Inflammatory Cells as a Source of Airspace Extracellular Superoxide Dismutase after Pulmonary Injury

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Extracellular superoxide dismutase (EC-SOD) is an antioxidant abundant in the lung. Previous studies demonstrated depletion of lung parenchymal EC-SOD in mouse models of interstitial lung disease coinciding with an accumulation of EC-SOD in airspaces. EC-SOD sticks to the matrix by a proteolytically sensitive heparin-binding domain; therefore, we hypothesized that interstitial inflammation and matrix remodeling contribute to proteolytic redistribution of EC-SOD from lung parenchyma into the airspaces. To determine if inflammation limited to airspaces leads to EC-SOD redistribution, we examined a bacterial pneumonia model. This model led to increases in airspace polymorphonuclear leukocytes staining strongly for EC-SOD. EC-SOD accumulated in airspaces at 24 h without depletion of EC-SOD from lung parenchyma. This led us to hypothesize that airspace EC-SOD was released from inflammatory cells and was not a redistribution of matrix EC-SOD. To test this hypothesis, transgenic mice with lung-specific expression of human EC-SOD were treated with asbestos or bleomycin to initiate an interstitial lung injury. In these studies, EC-SOD accumulating in airspaces was entirely the mouse isoform, demonstrating an extrapulmonary source (inflammatory cells) for this EC-SOD. We also demonstrate that EC-SOD knockout mice possess greater lung inflammation in response to bleomycin and bacteria when compared with wild types. We conclude that the source of accumulating EC-SOD in airspaces in interstitial lung disease is inflammatory cells and not the lung and that interstitial processes such as those found in pulmonary fibrosis are required to remove EC-SOD from lung matrix.

Keywords: inflammation; neutrophils; pneumonia; proteolysis; superoxide dismutase

Extracellular superoxide dismutase (EC-SOD) is a 135-kD catalytic antioxidant enzyme that scavenges the superoxide free radical (1). Although three SODs exist in mammalian tissues (copper/zinc, manganese, and extracellular), only EC-SOD exists primarily in extracellular fluids and matrices. EC-SOD is abundant in the lung, suggesting an important role for this antioxidant in protecting the lung from oxidative insults (1–3). Previous studies have demonstrated that EC-SOD is protective against a variety of lung injuries (4–8). EC-SOD has been shown to inhibit inflammation in bleomycin (4), hyperoxia (5), and endotoxin-induced pulmonary injuries (9), suggesting that it may play an important role in regulating inflammatory responses in the lung.

Each subunit of the EC-SOD homotetramer possesses a heparin-binding domain at the C-terminus that confers affinity for heparin and type I collagen in the extracellular matrix (1, 10) and regulates its tissue distribution (1, 11). Formed by a cluster of positively charged amino acids, these domains can be post-translationally cleaved from the enzyme by a number of proteases (12–14) without affecting enzymatic activity. Proteolysis of this heparin-binding domain occurs in a number of interstitial lung disease models, including bleomycin (15) and asbestos injury (16). These injuries lead to clearance of EC-SOD from the lung parenchyma coinciding with accumulation in the airspaces, suggesting that EC-SOD is redistributed from the lung parenchyma to the airspace. It is not known whether airspace EC-SOD originates from the lung or from another source.

Exposure of the lungs to bleomycin and asbestos in animals and humans leads to interstitial thickening of alveolar septa characteristic of pulmonary fibrosis (4, 16–19). Inflammation accompanies the disease process and affects the interstitium and the airspaces. Because full-length EC-SOD possessing the heparin-binding domain localizes to the lung parenchyma, we hypothesized that the interstitial inflammation and remodeling play an important role in the clearance of EC-SOD from the interstitium.

To determine if pulmonary inflammation limited to airspaces without significant interstitial effects can lead to the redistribution of EC-SOD in the lung, we examined endogenous EC-SOD in a pneumonia model caused by *Escherichia coli* inhalation. Six and twenty-four hours after inoculation with *E. coli*, mice developed an infiltration of leukocytes into the airspaces. Increased polymorphonuclear leukocytes (PMNs) were observed through manual cell counts and myeloperoxidase measurements. Increased matrix metalloproteinase (MMP)-9, which is produced by PMN (20), was observed in the airspaces. Analogous to our findings in bleomycin and asbestos lung injury, there was an increase in EC-SOD in the bronchoalveolar lavage fluid (BALF) at 24 h after exposure. EC-SOD also localized strongly to inflammatory cells in the airspaces by immunohistochemistry. In contrast to our earlier findings in interstitial lung diseases, we did not observe depletion of EC-SOD from the pulmonary interstitium. In experiments with mice with lung-specific expression of human EC-SOD, we did not find human EC-SOD in the airspaces after a stimulus for interstitial lung disease. These data reveal that airspace EC-SOD originates from inflammatory cells that release this antioxidant into the airspaces and is not secondary to diffusion of proteolyzed EC-SOD from the lung parenchyma into the airspaces. Our findings demonstrate that inflammation limited to the airspaces is insufficient to cause the depletion of EC-SOD from the pulmonary interstitium, as is seen with interstitial inflammation and remodeling. We also found that EC-SOD is functionally relevant in pulmonary inflammation, as EC-SOD knockout mice develop greater PMN infiltration into the airspaces in response to bleomycin and bacteria when compared with wild-type mice.

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MATERIALS AND METHODS

Animals

C57BL/6 breeders were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred in the vivarium of the VA Puget Sound Health Care Systems Research facility. Age- and sex-matched animals were used in all experiments. Animals were maintained in specific pathogen-free cages. Bacterial inhalation experiments were conducted in accordance with the Institutional Animal Care and Use Committee at the VA Puget Sound Health Care Systems and the University of Washington. Surfactant protein C EC-SOD transgenic mice were bred in the vivarium at the University of Pittsburgh Medical Center and have been previously described (5). EC-SOD knockout mice were also bred at the University of Pittsburgh Medical Center and have been previously described (4). Transgenic and knockout animal experiments were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Bacteria

E. coli, serotype K-1, was a clinical isolate obtained from the blood of a patient with biliary sepsis. The pathogenicity of *E. coli* in mice has been previously described (21). Methods used to pass and store the bacteria have been detailed elsewhere (22). An aliquot of frozen bacteria was thawed and inoculated into 50 ml of Luria broth (Gibco-BRL Laboratories, Gaithersburg, MD), which was incubated for 6 h at 37°C with shaking at 225 rpm. For the aerosol experiments, this culture was inoculated into 1 l of Luria broth and incubated for 14 h under the same conditions. The broth was centrifuged at 9,000 rpm, and the bacterial pellet was washed twice with sterile 0.9% NaCl. The washed bacterial pellet was resuspended in 22 ml 0.9% NaCl and divided equally into two nebulizers. Quantitative cultures were performed on the bacterial slurry. *Klebsiella pneumoniae* strain 43,816 (serotype 2) was from American Type Culture Collection (Manassas, VA).

E. coli Pneumonia Studies

The inhalation method of delivering live bacteria to mice has been previously described (21, 23). After bacterial delivery, the mice were killed immediately to establish the initial bacterial inoculum or at 24 h for measurements of bacterial burden. Mice were killed with 120 mg/kg pentobarbital and exsanguinated by direct cardiac puncture. The thoracic cavity was opened by midline incision. The trachea was exposed and cannulated with a 20-gauge catheter, which was secured with a 2-0 silk suture. The left mainstem bronchus was identified and divided at the hilum, and the entire left lung was placed in 1 ml sterile H_2O for subsequent homogenization. BAL was performed on the right lung using 0.9% NaCl containing 0.6 mM EDTA instilled in one aliquot of 0.6 ml followed by three aliquots of 0.5 ml. The right lung was fixed with 4% paraformaldehyde at an inflation pressure of 15 cm H_2O.

Klebsiella pneumoniae Studies

After 18 h of broth culture and 2 h log-phase culture in Tryptic Soy broth (Becton Dickinson Microbiology Systems, Sparks, MD), *K. pneumoniae* bacteria were heat killed at 100°C for 10 min. Bacteria (1×10^5 CFU) were intratracheally instilled under anesthesia into wild-type C57BL/6 or EC-SOD knockout mice. Mice were killed immediately (0 h) as a control or at 24 h. BALF was recovered by the instillation and recovery of 0.8 ml of 0.9% saline.

Asbestos and Bleomycin Exposures

Mice were exposed to asbestos by intratracheal instillation of saline vehicle or 0.1 mg crocidolite asbestos or 0.04 units of bleomycin under anesthesia as described previously (16). For the asbestos study, mice were killed at 14 d after exposure, and BALF was recovered as described previously for the *Klebsiella* experiments. For the bleomycin study, mice were killed at 3 d.

Sample Processing

Total cell counts in the BALF of *E. coli*–treated mice were performed using a Neubauer chamber. Cell differentials were performed by counting 200 consecutive cells from cytospin preparations stained with Diff-Quik. The remainder of the BALF was spun at $200 \times g$, and supernatants were stored at −70°C. The left lung homogenates were separated into aliquots for quantitative cultures, Western blot analysis, and myeloperoxidase (MPO) determinations. For the Western blots, the lung homogenate was added to a buffer containing 0.5% Triton-X-100, 150 mM NaCl, 15 mM Tris, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ (pH 7.40), incubated on ice for 30 min and spun at $10{,}000 \times g$ for 20 min. The supernatants were stored at −70°C. For the MPO measurements, the lung homogenate was added to a buffer containing 50 mM potassium phosphate (pH 6.0), 5 mM EDTA, and 5% wt/vol hexadecyltrimethyl ammonium bromide. The mixture was sonicated and spun at $12{,}000 \times g$ for 30 min, and the supernatants were stored at –70°C.

Cell counts of BALF from *Klebsiella*-, bleomycin-, and asbestos-treated mice were performed using a Z1 Coulter particle counter (Coulter, Fullerton, CA). Differentials were performed as described previously (16).

Bacterial Recovery Measurements

Quantitative cultures were performed on the lung homogenates obtained from each animal at the various times by spreading serial 10-fold dilutions of the lung homogenates in warm Luria-Bertani agar using the pour-plate method.

MPO Activity Assay

The Amplex Red peroxidase assay kit (Molecular Probes, Eugene, OR) was used to determine MPO activity in the lung homogenates. Lung tissue homogenates were prepared as described previously and mixed with reaction buffer, H_2O_2, and Amplex Red reagent according to the manufacturer's instructions. The Amplex Red reagent reacts with H_2O_2 in the presence of peroxidase to produce the fluorescent molecule resorufin. The fluorescence of each sample was measured using a cytofluorometer set at excitation wavelength 530 nm and emission wavelength 590 nm (CytoFluor II; PerSeptive Biosystems, Foster City, CA). The MPO activity was obtained by calculating resorufin units for each unknown lung homogenate sample using a resorufin standard curve.

Western Blot Analysis

Western blot analysis was performed as previously described (16). Briefly, 3 μg of protein from BALF or 10 μg from lung homogenates was subjected to SDS-PAGE and blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). EC-SOD was detected with a rabbit anti-mouse polyclonal antibody (1:10,000 dilution) against mouse EC-SOD (3). After visualization by enhanced chemiluminescence, densitometry was performed with lung homogenates standardized to mouse β-actin as a loading control (Sigma, St. Louis, MO). Densitometry was performed on bands on Kodak 1D software (Kodak, Rochester, NY).

EC-SOD Activity

BALF EC-SOD activity was determined as previously described (15). In this assay, superoxide was generated by the reaction of xanthine oxidase with xanthine at pH 10. EC-SOD activity was measured by inhibition of the reduction of partially acetylated cytochrome c by superoxide.

Gelatin Zymography

To quantify protein levels of MMP-9, BALF was subjected to gelatin zymography as previously described (24, 25). Briefly, equal protein samples were applied to 10% SDS-PAGE gels containing gelatin (Invitrogen, Carlsbad, CA). After SDS-PAGE, the gel was washed in Triton X-100 and incubated for 22 h at 37°C in pH 7.4 buffer containing calcium and zinc. After staining of the gel with Coomassie blue, areas of MMP activity appeared as clear bands. Purified mouse MMP-9 (Calbiochem, Temecula, CA) was used to identify the MMP-9 band. Densitometry was performed as described previously.

Histology and Immunohistochemistry

Standard hematoxylin and eosin staining was performed on 5-μm-thick lung sections as previously described (16). Immunohistochemistry was performed as previously described, with changes noted below (16, 26). An automated slide staining system was used (DakoCytomation, Carpinteria, CA). Briefly, slides were deparaffinized and incubated in 6% hydrogen peroxide in methanol to inactivate endogenous

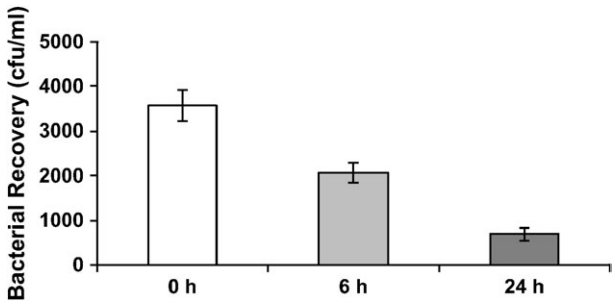

Figure 1. Bacterial recovery from the lungs of mice exposed to *E. coli*. Mice were killed immediately after bacterial exposure to determine the initial bacterial inoculation at 0 h ($n = 4$). Mice were killed at 6 h ($n = 6$) or at 24 h ($n = 10$) to determine remaining bacterial burden as the lungs cleared the infection. Results are representative of two separate experiments.

peroxidases and then 0.1% pepsin for antigen retrieval. After blocking with protein-blocking solution (Thermo Electron, Pittsburgh, PA), slides were incubated with the same antibody against mouse EC-SOD (1:500) used in Western blotting or with normal rabbit sera at the same dilution as a control for 60 min. A 30-min incubation in biotinylated donkey anti-rabbit secondary antibody (1:1,000) (Jackson Immunoresearch Laboratories, West Grove, PA) was followed by 30 min in ABC reagent (Vector Labs, Burlingame, CA). Slides were developed with the Nova Red staining kit (Vector Labs). All slides were stained on the same day and developed for the same amount of time. Photos of 10 random fields from each slide taken at 40× magnification were analyzed for EC-SOD staining intensity using Metamorph software (Molecular Devices, Sunnyvale, CA) as previously described (16). We have previously shown that quantification of EC-SOD staining in immunoperoxidase-stained slides using this method produces similar results compared with immunofluorescence staining (27).

Statistical Analyses

Data are provided as means ± SEM. All comparisons between the two groups were made using Student's *t* test; three or more groups were compared with one-way ANOVA with Tukey's post-test using Graphpad Prism 4 (Graphpad, San Diego, CA). A P value of ≤ 0.05 was considered statistically significant.

Results

Bacterial Inhalation Causes Airspace Inflammation

Wild-type C57BL/6 mice were exposed to *E. coli* serotype K1 through inhalation of nebulized bacteria. Mice were killed immediately at 0 h or at 6 or 24 h. The 0-h measurement is an indication of initial bacterial load, and the 24-h count shows the bacterial burden of the lungs over time. After the mice were killed, the left lung was homogenized and plated onto Luria-Bertani agar to determine bacterial load. The number of cfu were determined and expressed per milliliter of homogenate (Figure 1). Lungs at 0 h had an average of 3,567 cfu/ml; this number decreased to 700 CFU/ml at 24 h.

Histologic examination of the lungs revealed increased numbers of inflammatory cells in the airspaces at 6 and 24 h, as compared with 0 h (Figure 2). The majority of these cells were PMNs, although macrophages were present. In contrast to interstitial lung diseases, the lung parenchyma displayed only minor edema and lacked inflammation and fibrosis. BALF also showed significantly increased PMNs at 6 and 24 h (Figure 3A). Myeloperoxidase measurements, a surrogate marker for total neutrophil content in the lungs, were also elevated (Figure 3B). In addition, levels of MMP-9, a protease that is highly expressed by PMNs, were significantly increased in the BALF at 24 h (Figure 3C). Macrophage numbers were increased at 24 h, although only by a factor of 3, whereas PMNs were increased by a factor of 1,000 (Figure 3A). These data show that inoculation with *E. coli* bacteria induces an inflammatory response with a particularly large increase in PMN content.

EC-SOD Accumulates in the BALF of Mice with Pneumonia

To examine levels of EC-SOD in the airspaces and lung parenchyma, Western blots were performed on BALF and lung homogenates. An accumulation of EC-SOD was found in the airspaces of mice at 24 h after inhaled *E. coli*, as compared with 0 and 6 h after inhaled *E. coli* (Figures 4A and 4C). The majority of this EC-SOD was comprised of the lower-molecular-weight form lacking the heparin-binding domain, indicating proteolysis of this domain (Figure 4B). Activity assays of the BALF confirmed our Western blot results because EC-SOD activity was detected at 24 h (11.7 U/ml ± 3.07) with no detectable activity at 0 and 6 h.

Lung homogenates from mice were subjected to Western blotting to evaluate EC-SOD levels in the lung parenchyma (Figure 5). The lung homogenates did not show a depletion of EC-SOD at 24 h, in contrast to earlier results published for interstitial lung disease models. This indicates a difference in EC-SOD regulation in interstitial inflammatory injuries versus airspace bacterial pneumonias. When lung sections were examined by immunohistochemistry for EC-SOD, alveolar septa stained strongly in the 0-h and 24-h lungs (Figures 6A and 6B). No significant difference was found between the two groups (Figure 6F). In addition, PMNs and macrophages within the airspaces stained strongly for EC-SOD in the 24-h samples

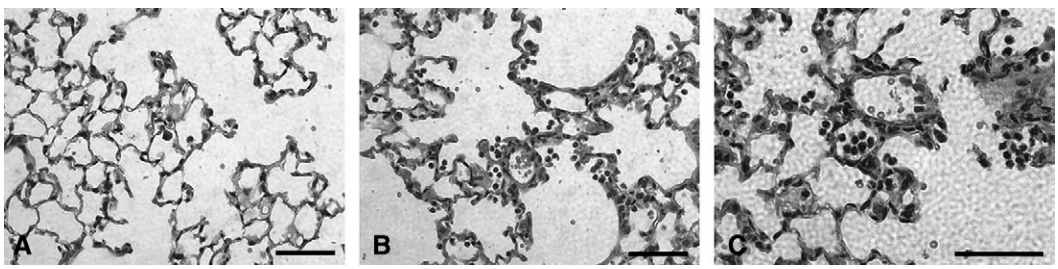

Figure 2. Bacterial inhalation induces airspace inflammation. Hematoxylin and eosin staining was performed on lung sections from mice exposed to inhaled *E. coli*. Mice killed at 0 h (*A*) showed only occasional airway macrophages. At 24 h post-treatment (*B*), increased numbers of white blood cells were found in the airspaces. The majority of these cells were neutrophils (*C*), although macrophages were also present. *Bar*, 50 μm.

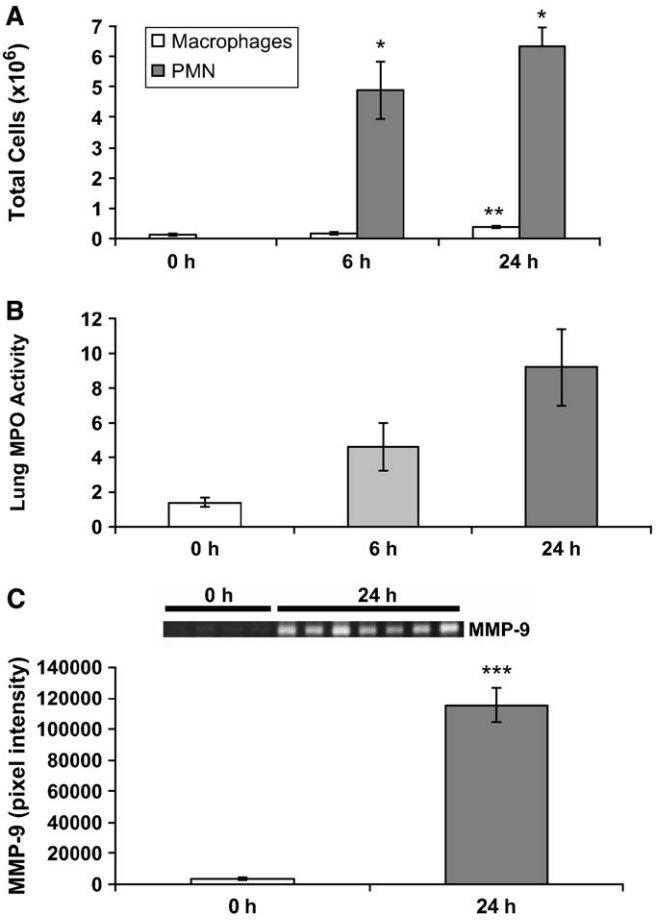

Figure 3. BALF PMNs and macrophages are increased after bacterial inoculation. BALF was recovered from mouse lungs as described in Materials and Methods, and total numbers of PMNs and macrophages (*A*) were determined by differential counting. MPO activity (*B*) and MMP-9 levels (*C*) were determined. $*P < 0.01$ compared with 0 h, ANOVA and Tukey's post-test. $**P < 0.05$ compared with 0 and 6 h, ANOVA and Tukey's post-test. $***P < 0.05$ compared with 0 h, Student's *t* test.

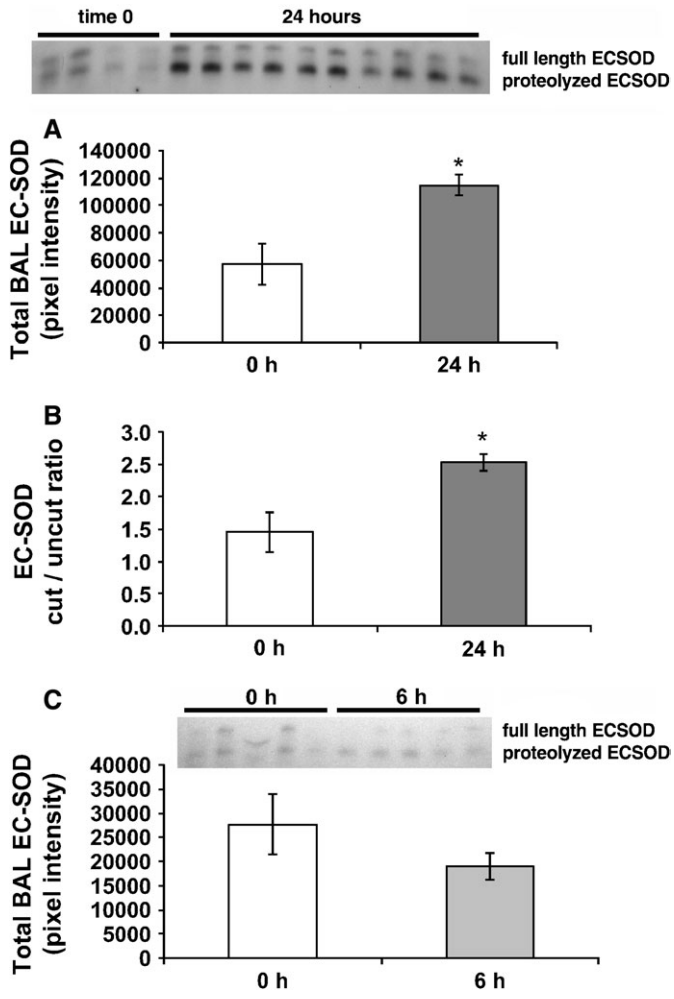

Figure 4. EC-SOD accumulates in the BALF 24 h after inoculation with *E. coli*. Western blots were performed on equivalent protein amounts of BALF. (*A*) Total ECSOD is increased in the BALF 24 h after inoculation. (*B*) The majority of accumulating EC-SOD is proteolyzed (lacks heparin-binding domain). (*C*) There was no significant difference in total EC-SOD at 6 h. Results are representative of two separate experiments. $*P < 0.05$, Student's *t* test.

(Figure 6E). This implies a role for these inflammatory cells in the release of EC-SOD in the airspaces, leading to its accumulation there.

To further examine the role of inflammatory cells in airspace EC-SOD accumulation, experiments were performed with a strain of transgenic mice possessing lung-specific expression of human EC-SOD. These mice possess the gene for human EC-SOD under the surfactant protein C (SPC) promoter and have been described previously (5). Inflammatory cells and all other tissues produce only mouse EC-SOD. Because human and mouse EC-SOD can be distinguished on reducing SDS-PAGE by size, these mice provided the opportunity to determine the source of airspace EC-SOD.

As determined by Western blotting, lung homogenates from saline-treated transgenic mice contained human and mouse EC-SOD, whereas wild-type mice expressed only the latter (Figure 7A). These mice were exposed to asbestos fibers or the drug bleomycin. These agents induced lung inflammation and interstitial lung disease and led to the depletion of EC-SOD from the lung parenchyma with concomitant EC-SOD accumulation in the airspaces as described previously (15, 16). These injuries led to the accumulation of mouse EC-SOD but not human EC-SOD in the BALF of transgenic mice (Figures 7B and 7C), strongly supporting an inflammatory cell origin of this EC-SOD.

Loss of EC-SOD Potentiates Neutrophilic Inflammation in the Lung

To determine the functional role of EC-SOD in these injuries, we performed experiments in mice that were null for EC-SOD. These knockout mice were intratracheally instilled with bleomycin (0.04 units per mouse) or with heat-killed bacteria (*K. pneumoniae*, 1×10^5 bacteria per mouse). The bacteria were heat killed to avoid potential confounding differences in bacterial load or propagation due to the lack of EC-SOD. Twenty-four hours after bleomycin treatment, EC-SOD knockout mice possessed significantly increased concentrations of PMNs compared with wild-type mice (Figure 8A). Similarly, 24 h after bacterial instillation, EC-SOD knockout mice showed a trend toward increased concentrations of PMNs compared with wild-type mice (Figure 8B). These studies show that EC-SOD has a functional role in inflammation in interstitial and airspace lung diseases.

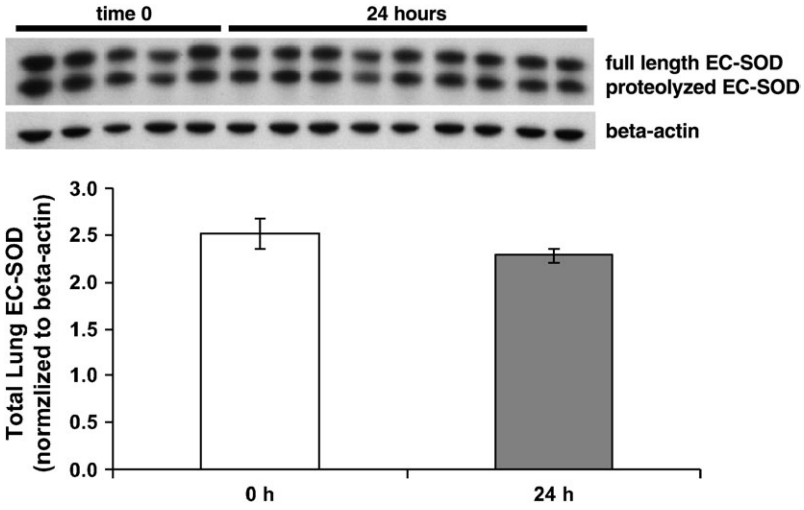

Figure 5. EC-SOD is not depleted from lung homogenates 24 h after bacterial inoculation. Western blots were performed on equivalent protein amounts of lung homogenate. Total EC-SOD in the lung is unchanged 24 h after inoculation ($P = 0.15$, Student's *t* test).

DISCUSSION

EC-SOD is an antioxidant enzyme that scavenges the potentially harmful superoxide free radical. Abundant in the lungs of humans and mice, EC-SOD has been shown to protect against a variety of pulmonary insults, including exposure to bleomycin (4), oil fly ash (28), hyperoxia (5, 7), and influenza (8). The overexpression of EC-SOD in mice exposed to LPS reduced neutrophilic inflammation in the lung, whereas EC-SOD knockout mice developed increased neutrophilic inflammation, demonstrating an important role in modulation of the immune system to bacterial infection (9). In addition, proteolytic processing of the heparin-binding domain of EC-SOD, accompanied by depletion of this antioxidant from the lung parenchyma and accumulation in the airspaces, was observed in bleomycin-mediated (15) and asbestos-mediated (16) lung disease. A similar depletion from the lungs was observed in response to hyperoxia (27). Because these disease processes are characterized by interstitial inflammation and remodeling, we hypothesized that these interstitial processes are responsible for the proteolysis and redistribution of EC-SOD. To test this hypothesis, we examined the proteolysis and localization of EC-SOD in a bacterial pneumonia model that lacks interstitial remodeling events and in which inflammation is limited to the airspaces.

Our studies show that challenge of mice with inhaled *E. coli* leads to an inflammatory reaction 6 and 24 h later that is localized primarily to the airspaces. PMNs were increased as determined by histology, BALF cell counts, myeloperoxidase, and MMP-9 activity (29). This neutrophilic influx is also observed in interstitial lung disease (16).

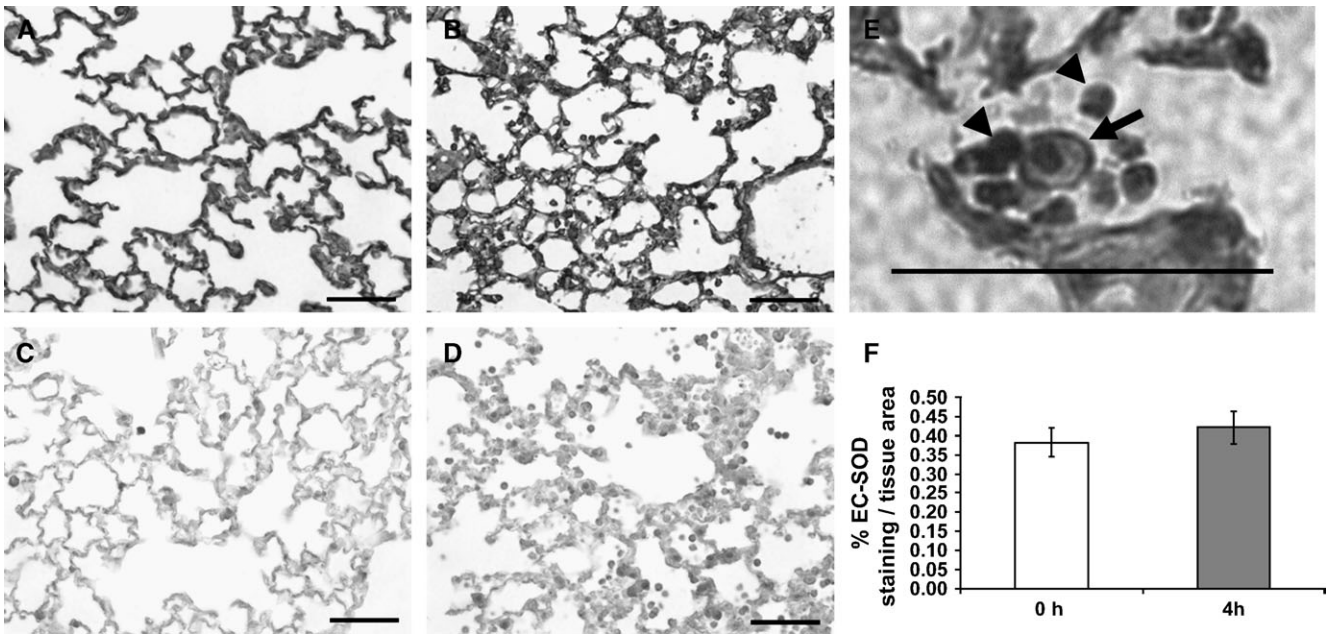

Figure 6. EC-SOD levels in the lung parenchyma are unchanged after *E. coli* inoculation. Paraffin-embedded lung sections (5 μm thick) from mice killed at 0 h (*A* and *C*) or at 24 h (*B* and *D*) were stained with antibody against EC-SOD (*A* and *B*) or with normal rabbit serum (*C* and *D*). (*E*) Macrophages (*arrow*) and PMN (*arrowheads*) stained strongly for EC-SOD. (*F*) EC-SOD labeling intensity is unchanged 24 h after inoculation ($P = 0.54$, Student's *t* test). *Bar*, 50 μm.

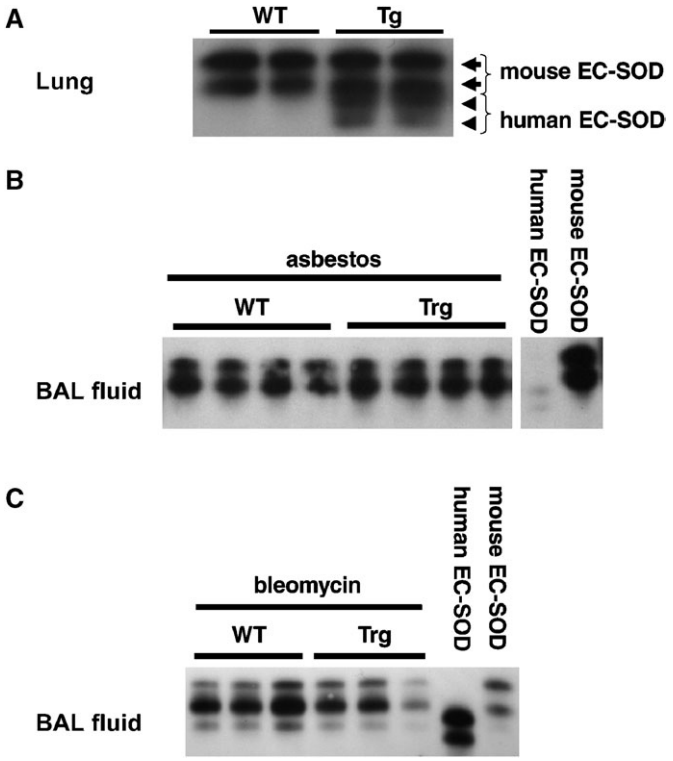

Figure 7. Human EC-SOD produced under the SPC promoter in transgenic mice is not found in BALF of asbestos-treated transgenic mice. Western blots for EC-SOD were performed on lung homogenates (*A*) and BALF (*B*) from SPC EC-SOD transgenic (Trg) and wild-type (WT) mice. (*A*) Trg mice produce the human and mouse forms of EC-SOD (distinguishable on reducing SDS-PAGE) in the lung, whereas inflammatory cells express only mouse EC-SOD. Fourteen days after exposure to asbestos (*B*) or 3 d after exposure to bleomycin (*C*), there is BALF accumulation of only mouse EC-SOD in the Trg mice, suggesting an extrapulmonary source of EC-SOD (i.e., infiltrating inflammatory cells).

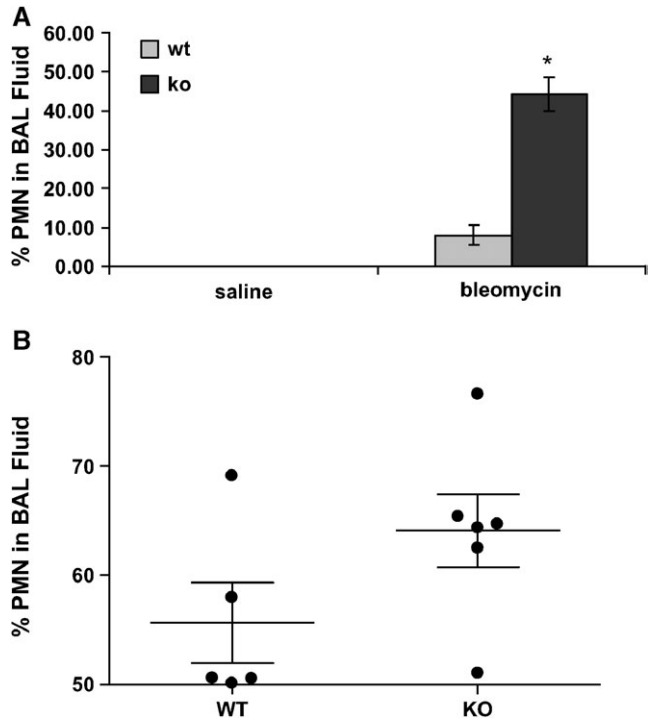

Figure 8. EC-SOD knockout mice have increased neutrophils in response to inflammatory lung injury. C57BL/6 wild-type (WT) mice and EC-SOD knockout (KO) mice were intratracheally instilled with 0.04 units bleomycin (*A*) or with 1×10^5 bacteria (*B*). Bacteria consisted of heat-killed *K. pneumoniae*. Percentages of neutrophils in the BALF were determined through differential counting. $^*P < 0.05$, one-way ANOVA and Tukey's post test, bleomycin-treated EC-SOD KO mice compared with bleomycin-treated wild-type mice.

We found a significant increase in EC-SOD levels in the airspaces at 24 h as measured in the BALF. This accumulation is similar to that seen in mouse models of interstitial lung disease (15, 16). However, in contrast to findings in interstitial lung diseases that show a loss of EC-SOD from the lung parenchyma, lung homogenates and immunohistochemistry did not show any change in EC-SOD in the lung parenchyma after bacterial pneumonia, in which the inflammatory response is limited to the airspaces.

Previously, we had observed a depletion of EC-SOD from the lung parenchyma in mouse models of bleomycin (15), asbestos (16), and hyperoxia (27). These disease models have effects on the interstitium (inflammation and fibrosis or interstitial thickening) and the airspaces (inflammation). In contrast, bacterial pneumonias cause inflammation primarily in the airspaces. This difference may underlie the lack of EC-SOD depletion from the lung parenchyma in bacteria-exposed mice. Interstitial inflammation or remodeling processes may lead to the elaboration of proteases that cleave the heparin-binding domain of EC-SOD in the lung parenchyma. Without a significant interstitial component to the disease, as in the pneumonia model, EC-SOD remains at normal levels in the lung parenchyma.

We observed EC-SOD accumulation in the airspaces during bacterial pneumonia in spite of the retention of EC-SOD in the lung parenchyma. PMNs and macrophages are present in the airspaces in interstitial lung diseases and pneumonia, and these cells possess large amounts of EC-SOD (Figure 6E) (3, 30). We observed that although neutrophils are significantly increased at 6 h, significant EC-SOD accumulation in BALF does not occur until 24 h. It is likely that a period of time is required before enough EC-SOD accumulates to be detected by Western blotting and the activity assay. We cannot rule out a role for macrophages because they are significantly elevated at 24 h but not at 6 h. However, the large influx of neutrophils compared with macrophages implies a greater neutrophilic contribution to airspace EC-SOD.

In experiments with transgenic mice producing human and mouse EC-SOD in the lung (Figure 7A) and mouse EC-SOD in all other cells and tissues, we found only mouse EC-SOD in the BALF after a stimulus for inflammation and interstitial lung injury (Figure 7B). This finding strongly suggests that inflammatory cells transport and release EC-SOD into the airspaces because a lung origin would lead to human and mouse isoforms in the airspace. Other authors have suggested a role for neutrophils and macrophages in transporting EC-SOD into the lung (30). However, we cannot rule out the less likely possibility that only mouse EC-SOD, but not human EC-SOD, is being redistributed to the airspace.

It is not known whether this increased EC-SOD in the BALF represents a protective response. Proteolyzed EC-SOD retains its catalytic antioxidant capacity. Its release in sites of PMN infiltration may balance the large pro-oxidant activities of neutrophils. The release of EC-SOD may prevent widespread oxidative damage to alveolar airspace contents, such as surfactant and

epithelial cells. EC-SOD is also known to inhibit neutrophil influx in response to hyperoxia (5), LPS (9), oil fly ash (28), and asbestos (31). Therefore, release of EC-SOD from neutrophils may serve as a mechanism to limit further neutrophil influx. Our results support this hypothesis: Exposure of EC-SOD knockout mice to bleomycin or bacteria leads to increased PMNs in the BALF (Figures 8A and 8B). EC-SOD therefore attenuates PMN-dominated inflammation in the lung.

In conclusion, EC-SOD accumulates in the BALF of mice after inhaled *E. coli* challenge. This is not accompanied by any depletion of EC-SOD from the lung parenchyma, indicating that PMNs and macrophages release this antioxidant into the airspaces. This pattern differs from that seen in interstitial lung diseases, where the presence of interstitial inflammation and remodeling may contribute to the depletion of EC-SOD from the pulmonary interstitium. Our data suggest differential regulation of this antioxidant in the progression of interstitial lung disease and bacterial pneumonia and that inflammatory cells are a source of airspace EC-SOD during pulmonary inflammatory responses.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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