High Mobility Group Box-1 Mediates Hyperoxia-Induced Impairment of *Pseudomonas aeruginosa* Clearance and Inflammatory Lung Injury in Mice

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Mechanical ventilation with supraphysiological concentrations of oxygen (hyperoxia) is routinely used to treat patients with respiratory distress. However, a significant number of patients on ventilators exhibit enhanced susceptibility to infections and develop ventilator-associated pneumonia (VAP). *Pseudomonas aeruginosa* (PA) is one of the most common species of bacteria found in these patients. Previously, we demonstrated that prolonged exposure to hyperoxia can compromise the ability of alveolar macrophages (AMs), an essential part of the innate immunity, to phagocytose PA. This study sought to investigate the potential molecular mechanisms underlying hyperoxia-compromised innate immunity against bacterial infection in a murine model of PA pneumonia. Here, we show that exposure to hyperoxia ($\geq 99\% \text{O}_2$) led to a significant elevation in concentrations of airway high mobility group box-1 (HMGB1) and increased mortality in C57BL/6 mice infected with PA. Treatment of these mice with a neutralizing anti-HMGB1 monoclonal antibody (mAb) resulted in a reduction in bacterial counts, injury, and numbers of neutrophils in the lungs, and an increase in leukocyte phagocytic activity compared with mice receiving control mAb. This improved phagocytic function was associated with reduced concentrations of airway HMGB1. The correlation between phagocytic activity and concentrations of extracellular HMGB1 was also observed in cultured macrophages. These results indicate a pathogenic role for HMGB1 in hyperoxia-induced impairment with regard to a host's ability to clear bacteria and inflammatory lung injury. Thus, HMGB1 may provide a novel molecular target for improving hyperoxia-compromised innate immunity in patients with VAP.

Keywords: hyperoxia; bacterial infection; macrophage function; HMGB1

Oxygen therapy using mechanical ventilation (MV) with supra-physiological concentrations of oxygen (i.e., hyperoxia) is a life-saving intervention for patients with respiratory distress. During an average day, MV is administered to at least 40% of the patients in an American intensive care unit (1). However, the application of MV is associated with many adverse effects on the respiratory system, including increased airway injury, alveolar damage, and susceptibility to microbial infections (2–4). Ventilator-associated pneumonia (VAP) has been defined as pneumonia occurring more than 48 hours after the initiation of endotracheal intubation

and MV (5–7). VAP affects 8 to 28% of patients receiving MV (8, 9). According to the National Nosocomial Infection Survey, 86% of nosocomial pneumonia is associated with MV (10). VAP accounts for up to 60% of all deaths from healthcare-associated infections in United States (7), and continues to be a major cause of high morbidity and mortality for patients on ventilators (11–14).

Bacterial infections comprise one of the major contributors to VAP (8, 15). Both gram-negative and gram-positive strains of bacteria are frequently found in patients with VAP (8, 15). *Pseudomonas aeruginosa* (PA), a gram-negative aerobic bacterium, was reported to be associated with 21% of all nosocomial pneumonia cases (10). The overall prevalence of PA infections has been reported at approximately 0.4% in United States hospitals (<http://www.cdc.gov/>). Although antibiotics are routinely used, the management of PA infections in VAP remains difficult and complex because of their resistance to antibiotics (8, 15–17). Therefore, novel approaches are needed to enhance the efficacy of VAP treatment.

Corresponding to the poor clinical outcomes for patients with VAP, the mechanisms underlying the pathogenesis of VAP are not well elucidated. Invading microorganisms are cleared by host defenses, including innate immunity (18, 19). Both resident and recruited phagocytes are involved in the innate immunity to clear bacteria from the lungs and airways (20, 21). Alveolar macrophages (AMs) are professional phagocytes that reside in the airways (18). By engulfing and killing the invading pathogens, AMs form the first line of cell-mediated defense in the respiratory tract (19, 22, 23). We and others have previously shown that exposure to prolonged hyperoxia, which is routinely used during MV (24, 25), can compromise the ability of AMs to phagocytose PA (26, 27) and other bacteria, including *Klebsiella pneumoniae* (28, 29). Despite identifying the involvement of reactive oxygen species (ROS) (26), little is known about the downstream events that lead to the deleterious effects of prolonged hyperoxia on macrophage functions and the host defense system, and whether compromised macrophage function results in abridged survival in PA pneumonia.

We recently reported on the role of high mobility group box (HMGB)-1 in the phagocytic activity of AMs and host defense (30). HMGB1 belongs to the high mobility group family of nuclear proteins (31). In the nucleus, HMGB1 acts as a cotranscriptional factor and is implicated in stabilizing nucleosomes and regulating transcription and DNA repair (32–34). However, HMGB1 can be released into the extracellular milieu from immune cells in response to exogenous bacterial endotoxins or endogenous proinflammatory cytokines (35, 36). Once released, extracellular HMGB1 acts as an inflammatory cytokine, leading to lung injury and multiple organ failure (34, 35). In addition to its role as a proinflammatory cytokine, HMGB1 has been shown to play a role in bacterial pneumonia (30). Pronounced PA infection, a hallmark of cystic fibrosis (CF), occurs in the majority of adult patients (37, 38). We found that concentrations of airway HMGB1 were markedly increased in patients with CF, and elevated concentrations of airway HMGB1

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can directly diminish the phagocytic activity of AMs (30). Using a murine model of PA pneumonia and cultured murine macrophages, we investigated in this study whether (1) exposure to hyperoxia induces an elevation in concentrations of airway HMGB1, and (2) HMGB1 plays a role in hyperoxia-compromised host defense in clearing bacteria and macrophage phagocytic function.

MATERIALS AND METHODS

Special Reagents

Neutralizing anti-HMGB1 monoclonal and polyclonal antibodies were generated as described previously (35, 39). Briefly, recombinant HMGB1 with a purity of less than 0.1 $\mu\text{g}/\mu\text{g}$ endotoxin was generated using rat HMGB1 cDNA, which was cloned onto a pCAL-n vector (Stratagene, La Jolla, CA) and expressed in *Escherichia coli* BL21(DE3)pLysS cells (35, 36, 40). Contaminating endotoxin was removed from HMGB1 preparations by Triton X-114 extraction (41). The extent of endotoxin contamination was assessed using the chromogenic *Limulus* amoebocyte lysate assay (Endochrome; Charles River, Charleston, SC). Green fluorescent protein–PAO1, a nonmucoid strain of *Pseudomonas aeruginosa*, was cultured as described previously (42, 43).

Bronchoalveolar Lavage

Murine bronchoalveolar lavage (BAL) fluid was obtained as described previously (26, 44, 45). Briefly, mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (120 mg/kg). After a 1- to 2-cm incision was made on the neck, the trachea was dissected, and a 20-gauge $\times$ 1.25-inch intravenous catheter was inserted caudally into the lumen of the exposed trachea. The lungs were gently lavaged twice with 1 ml sterile, nonpyrogenic PBS solution (Mediatech, Inc., Hendon, VA). The BAL samples were centrifuged, and the resultant supernatants were stored in a freezer at -80°C for analyzing concentrations of HMGB1 and the total protein content, using Western blot analysis and a bicinchoninic acid assay.

Animal Studies

The experimental use of animals presented in this study was approved by the Institutional Animal Care and Use Committees of St. John's University. The mice were housed in a specific pathogen-free environment. C57BL/6 mice obtained from the Jackson Laboratory (Bar Harbor, ME) were inoculated with 5×10^8 colony-forming units (CFUs) of PA (46) via intranasal aspiration after brief anesthesia with isoflurane. Mice were randomized to receive either neutralizing anti-HMGB1 (α -HMGB1) monoclonal antibody (mAb) or an isotypic control mAb, administered by intraperitoneal injection 24 hours before bacterial inoculation, during hyperoxia. Twenty-four hours after bacterial inoculation, mice were anesthetized with intraperitoneal sodium pentobarbital (120 mg/kg) to obtain BAL and lung tissues, as depicted in Figure 1. In brief, mice were anesthetized and tracheas were dissected and cannulated, as already described. After lavage with PBS, the lungs were excised and immediately placed into 1 ml of cold PBS and homogenized.

Exposure to Hyperoxia

Male C57BL/6 mice were placed in micro-isolator cages (Allentown Caging Equipment Co., Inc., Allentown, NJ), which were kept in a Plexiglas chamber (BioSpherix, Laconia, NY) and exposed to $\geq 99\%$ O_2 for up to 96 hours. The exposure of murine macrophage-like RAW 264.7 cells (American Type Culture Collection, Manassas, VA) was achieved in sealed, humidified Plexiglas chambers (Billups-Rothenberg, Inc., Del Mar, CA) flushed with 95% O_2 /5% CO_2 at 37°C . An oxygen analyzer (MSA; Medical Products, Pittsburgh, PA) was used to monitor the O_2 concentration in the chamber.

Phagocytosis Assay

The phagocytic activity of leukocytes was determined as described previously, with minor modifications (26). Male C57BL/6 mice were exposed to $\geq 99\%$ O_2 for 48 hours and treated with 50 $\mu\text{g}/\text{mouse}$ of either anti-HMGB1 mAb (α -HMGB1) or isotype control mAb, 24 hours before inoculation with PA and opsonized FITC-labeled latex beads (Polysciences, Warrington, PA). Isolated leukocytes were then treated with 0.04%

Figure 1. Antibody treatment protocol for mice with *Pseudomonas aeruginosa* (PA) infection. Male C57BL/6 mice were exposed to $\geq 99\%$ O_2 for 48 hours, followed by inoculation with PA (5×10^8 colony-forming units [CFUs]) via intranasal aspiration, and returned to 21% O_2 after inoculation. These mice were randomized to receive either neutralizing anti-HMGB1 (α -HMGB1) monoclonal antibody (mAb) or an isotypic control mAb intraperitoneally after 24 hours during hyperoxic exposure. Bronchoalveolar lavage (BAL) and lung tissues were harvested 24 hours after infection. The total protein content in BAL was measured using the bicinchoninic acid protein assay. The numbers of viable bacteria in the BAL and lungs were determined by plating serial dilutions of BAL and homogenized lung tissue. HMGB1, high mobility group box-1.

trypan blue and washed with cold (4°C) PBS to quench the extracellular adherent particles. To visualize the uptake of PA and FITC-labeled latex beads, leukocytes were fixed with 2% paraformaldehyde, washed with PBS, and stained with Texas Red X-phalloidin (Molecular Probes, Eugene, OR) in 1% BSA. The slides were analyzed using an epifluorescence microscope (Nikon, Melville, NY). The uptake of beads by leukocytes was quantified by counting at least 100 leukocytes per slide.

Measurement of HMGB1

Concentrations of HMGB1 in BAL were determined using an immunoblotting analysis with anti-HMGB1 antibody, as described previously (35). In brief, samples were separated on SDS-PAGE. Proteins were electrotransferred to a polyvinylidene difluoride membrane, and then blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween-20. The membrane was incubated with anti-HMGB1, and then with anti-rabbit horseradish peroxidase-coupled secondary antibodies (Bio-Rad, Hercules, CA). After washing, antibody binding was detected by enhanced chemiluminescence plus Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Western blots were scanned with a UVP Biospectrum 600 Imaging System (Vision Works LS, Upland, CA), and band intensities were quantified using Image acquisition and analysis software, version 6.8 (Vision Works LS, Upland, CA).

Quantitative Bacteriology

Viable bacterial counts in airways and lungs were determined using a CFU assay by plating serial dilutions of the BAL and lung homogenates, respectively, onto *Pseudomonas* Isolation Agar (Difco, Sparks, MD), and culturing at 37°C as already described.

Statistical Analysis

Results are presented as means $\pm$ SEMs from at least three independent experiments. The data were analyzed for statistical significance according to paired and unpaired *t* tests, ANOVA, or Kaplan-Meier analysis, using Microsoft Excel (Microsoft Corp., Seattle, WA). $P \leq 0.05$ was considered statistically significant.

RESULTS

Exposure to Hyperoxia Increases the Mortality of Mice with PA Pneumonia

Previously, we have shown that exposure to hyperoxia reduces the phagocytic ability of AMs to engulf PA (26). To determine

whether the hyperoxia-compromised phagocytic function of AMs affects an animal's host defense against PA infection, C57BL/6 mice were exposed to $\geq 99\%$ O₂ (hyperoxia) for 72 hours, and then intranasally inoculated with PA. Mice exposed to hyperoxia exhibited clinical signs of severe illness, including lethargy and huddling together in the corners of their cages (data not shown). Seventy percent of the mice in this group died within the first 4 hours after inoculation, and none had survived by the end of 24 hours (Figure 2). In contrast, mice remaining at 21% O₂ before inoculation with PA were relatively active, and all continued to survive 24 hours after infection (Figure 2). In addition, exposure to hyperoxia for up to 96 hours without PA infection did not induce any lethality in C57BL/6 mice (data not shown). These results indicate that exposure to hyperoxia not only reduces the ability of AM to phagocytose bacteria, but also compromises the survival of animals with PA infection.

Exposure to Hyperoxia Reduces Bacterial Clearance from the Lung in PA Pneumonia

To determine the mechanism underlying hyperoxia-increased mortality in PA pneumonia, bacterial loads in the lungs were assessed in mice exposed to $\geq 99\%$ O₂ (48 hours) and inoculated with PA. The bacterial load in lung lavage fluids was 6-fold greater ($149.33 \pm 37.92 \times 10^4$ CFUs/ml versus $24.91 \pm 6.92 \times 10^4$ CFUs/ml of BAL, $P < 0.01$; Figure 3A) in mice exposed to hyperoxia than in mice remaining at 21% O₂. Similar results were observed in the lung tissue of these mice ($61.77 \pm 9.51 \times 10^4$ CFUs/ml versus $8.78 \pm 2.16 \times 10^4$ CFUs/ml, $P < 0.01$; Figure 3B). These results indicate that mice exposed to hyperoxia have a compromised ability to clear PA from the lung and airways, suggesting that higher bacterial loads in the lung contribute to the increased mortality of these animals.

Exposure to Hyperoxia Increases Concentrations of Airway HMGB1

Recent studies in our laboratory revealed a novel role for extracellular HMGB1 in impairing the function of AMs in phagocytosis (30). To investigate whether extracellular HMGB1 is responsible for hyperoxia-suppressed bacterial clearance in PA pneumonia, concentrations of airway HMGB1 were determined in lung lavage

fluids isolated from mice exposed to hyperoxia. No concentration of HMGB1 was detectable in mice that remained at 21% O₂. However, exposure to hyperoxia resulted in an incremental elevation in concentrations of airway HMGB1 in the BAL of mice exposed to $\geq 99\%$ O₂ for up to 96 hours ($162\% \pm 8\%$, $262\% \pm 107\%$, $347\% \pm 103\%$, and $437\% \pm 73\%$ at 24 hours, 48 hours, 72 hours, and 96 hours, respectively; Figure 4).

Pretreatment with Neutralizing Anti-HMGB1 mAb Reduces Bacterial Load in the Lung and Ameliorates Acute Lung Injury

To determine whether elevated concentrations of airway HMGB1 play a causal role in hyperoxia-compromised bacterial clearance, neutralizing anti-HMGB1 mAb was administered to mice. During exposure to $\geq 99\%$ O₂, C57BL/6 male mice were treated with either neutralizing anti-HMGB1 mAb or isotype control mAb (control group) at 24 hours (Figure 1). Treatment with anti-HMGB1 mAb significantly reduced bacterial counts in the airways ($0.21 \pm 0.04 \times 10^6$ CFUs/ml versus $15.07 \pm 0.23 \times 10^6$ CFUs/ml of BAL, $P < 0.01$; Figure 5A) and in the lungs ($0.41 \pm 0.04 \times 10^6$ CFUs/ml versus $40.20 \pm 2.82 \times 10^6$ CFUs/ml, $P < 0.01$; Figure 5B). In addition, anti-HMGB1 mAb also significantly lowered the total protein content (a marker of lung injury) in the airways, compared with the total protein content of mice treated with control mAb ($6.46 \pm 1.7 \times 10^2$ μ g/ml versus $38.66 \pm 5.8 \times 10^2$ μ g/ml, $P < 0.01$; Figure 6A). These data suggest that HMGB1 plays a critical role in hyperoxia-impaired bacterial clearance in PA pneumonia and bacteria-elicited lung injury.

Pretreatment with Neutralizing Anti-HMGB1 mAb Reduces Numbers of Leukocytes in the Lung

Leukocyte recruitment is important in clearing bacterial infections from the lung (43, 47–49). To determine the cellular mechanisms underlying the HMGB1-mediated impairment of host defense in PA pneumonia, numbers of leukocytes in the lung were assessed. Histological examination showed fewer leukocytes in the lungs of mice treated with anti-HMGB1 mAb (Figure 6B). In addition, the numbers of leukocytes in lung lavage fluids were significantly reduced in mice treated with anti-HMGB1 mAb compared with control mice ($850.48 \pm 117.1 \times 10^4$ versus $107.69 \pm 36.75 \times 10^4$, $P < 0.01$; Figure 6C). Furthermore, numbers of neutrophils were markedly lower in airways of mice that received anti-HMGB1 mAb ($347.07 \pm 80.32 \times 10^4$ versus $35.51 \pm 15.97 \times 10^4$, $P < 0.01$; Figure 6C). Therefore, the improved host defense against bacterial infection in mice treated with anti-HMGB1 mAb is not associated with increased numbers of leukocytes, especially neutrophils, in the lungs.

Anti-HMGB1 mAb Attenuates Hyperoxia-Compromised Leukocyte Phagocytic Activity and Reduces the Concentrations of Airway HMGB1

To investigate further the potential cellular mechanisms underlying the improved host defense against PA infection with reduced numbers of neutrophils in the lungs of mice receiving anti-HMGB1 mAb, the phagocytic activity of lung leukocytes was assessed. The phagocytic activity of leukocytes in lung lavage fluids was significantly elevated in mice treated with anti-HMGB1 mAb, compared with control mice (0.42 ± 0.03 versus 13.28 ± 3.5 beads/cell, $P < 0.05$; Figure 7A). These data suggest that the HMGB1-mediated impairment of host defense is triggered by altering leukocyte function in bacterial clearance. Interestingly, the increased phagocytic activity of leukocytes in mice treated with anti-HMGB1 mAb was associated with a decreased accumulation

Figure 2. Exposure to hyperoxia increases the mortality of mice with bacterial pneumonia. Male C57BL/6 mice were exposed to $\geq 99\%$ O₂ for 72 hours (or 21% O₂ in control mice). After the exposure, mice in both groups were intranasally inoculated with PA (5×10^8 CFUs/mouse), returned to 21% O₂, and observed for survival up to 24 hours. Data were analyzed using Kaplan-Meier analysis ($n = 9$ mice per group). * $P < 0.05$, compared with 21% O₂/PA mice.

Figure 3. Exposure to hyperoxia reduces bacterial clearance in the lung. Male C57BL/6 mice were exposed to $\geq 99\%$ O₂ for 48 hours (or 21% O₂ in control mice), and then inoculated with PA (5×10^8 CFUs/mouse). Mice were killed 24 hours after inoculation, and the BAL and lungs were harvested. Viable bacteria in airways and lungs were quantified by plating serial dilutions of BAL (A) and homogenized lung (B), and are expressed as CFUs/ml of BAL and CFUs/lung, respectively. Data represent means $\pm$ SEMs from three independent experiments ($n = 9$ mice per group). ** $P < 0.01$, compared with 21% O₂/PA mice.

of airway HMGB1 (80% less than that of control mice, $P < 0.05$; Figure 7B). Previously, we demonstrated that AMs isolated from mice exposed to hyperoxia exhibit significantly reduced phagocytic activity (26). To determine whether elevated concentrations of extracellular HMGB1 contribute to the molecular mechanisms underlying hyperoxia-compromised macrophage function, the phagocytic activity and concentrations of extracellular HMGB1 were analyzed in cultured macrophages. The phagocytic activity of RAW 264.7 cells, after exposure to 95% O₂ for 24 hours, was reduced by approximately 3-fold (6.08 ± 0.21 versus 2.25 ± 0.11 , $P < 0.01$; Figure 7C), whereas concentrations of extracellular HMGB1 in the culture media were 35-fold higher compared with those of cells remaining at 21% O₂. These results suggest that HMGB1 mediates hyperoxia-impaired macrophage function and host defense in bacterial clearance during PA pneumonia.

DISCUSSION

We have previously shown that prolonged exposure to hyperoxia can compromise the function of macrophages to phagocytose PA. In this study, we further investigated the effects of hyperoxia on animal survival in PA pneumonia, and explored the potential underlying cellular and molecular mechanisms. We demonstrate that prolonged exposure to hyperoxia results in both a marked increase in animal mortality from PA pneumonia and an impaired host defense to effectively clear PA from the lung. Hyperoxia-compromised host defense was associated with a substantial elevation in concentrations of airway HMGB1. Interestingly, not only did treatment of mice with anti-HMGB1 mAb effectively reduce both leukocyte counts in the lung and the extent of inflammatory lung injury, but it also significantly enhanced bacterial clearance in the lung. The increased bacterial clearance in mice treated with anti-HMGB1 mAb was associated with both enhanced phagocytic activities in airway leukocytes and reduced concentrations of airway HMGB1. The critical role of extracellular HMGB1 in hyperoxia-compromised phagocytic activity was also confirmed in cultured macrophages. Thus, the experiments in this report indicate that HMGB1 mediates hyperoxia-impaired host defense against bacterial infection, at least in part, by inhibiting phagocyte functions.

Patients on MV are known to be more susceptible to bacterial infections and prone to develop VAP (6). Either the mechanical stretching caused by MV or exposure to the hyperoxia applied during MV may independently predispose subjects to VAP (28, 50). In this study, we examined the effects of pre-exposure to hyperoxia alone on animal survival in a murine model of PA pneumonia. The results presented in Figure 2 demonstrate that prolonged exposure to hyperoxia ($\geq 99\%$ O₂) can cause a marked increase in lethality of mice with PA pneumonia (100% versus 0% without hyperoxic exposure). Similarly, the exposure of animals with *Klebsiella* infection to hyperoxia was also reported to increase animal lethality significantly (28). Thus, prolonged exposure to hyperoxia alone can lead to fatal outcomes in bacterial

pneumonia. In addition, Kikuchi and colleagues reported that the PA infection of mice before exposure to 90% O₂ increased bacterial dissemination to organs other than the lung, such as the liver (51). Baleeiro and colleagues demonstrated that mice infected with *Klebsiella pneumoniae* during hyperoxic exposure ($> 95\%$ O₂) manifested an increased bacterial burden in both the lung and liver (28). Together, these studies suggest that hyperoxia directly contributes to adverse clinical outcomes, leading to the high mortality and morbidity observed in patients with VAP (5).

To determine the mechanisms underlying hyperoxia-increased mortality in PA pneumonia, we further investigated whether animals pre-exposed to hyperoxia can effectively clear PA from their lungs. The results presented in Figure 3 show that exposure to hyperoxia increased the bacterial burden in airways and lungs by more than 6-fold. The increased bacterial burden in lung lavage fluids of hyperoxia-exposed mice was accompanied by severe lung injury (Figure 6A). Hyperoxia-compromised bacterial clearance was also observed by others in several murine models of bacterial pneumonia (28, 52). Baleeiro and colleagues investigated the effects of exposure to 95% O₂ on innate immunity in animals with *K. pneumoniae* infection. In their study, mice were inoculated with *K. pneumoniae* on the third day of hyperoxic exposure, and the exposure was continued for another 24 hours after the inoculation (28). The authors observed that hyperoxia-exposed mice exhibited increased mortality, with an increased bacterial burden in the lungs, suggesting that hyperoxia was responsible for the reduced bacterial clearance. In another study, Dunn and Smith exposed mice to 100% O₂ for 48 hours and inoculated them with PA. They found that these

Figure 4. Exposure to hyperoxia increases concentrations of airway HMGB1. Male C57BL/6 mice were exposed to $\geq 99\%$ O₂ for different periods of time (24, 48, 72, and 96 hours). Mice were killed at the end of exposure to hyperoxia, and BAL fluids and lungs were harvested. HMGB1 concentrations in the BAL were measured by Western blot analysis. Data represent the means $\pm$ SEMs from three independent experiments ($n = 4-6$ mice per group). * $P < 0.05$, compared with mice remaining at 21% O₂.

Figure 5. Anti-HMGB1 mAb reduces the bacterial load in lungs during PA pneumonia. The experiment with antibody treatment was performed as described in Figure 1. Viable bacteria in the airways and lungs were quantified by plating serial dilutions of BAL (A) and homogenized lung (B), and are expressed as CFUs/ml of BAL and CFUs/lung, respectively. Data represent the means $\pm$ SEMs from three independent experiments ($n = 6$ mice per group). ** $P < 0.01$, compared with mice receiving control mAb.

mice manifested an elevated bacterial burden in their lungs, 4 hours after inoculation (52). Thus, the hyperoxia-increased mortality in mice with PA pneumonia is caused, at least in part, by impaired host defense to clear bacteria from both the airways and lungs.

Few published studies have sought to elucidate the molecular mechanisms underlying the observed deleterious effects of hyperoxia on bacterial pneumonia. The role of inflammatory cytokine TNF- α in host defense against bacterial infection has been examined under hyperoxic conditions (28, 53). The production of TNF- α was found to be profoundly suppressed in AMs isolated from mice exposed to hyperoxia and then subjected to *Klebsiella* or *Legionella* infection, suggesting that TNF- α contributes to bacterial clearance under hyperoxic conditions (28, 53). However, the administration of TNF- α did not improve bacterial clearance in hyperoxia-exposed animals (53). Thus, the role of proinflammatory cytokines in bacterial clearance among animals exposed to hyperoxia requires further investigation.

Here, we provide compelling evidence that the proinflammatory cytokine HMGB1 plays a critical role in hyperoxia-compromised host defense during PA pneumonia. The results shown in Figure 4 demonstrate that hyperoxia induces an accumulation of high concentrations of HMGB1 in murine airways. Interestingly, neutralizing HMGB1 in hyperoxia-exposed mice with an anti-HMGB1 mAb significantly increased bacterial clearance in both the airways and lungs by 75-fold and 100-fold, respectively (Figures 5A and 5B). The increased bacterial clearance was associated with substantially reduced concentrations of airway HMGB1 (80%; Figure 7B) in these mice. Thus, hyperoxia-induced high concentrations of airway HMGB1 contribute to the molecular mechanisms underlying hyperoxia-compromised bacterial clearance. In addition, PA infection without previous exposure to hyperoxia also increased concentrations of airway HMGB1, although it is difficult to quantify which treatment alone induces higher concentrations of HMGB1 release,

which is dependent on the dose and length of exposure (unpublished observations). Regardless, the combination of hyperoxia and PA induces the release of more HMGB1 than either treatment alone (unpublished observations). Interestingly, it takes more than 48 hours for patients on MV to develop VAP. This time window coincides with the significant increase in concentrations of airway HMGB1 among animals exposed to hyperoxia (Figure 4). HMGB1 is ubiquitously expressed and highly conserved throughout the animal kingdom, and has been shown to play essential roles in a number of animal models of inflammatory diseases as well as in human subjects (54). Recently, we demonstrated that high concentrations of airway HMGB1 in cystic fibrosis transmembrane conductance regulator (CFTR) $^{-/-}$ mice compromise the functions of alveolar macrophages against PA infection. Importantly, highly elevated concentrations of airway HMGB1 in lung lavage samples from patients with CF were also found to suppress macrophage functions (30). Thus, these findings suggest that the critical roles of airway HMGB1 in pathogenesis of PA infection in murine models may be applicable to human subjects. Taken together with the clinical observation of an increased incidence of bacterial infection in patients undergoing hyperoxia, our findings on the role of airway HMGB1 in host defense against PA infection warrants the recommendation to wean patients off of the relatively high concentrations of oxygen as quickly as possible.

We further explored the mechanisms underlying HMGB1-impaired host defense in PA infection under hyperoxic conditions. AMs play important roles as the first line of defense against invading pathogens in the lung (55, 56). However, AMs that were isolated from mice exposed to hyperoxia exhibited significantly decreased phagocytic activities (26, 28). Previous experiments performed in our laboratory, using antioxidants, demonstrated the essential role of ROS in hyperoxia-compromised phagocyte function (26). In that earlier study, experiments were designed to further explore downstream events leading to the macrophage dysfunction induced by oxygen toxicity. The results presented

Figure 6. Anti-HMGB1 mAb reduces lung injury and leukocyte infiltration into the lung. The experiment with antibody treatment was performed as described in Figure 1. The total protein content in the BAL (A) was measured as a marker of lung injury. Total leukocyte counts in the BAL (C) and in lung sections with hematoxylin and eosin staining (B) were analyzed for leukocyte infiltration. Arrows indicate leukocytes. Data represent the means $\pm$ SEMs from three independent experiments ($n = 4-9$ mice per group). Ab, antibody; PMNs, polymorphonuclear leukocytes. # $P < 0.05$, compared with 21% O_2 /PA mice. *** $P < 0.01$, compared with mice receiving control mAb.

Figure 7. Extracellular HMGB1 compromises the leukocyte function of phagocytosis. The experiment with antibody treatment was performed as described in Figure 1. Airway leukocytes were isolated from BAL samples. RAW 264.7 cells were exposed to 95% O₂ and incubated with fluorescent latex beads. Phagocytosis by leukocytes (*ex vivo*) (A) and phagocytosis by RAW cells (*in vitro*) (C) were expressed as beads/cell. At least 100 cells/slide were counted. HMGB1 concentrations in the BAL (B) and cell culture media (D) were measured by Western blot analysis. Data represent the means $\pm$ SEMs from three independent experiments ($n = 4-6$ mice per group). * $P < 0.05$, compared with mice receiving control mAb. ** $P < 0.01$, compared with cells remaining at 21% O₂ control level.

in Figure 4 demonstrate that concentrations of HMGB1 in airways of mice exposed to prolonged hyperoxia (48 h) were much higher than those of the mice remaining at 21% O₂. Similarly, the concentrations of extracellular HMGB1 were substantially increased (35-fold) in cultured macrophages exposed to hyperoxia (Figure 7D). We have recently shown that HMGB1 can directly impair the ability of macrophages to phagocytose PA (30). The association between the elevated concentrations of extracellular HMGB1 and the hyperoxia-compromised phagocytic ability of macrophages in both a murine model of PA infection and cultured macrophages (Figures 7C and 7D) implicates the role of HMGB1 in impairing phagocyte functions under hyperoxic conditions. The improved phagocytic function of leukocytes, including macrophages, in hyperoxic animals treated with anti-HMGB1 mAb helps to establish a causal role for HMGB1 in hyperoxia-induced phagocytic dysfunction. Indeed, enhanced phagocyte activities were associated with attenuated accumulations of HMGB1 in the airways of anti-HMGB1 mAb-treated mice (Figure 7B). These results demonstrate that hyperoxia-induced elevations in concentrations of airway HMGB1 significantly compromise phagocytic function, resulting in suppressed host defense, which can lead to increased pulmonary bacterial burden and mortality in animals with PA infection.

The inhibition of airway HMGB1 may also improve clinical outcomes by modulating neutrophilic inflammation with PA infection under hyperoxic conditions. Although the inflammatory response with leukocytic recruitment is important for the effective clearance of invading microorganisms (57–59), a prolonged presence of neutrophils in the lung may cause tissue injury by releasing excessive ROS and hydrolytic enzymes (43, 47–49). The roles of excessive neutrophilic accumulation in mediating hyperoxia-induced lung injury have been examined in both neonatal and adult animal models (60–62). The infiltration of neutrophils into the lung is directed by cytokines such as IL-8, one of the most studied cytokines (63). However, the upstream events leading to hyperoxia-mediated neutrophil recruitment are not well understood. HMGB1 is a potent chemoattractant for neutrophils (64). The intratracheal instillation of recombinant HMGB1 has been shown to markedly increase the secretion of IL-8 and cause lung injury with pronounced neutrophil infiltration (64). Blocking the α -chemokine receptor, one of the receptors for IL-8 (65), significantly attenuated HMGB1-mediated inflammatory lung injury by inhibiting neutrophil infiltration (45).

In addition, HMGB1 can hamper the removal of neutrophils by impairing the clearance of apoptotic neutrophils by macrophages (66). The accumulated apoptotic neutrophils can release more HMGB1 into the airways to attract more neutrophils to the lungs, establishing a vicious cycle that results in severe lung injury. In this study, we show that the exposure of mice to hyperoxia markedly induced concentrations of airway HMGB1 (Figure 4). The elevated concentrations of airway HMGB1 in hyperoxic animals may result in a significant accumulation of apoptotic neutrophils, further aggravating PA-elicited lung injury. Thus, inhibiting airway HMGB1 in these animals with neutralizing anti-HMGB1 mAb was anticipated to reduce neutrophil counts by inhibiting their recruitment and enhancing the clearance of apoptotic neutrophils. Indeed, inhibiting HMGB1 by the anti-HMGB1 mAb significantly reduced neutrophil counts and tissue injury in the lung (Figure 6). Therefore, these results suggest that HMGB1 plays an important role in mediating inflammatory lung injury in hyperoxic animals with PA infection.

In conclusion, the experiments in this report demonstrate that the elevation in concentrations of airway HMGB1 induced by prolonged exposure to hyperoxia is critical in both compromising host immunity against PA infection and mediating inflammatory lung injury. In addition to improving phagocytic function against bacterial infection, the inhibition of HMGB1 breaks the vicious cycle, wherein HMGB1 induces both neutrophil recruitment and the accumulation of apoptotic neutrophils that subsequently result in the further accumulation of HMGB1. Therefore, HMGB1 provides a novel target in modulating hyperoxia-compromised innate immunity, and understanding its regulation may help to devise therapeutic interventions for patients with VAP.

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