in WT mice, lung TNF-α was not detectable in TNF KO mice after LPS treatment. Data are expressed as means ± SE; n = 5 in each group. *P < 0.05 and **P < 0.01 vs. sham.

Effects of TNF KO on pulmonary recruitment of neutrophils and lung leak. To determine the role of TNF-α in lung neutrophil recruitment after hemorrhagic shock, we examined neutrophil count in lungs of TNF KO mice 4 h after blood removal or sham treatment. Although TNF KO did not influence lung neutrophil count after sham treatment, lung neutrophil count was greatly reduced after hemorrhagic shock in mice lacking TNF-α compared with that of wild-type animals (Fig. 5). Intravenous administration of a small dose of recombinant murine TNF-α to TNF KO mice immediately before blood removal restored lung neutrophil recruitment, although this dose of TNF-α had a minimal influence on lung neutrophil count after sham treatment (Fig. 5). At 30 min after hemorrhagic shock, lung neutrophil count of TNF KO mice was 187 ± 39.0/mm² (n = 3). This value was also significantly lower than that of wild-type animals (385 ± 46.7/mm²) at the same time point after hemorrhagic shock (P < 0.05).

To examine whether the absence of endogenous TNF would attenuate lung injury after hemorrhagic shock, TNF KO mice were subjected to hemorrhagic shock or sham treatment, and lung leak was assessed by EBD assay. TNF KO did not influence lung EBD concentration after sham treatment, but mice lacking TNF-α had reduced lung EBD concentration compared with wild-type animals (Fig. 6). Similarly, TNF KO mice exhibited a lung EBD concentration comparable to that of wild-type mice when they received an intravenous ad-
neutrophil recruitment and lung injury, we subjected p55 TNFR KO and p75 TNFR KO mice to hemorrhagic shock or sham treatment. Lung neutrophil count after sham treatment was not influenced by either p55 TNFR KO or p75 TNFR KO (Fig. 7). However, lung neutrophil count in mice lacking the p55 TNF-α receptor was greatly reduced ($P < 0.01$ vs. wild type) 4 h after hemorrhagic shock and was comparable to that of mice lacking TNF-α. In contrast, the absence of the p75 TNF-α receptor had no influence on hemorrhagic shock-induced lung neutrophil recruitment (Fig. 7).

Similarly, lung EBD concentration after sham treatment was not influenced by p55 TNFR KO or p75 TNFR KO (Fig. 8). Mice lacking the p55 TNF-α receptor had a reduced lung EBD concentration 4 h after hemorrhagic shock ($P < 0.01$ vs. wild type), which was comparable to that of TNF KO mice. Lung EBD concentration after hemorrhagic shock in mice lacking the p75 TNF-α receptor was not different from that of wild-type mice (Fig. 8).

**DISCUSSION**

In this study, we found that hemorrhagic shock induced a transient increase in lung TNF-α production and a time-dependent lung neutrophil accumulation associated with lung leak in wild-type mice. Lung neutrophil accumulation appeared before an increase in lung TNF-α level. Despite this temporal discordance, TNF KO mice exhibited attenuated lung neutrophil accumulation and lung leak after hemorrhagic shock, and both lung neutrophil accumulation and lung leak were restored in TNF KO mice by administration of recombinant murine TNF-α before hemorrhage. More-
over, lung neutrophil accumulation and lung leak induced by hemorrhagic shock were similarly attenuated in mice lacking the p55 TNF-α receptor, whereas knockout of the p75 TNF-α receptor had no influence on either lung neutrophil accumulation or lung leak. These results suggest that TNF-α provokes the pulmonary inflammatory response to hemorrhagic shock and that the p55 TNF-α receptor mediates the proinflammatory effect of TNF-α in the lung after hemorrhagic shock.

Neutrophils are the major cellular elements involved in acute lung inflammation after resuscitated hemorrhagic shock (32, 45). Our recent studies in a murine model found that neutrophil accumulation and tissue injury occur in the lung during hemorrhagic shock without resuscitation (1, 39) and that neutrophil depletion attenuates acute lung injury in this model of hemorrhagic shock (1). Thus pulmonary neutrophil recruitment is an important factor contributing to acute lung injury regardless of reperfusion. Although neutrophil accumulation plays a central role in acute lung injury, the factor that provokes the pulmonary inflammatory response to hemorrhagic shock remains to be defined. TNF-α is a proinflammatory cytokine and induces chemokines and adhesion molecules in a variety of cell types (18, 19). Previous studies demonstrated that a large dose (200 ng/g body wt or greater) of recombinant TNF-α can induce lung neutrophil accumulation and lung injury (17, 42). Indeed, hemorrhagic shock induces pulmonary expression of TNF-α (1, 3), and neutralization of TNF-α with a monoclonal antibody attenuates lung injury after hemorrhagic shock (2). These studies suggest that endogenous TNF-α contributes to hemorrhagic shock-induced lung injury. It remains unclear, however, whether TNF-α provokes lung neutrophil recruitment.

In the present study, lung neutrophil count was elevated significantly at 30 min and continued to increase for 4 h, whereas the twofold increase in the lung TNF-α level did not occur until 1 h after hemorrhagic shock. The temporal discordance between pulmonary neutrophil recruitment and the increase in lung TNF-α level implies that the initial pulmonary neutrophil recruitment after hemorrhagic shock does not require an increase in lung TNF-α production. It is possible that a basal level of lung TNF-α is sufficient to mediate the pulmonary inflammatory response to hemorrhagic shock. Alternatively, some other factor(s) is responsible for the initial pulmonary neutrophil recruitment. A slight increase in lung TNF-α level at 1 h is associated with a further accumulation of neutrophils between 1 and 4 h. However, lung TNF-α level does not correlate with the magnitude of neutrophil accumulation, since LPS-induced lung neutrophil accumulation was comparable to that of hemorrhagic shock, but it induced an 80-fold increase in lung TNF-α. Together these data do not appear to support the hypothesis that TNF-α is an important factor in hemorrhage-induced lung neutrophil recruitment.

Unexpectedly, pulmonary neutrophil accumulation 4 h after hemorrhagic shock was greatly reduced in mice lacking TNF-α compared with wild-type mice, although TNF KO had no influence on lung neutrophils after sham treatment. Moreover, lung neutrophil count was also significantly lower in TNF KO mice at 30 min after hemorrhage, a time point before the slight increase in lung TNF-α in wild-type mice. These results suggest that a low level of endogenous TNF-α is required for the initiation of the pulmonary inflammatory response. Similarly, lack of endogenous TNF-α production also significantly attenuated lung leak after hemorrhage. Thus TNF-α mediates hemorrhage-induced lung neutrophil accumulation and lung leak, although lung TNF-α remains at a low level after hemorrhagic shock.

To further determine the role of TNF-α in provoking neutrophil accumulation and tissue injury during hemorrhagic shock, we administered a small dose of recombinant murine TNF-α (80 pg/g body wt iv) to TNF KO mice before subjecting them to hemorrhagic shock. Assuming that exogenously administered TNF-α distributes evenly throughout the body, this dose of TNF-α would result in tissue TNF-α levels comparable to that in lungs of sham-treated wild-type mice. The hyporesponsiveness to hemorrhagic shock in TNF KO mice was reversed by administration of recombinant TNF-α, although this dose of TNF-α was insufficient to induce significant lung neutrophil accumulation or lung leak in the absence of hemorrhagic shock. Together these results demonstrate that a low level of TNF-α is required for hemorrhagic shock to provoke acute pulmonary inflammation and injury. Because
TNF-α signaling is mediated by two types of cell surface receptors, the p55 TNF-α receptor and the p75 TNF-α receptor (15). The p55 TNF-α receptor is the dominant effector in TNF-α biology and mediates most of the proinflammatory effects of TNF-α (25, 27, 28, 38). The p75 TNF-α receptor has been found to play an important role in ligand passing and thus enhances p55 receptor signaling (41). Indeed, lung neutrophil infiltration in response to systemic TNF-α requires p55 TNF-α receptor signaling (25). Although the role of TNF-α receptors in pulmonary recruitment of neutrophils and tissue injury remains to be defined, both p55 and p75 TNF-α receptors have been reported to be involved in neutrophil recruitment in response to LPS (43). In the present study, mice lacking the p55 TNF-α receptor exhibited reduced lung neutrophil accumulation and attenuated lung injury after hemorrhagic shock, and the effects of TNF KO were mimicked by p55 TNFR KO. In contrast, absence of the p75 TNF-α receptor had no influence on either lung neutrophil accumulation or lung injury. These results provide in vivo evidence that the p55 TNF-α receptor mediates lung neutrophil accumulation and acute lung injury in hemorrhagic shock. It is likely that TNF-α serves as a proximal mediator to initiate a pulmonary inflammatory cascade during hemorrhagic shock, resulting in recruitment of neutrophils and tissue injury, and that the p55 TNF-α receptor plays a critical role in this process. The results of the present study suggest that antagonization of p55 TNF-α receptor may be a therapeutic approach for protection of the lung against hemorrhagic shock-induced injury.

TNF-α has been demonstrated to induce factors involved in tissue neutrophil accumulation, such as chemokines (12, 37, 38), integrins (40), and adhesion molecules (11, 18, 25). Studies using animal models of LPS-induced lung injury suggest that TNF-α serves to signal the expression of adhesion molecules and chemokines (10). Thereafter, lung injury occurs as the result of the release of reactive oxygen species and proteases from sequestered intrapulmonary neutrophils (7, 33, 44). Although resuscitation after hemorrhagic shock induces lung intercellular adhesion molecule (ICAM)-1 expression, our previous work has suggested that pulmonary neutrophil recruitment occurs during unresuscitated hemorrhagic shock independent of lung ICAM-1 expression, since lung ICAM-1 levels were unchanged and pulmonary neutrophil recruitment was not affected in ICAM-1 knockout mice (39). The results of the present study suggest a role for TNF-α and the p55 TNF receptor in mediating lung neutrophil accumulation and lung injury after hemorrhagic shock. The downstream mediators of this phenomenon, however, remain to be determined.

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