Sublethal Hyperoxia Impairs Pulmonary Innate Immunity

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*J Immunol* 2003; 171:955-963; doi: 10.4049/jimmunol.171.2.955

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Supplemental oxygen is often required in the treatment of critically ill patients. The impact of hyperoxia on pulmonary host defense is not well-established. We hypothesized that hyperoxia directly impairs pulmonary host defense, beyond effects on alveolar wall barrier function. C57BL/6 mice were kept in an atmosphere of >95% O2 for 4 days followed by return to room air. This exposure does not lead to mortality in mice subsequently returned to room air. Mice kept in room air served as controls. Mice were intratracheally inoculated with *Klebsiella pneumoniae* and followed for survival. Alveolar macrophages (AM) were harvested by bronchoalveolar lavage after 4 days of in vivo hyperoxia for ex vivo experiments. Mortality from pneumonia increased significantly in mice exposed to hyperoxia compared with infected mice in room air. Burden of organisms in the lung and dissemination of infection were increased in the hyperoxia group whereas accumulation of inflammatory cells in the lung was impaired. Hyperoxia alone had no impact on AM numbers, viability, or ability to phagocytize latex microbeads. However, following in vivo hyperoxia, AM phagocytosis and killing of Gram-negative bacteria and production of TNF-α and IL-6 in response to LPS were significantly reduced. AM surface expression of Toll-like receptor-4 was significantly decreased following in vivo hyperoxia. Thus sublethal hyperoxia increases Gram-negative bacterial pneumonia mortality and has a significant adverse effect on AM host defense function. Impaired AM function due to high concentrations of supplemental oxygen may contribute to the high rate of ventilator-associated pneumonia seen in critically ill patients.


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**Materials and Methods**

**Animals**

We obtained specific pathogen-free, 6- to 12-wk-old, wild-type C57BL/6 mice from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in isolator cages within the Animal Care Facilities at the Veterans Affairs Research Laboratories until the day of experimentation. The animal care

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Received for publication February 5, 2003. Accepted for publication May 14, 2003.

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1 This work was supported by grants (to R.P.) from the Department of Veterans Affairs (Veterans Affairs Merit Review) and the National Heart, Lung, and Blood Institute (HL 64558).

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3 Abbreviations used in this paper: AM, alveolar macrophage; IT, intratracheally; BAL, bronchoalveolar lavage; PI, phagocytic index; GI, growth index; MIP, macrophage inflammatory protein; m, murine; TLR, Toll-like receptor; FA, fluorescent Ab.
committee at the Veterans Affairs Medical Center (Ann Arbor, MI) approved the experimental protocols. Mice received food and water ad libitum.

Sublethal hyperoxia (hyperoxia)

C57BL/6 mice exposed to 95% oxygen for 4 days experienced little or no mortality and, if allowed to recover in room air, were indistinguishable from their matched controls. Mice were exposed to hyperoxia in a Flexiglas chamber with a continuous oxygen supply (Covox model 110; Reming Bioinstruments, Redfield, NY) in the Veterans Affairs Medical Center animal housing facility, where they continued to receive food and water as above and were checked daily for any evidence of disease. CO2 in the chamber was monitored and remained <5%.

Preparation of Klebsiella pneumoniae

K. pneumoniae strain 43816, serotype 2 (American Type Culture Collection, Manassas, VA) was used for these studies. This strain is known to produce clinically significant pneumonia in mice (26, 27). Bacteria were grown in tryptic soy broth (Difco, Detroit, MI) for 18 h at 37°C. The concentration of bacteria in broth was calculated by measuring absorbance at 600 nm. Standard values based on known CFU previously established in our laboratory were used to calculate inoculum concentration. Final concentration was confirmed by quantitative culture of the inoculum in serial 10-fold dilutions on soy-base blood-agar.

Inoculation of mice with K. pneumoniae

C57BL/6 mice were kept in standard conditions or in hyperoxia as described above. After 3 days in hyperoxia, mice were intratracheally (IT) inoculated with K. pneumoniae. Three days of exposure to hyperoxia was chosen as an interval that allowed return to hyperoxia after inoculation with minimal perioperative mortality. Mice kept under similar housing conditions in room air were used as controls. Mice were anesthetized with pentobarbital sodium (Abbott Laboratories, North Chicago, IL) at 50 mg/kg i.p. The trachea was exposed and K. pneumoniae, in a final volume of 50 μl in sterile normal saline solution, was inoculated using a 26-gauge needle. Uninfected control mice were inoculated with the same volume of sterile normal saline solution. The skin incision was closed with 4-0 surgical prolene with simple stitches. Mice were allowed to recover from the procedure and were returned to appropriate housing. Mice in the hyperoxia group were returned to hyperoxia to complete 4 days of total exposure. For the survival studies, mice in the hyperoxia were returned to room air after completion of 4 days of exposure and both groups were followed for survival for 14 days. For the survival studies, a dose of 5 × 10^5 CFU was used. Previous studies indicated this dose would cause intermediate mortality in control mice. For the CFU counts and in subsequent experiments, 10 5 cells per well, stained with a modified Wright-Giemsa stain (Diff-Quik; Baxter, McGaw Park, IL) for differential counts. From these stained cytospin preparations, the percentages of mononuclear cells and neutrophils were determined by microscopic counting of 200 cells per slide.

Metabolic activity of AM

AM were harvested by BAL as above and total AM counts were established for uninfected mice. Viability from AM was confirmed by trypan blue exclusion as a dye marker of cell membrane integrity. Harvested AM were plated on 96-well microtiter plates (Corning, New York, NY) at 1 × 10^5 cells per well, in DMEM (Life Technologies, Grand Island, NY), supplemented with penicillin-streptomycin (Life Technologies) and 10% FBS (Sigma-Aldrich, St. Louis, MO) (cell culture medium). AM were allowed to adhere for 45 min at 37°C and then gently washed with warm PBS to remove nonadherent cells. Cell viability was assessed with a commercially available MTT Cell Proliferation assay (Roche Diagnostics, Mannheim, Germany) (28). Adherent AM were coincubated in medium with MTT for 4 h at 37°C. Viable cells convert the MTT to formazan salts, which are not soluble. A solubilization solution (10% SDS in 0.1 M HCI) was added and cells were incubated overnight. Spectrophotometric absorbance of the samples was measured with an ELISA plate reader (550-nm wavelength filter) with OD values directly related to cell viability.

Ex vivo AM phagocytic ability

AM were harvested by BAL as above and plated in eight-well Lab-Tek slides (Nunc, Naperville, IL) at 1 × 10^5 cells/well in DMEM, allowed to adhere for 45 min at 37°C, and were then gently washed with warm PBS to remove nonadherent cells. Medium without penicillin-streptomycin was used for bacterial clearance experiments. To measure phagocytosis of FITC-labeled latex minibeads (Polysciences, Warrington, PA), minibeads in DMEM were added to each well at a concentration of 10^6 beads/well. After incubation for 1 h at 37°C, each well was gently washed with warm PBS to remove nonphagocytosed minibeads and fixed with methanol at −20°C followed by dehydration with acetone. Phagocytosis of bacteria was measured by adding BODIPY-labeled heat-killed Escherichia coli (Molecular Probes, Eugene, OR) in DMEM with 5% rat serum used as an opsonin, at a concentration of 10^5 bacteria/well. After incubation for 1 h at 37°C, wells were gently washed with warm PBS to remove nonphagocytized bacteria, and fixed as above. Trypan blue, at a concentration of 0.04%, was added to quench the fluorescence of extracellular beads and bacteria. Phagocytosis of either minibeads or bacteria was determined by microscopic counting of cells containing beads or bacteria in quadruplicate wells. Two-hundred consecutive cells were counted for each well. Results are expressed as percentage of AM-containing beads or bacteria and as a phagocytic index (PI), calculated from the number of phagocytosed beads or bacteria (PI = (proportion of AM with particles) × (mean number of particles per positive AM)) (18). The PI is directly and representatively indicative of both the number of AM containing the phagocytic target as well as the mean number of phagocytosed particles per AM. The PI incorporates both parameters into a single index.

AM killing of K. pneumoniae

AM were harvested by BAL, resuspended in DMEM without penicillin-streptomycin and plated on eight-well Lab-Tek slides (Nunc) as described above. AM (1 × 10^5 cells/well) were allowed to adhere for 45 min at 37°C and then washed with warm PBS to remove nonadherent cells. For determination of killing of K. pneumoniae, AM were coincubated for 1 h with K. pneumoniae at a ratio of 10 bacteria:AM. Wells were gently washed with warm PBS to remove nonphagocytosed bacteria. AM were lysed with sterile distilled water and serial 10-fold dilutions were made to 10^-8. Ten microliters of each dilution were plated on soy-base blood agar plates (Difco) and incubated for 24 h at 37°C. Colony counts were then determined for each well. Bacterial growth was assessed from the number of live bacteria associated with AM normalized for the PI and expressed as a bacterial growth index (GI) according to the following formula: GI = (bacterial colonies per milliliter AM cell lysate/PI) × 100 (29). Thus, the GI normalizes the number of viable organisms recovered from the AM (CFU) to the relative number of organisms initially taken up by the AM (PI) and provides an indicator of intracellular bacterial killing.

Cytokine and chemokine measurements

For measurement of ex vivo production of cytokines, AM were harvested as above, resuspended in DMEM, plated on 96-well plastic plates (Corning) at 1 × 10^5 cells per well, allowed to adhere for 45 min at 37°C and washed with warm PBS to remove nonadherent cells. AM were incubated overnight in DMEM with penicillin-streptomycin with or without LPS (1.25 μg/ml, derived from E. coli O26:B6; Sigma-Aldrich) at doses of 1, 10, and 100 ng/ml. After 24 h in culture, cell-free supernatants were collected,
centrifuged to remove cellular debris, and assayed for cytokine levels by ELISA. For in vivo measurement of cytokines and chemokines, cell-free BAL fluid, whole lungs and serum were collected from infected and uninfected mice from hyperoxia and control. Whole lungs were harvested and homogenized with a tissue homogenizer (Biospec Products) in 500 μl of a solution of complete Protease Inhibitor (50 μl of PBS; Roche Diagnostics). We measured the levels of TNF-α, IL-6, IL-10, macrophage inflammatory protein (MIP)-2, and KC from in vivo samples using murine Quantikine kits (R&D Systems, Minneapolis, MN), as directed by the manufacturer. TGF-β was measured from the same samples using a Quantikine kit (R&D Systems), after treatment of samples with HCl to activate latent TGF-β for measurement of total levels, according to the manufacturer’s protocol.

Measurement of LPS-induced TNF-α mRNA
AM were harvested by BAL as above, resuspended in DMEM, plated on 96-well plastic plates (Corning) at 1 × 10^5 cells per well, allowed to adhere for 45 min at 37°C and washed with warm PBS to remove nonadherent cells. AM were incubated in DMEM with and without LPS (100 ng/ml) for 1 h. Supernatants were harvested and RNA was extracted from AM using a commercial TRizol reagent (Life Technologies) for a total of 5 × 10^5 cells per condition. Measurement of gene expression was performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers and probes for β-actin and TNF-α were designed using Shortcat to Primer Express software (Applied Biosystems). The primers, placed in different exons, were confirmed not to amplify genomic DNA. Primer and probe nucleotide sequences for murine (m) TNF-α (GenBank accession number M21555) were: forward primer 5'-CAG CGG ATG GGT TGT ACC TT-3', reverse primer 5'-TGT GGG TGA GGA ATG GGT TGT ACC TT-3', TaqMan probe 5'-FAM-TCC CAG GTT CTC TTC GCA CGT AGT-3', forward primer 5'-AAAG GGA CAA GGC-(TAMRA)-3'. Primer and probe nucleotide sequences for mβ-actin (GenBank accession number M12481) were: forward primer 5'-CCG TGA AAA GAT GAC CCA GAT C-3', reverse primer 5'-CAC AGC CTG GCC TAC GT-3', TaqMan probe 5'-(FAM)-TTT GAG ACC TTC AAC ACC CCA GCC A-TAMRA-3' (Applied Biosystems). Thermal cycling parameters for use with the TaqMan One-Step RT-PCR Master Mix Reagents kit included systems Custom Oligo Synthesis Service). Thermal cycling parameters for use with the TaqMan One-Step RT-PCR Master Mix Reagents kit included 30 min at 48°C, 10 min at 95°C, and 40 cycles involving denaturation at 95°C for 15 s, annealing/extension at 60°C for 1 min. These experiments were performed in duplicate. To normalize the amount of total RNA present in each reaction, the housekeeping gene β-actin was amplified. The amount of TNF-α mRNA was measured by normalizing it to β-actin and relative to a calibrator. This method is based on the assumption that the target TNF-α and β-actin display equal amplification efficiencies. To verify this, variations were assessed according to template dilution. To this end, a standard curve was generated composed of five different dilutions of total RNA, corresponding to 50, 25, 12.5, 6.25, and 3.125 ng. The slope of this curve was 0.0989. To assure the appropriate amplification efficiency, the slope of the standard curve should be <0.1.

Surface expression of Toll-like receptor (TLR)-4 on AM
AM were harvested by BAL as above and resuspended at 1 × 10^5 cells per 100 μl in cold fluorescent Ab (FA) buffer (Difco) containing 1% BSA and 0.1% sodium azide. AM were incubated for 30 min with PE-labeled anti-μm TLR-4 (BD PharMingen, San Diego, CA) or a nonspecific PE-labeled IgG2b (BD PharMingen, San Diego, CA) both diluted to a final concentration of 0.4 μg/100 μl (30). AM were washed with FA buffer to remove nonadherent Abs and fixed with FA buffer containing 1% formaldehyde. Flow cytometry was performed using a FACScan flow cytometer (BD Biosciences, Mountain View, CA); 20,000 events were counted for each condition and analysis was done using the CellQuest software package (BD Biosciences). The complete cell population was gated to include the AM. Thresholds for positive staining were determined from the isotype-matched treated samples from within the AM population. The thresholds were established visually from each individual control and used to calculate the percentage of treated AM above this threshold. Results are described as the percentage of AM with TLR-4 expression above this threshold.

Statistical analysis
Survival data were analyzed using a two-tailed Fischer’s test. All other data are expressed as means ± SEM. Data were compared using a two-tailed Student’s t test or ANOVA, if more than two groups were compared, with the InStat software package from GraphPad Software (San Diego, CA). Differences were considered statistically significant if p values were <0.05.

Results
Mortality due to bacterial pneumonia
We developed a model of sublethal hyperoxia to determine the impact of hyperoxia on susceptibility to lethal bacterial Gram-negative pneumonia. C57BL/6 mice in the hyperoxia group and matched controls in room air were IT inoculated with K. pneumoniae or saline and both groups were followed for survival. Infected mice in room air and uninfected mice in the hyperoxia group served as controls. Mortality in the uninfected mice in the hyperoxia group was not different from uninfected mice in room air (data not shown). Mortality was significantly increased in the infected mice in the hyperoxia group (Fig. 1) compared with infected mice in room air. Six days after inoculation, 52% of the infected mice in room air had died, while 92% of the mice in hyperoxia had died. Thus, prior exposure to hyperoxia resulted in greatly increased mortality in mice inoculated with K. pneumoniae.

In vivo clearance of K. pneumoniae and dissemination of infection
Mortality associated with pneumonia was significantly increased following hyperoxia. Infected mice in the hyperoxia group appeared ill, were less active, and huddled together. We performed experiments to explore the cause of increased mortality. Pulmonary burden of organisms was determined from lungs harvested after IT inoculation with K. pneumoniae. Lung burden of infection was greater in the hyperoxia group at all time points. CPU counts of K. pneumoniae from lungs of mice in the hyperoxia group were increased by 2.5 log when compared with those from mice in room air (Fig. 2A). Infected mice in the hyperoxia group also had a much higher rate of dissemination of infection from the lung as indicated by culture of organisms from spleens. One-hundred percent of infected mice in the hyperoxia group had positive spleen cultures while only 40% of the mice in the room air group had positive

FIGURE 1. Mortality due to bacterial pneumonia. Mice kept in hyperoxia for a total of 4 days and matched controls in room air were IT-inoculated with K. pneumoniae on day 3. Mice in the hyperoxia group were returned to hyperoxia to complete 4 days of total hyperoxia exposure and were returned to room air. Both groups were followed for survival. Mortality was significantly increased in the hyperoxia group. Fifty-two percent of the infected mice in room air compared with 92% of the hyperoxia group mice had died, * p = 0.01 by two-sided Fischer’s test on day 6 compared with room air group (25 mice in each group; representative experiment of four experiments).
cultures (Fig. 2B). Thus, prior exposure to hyperoxia was associated with increased pulmonary burden of organisms and septic dissemination of infection in the setting of bacterial pneumonia.

**Inflammatory cell accumulation in response to K. pneumonia**

Recruitment and accumulation of inflammatory cells are essential events orchestrated by the pulmonary innate immune system to contain and clear infection. We performed BAL in mice from the hyperoxia and room air groups to determine the number of inflammatory cells 12 and 24 h after IT inoculation with *K. pneumoniae*. Mice in the hyperoxia group had a blunted response to infection, with decreased numbers of inflammatory cells. The number of mononuclear cells and macrophages in BAL increased significantly in the room air group but failed to increase in the hyperoxia group (Fig. 3). Mononuclear cells were 1.7-fold more numerous in the room air group at 24 h. Similarly, the number of neutrophils in BAL increased significantly more in the room air group compared with the mice in hyperoxia. Neutrophil counts were 1.8-fold greater in the room air group at 24 h. Thus prior exposure to hyperoxia caused a modest but significant impairment of inflammatory cell accumulation in response to pneumonia.

**Expression of chemokines**

We performed experiments to determine the impact of sublethal hyperoxia on the expression of the potent neutrophil chemoattractants MIP-2 and KC after we demonstrated that inflammatory cell accumulation in the lungs was impaired following hyperoxia. Following IT inoculation with *K. pneumoniae*, significant levels of MIP-2 and KC were recovered from lung homogenates. MIP-2 expression was significantly reduced by prior exposure to hyperoxia, while KC expression was not changed by this exposure (Fig. 4). Exposure to hyperoxia resulted in both decreased expression of a potent neutrophil chemoattractant and reduced neutrophil accumulation in response to bacterial pneumonia.

**Anti-inflammatory cytokines**

The impaired accumulation of inflammatory cells was associated with decreased expression of a neutrophil chemoattractant. Anti-inflammatory cytokines such as IL-10 and TGF-β are known to impact AM production of proinflammatory cytokines and might account for these findings. Levels of IL-10 and TGF-β were measured in BAL fluid, total lung homogenates and serum from mice from the hyperoxia and room air groups with and without IT inoculation with *K. pneumoniae*. Levels of IL-10 and TGF-β were not significantly altered following exposure to in vivo hyperoxia (data not shown).
Number and viability of AM

The decreased accumulation of inflammatory cells in response to infection might be a result of loss of viable AM due to increased cell injury or death. BAL was performed in uninfected mice at 2, 3, and 4 days of hyperoxia exposure and in matched controls in room air. The number of AM recovered by BAL did not change after exposure to hyperoxia for up to 4 days (Fig. 5A). We confirmed >95% viability of AM from mice in the hyperoxia group by trypan blue exclusion and their metabolic activity was confirmed with a MTT assay following exposure to hyperoxia for up to 4 days (days 2 and 3 not shown) were not significantly changed. Trypan blue exclusion confirmed >95% viability of AM from mice in the hyperoxia group harvested by BAL and their metabolic activity was confirmed with a MTT assay following exposure to hyperoxia for up to 4 days (days 2 and 3 not shown). B, Conversion of MTT to formazan as a marker of metabolic activity was not different between the two groups (data shown as raw OD). Total BAL cell counts grouped for 80 mice per group. MTT assay was performed in duplicate for five mice per group, repeated three times.

AM phagocytosis and bactericidal activity

To assess AM phagocytic activity following exposure to in vivo hyperoxia, AM were incubated ex vivo with FITC-labeled latex minibeads or bacteria. AM phagocytic ability was calculated as both the percentage of AM containing phagocytized particles (beads or bacteria), as well as a PI. AM phagocytosis of latex minibeads was not significantly different between mice in the hyperoxia and the room air groups (data not shown). However, phagocytosis of bacteria was significantly impaired in the hyperoxia group compared with the room air group, with a reduction in both the percentage of AM with bacteria and the PI (Fig. 6). Thus, in vivo hyperoxia resulted in a selective defect in phagocytosis of bacteria ex vivo. To determine the effect of hyperoxia on bacterial killing, AM were coincubated ex vivo with K. pneumoniae. The bacterial CFU counts recovered from the AM from the hyperoxia group were greater than the bacterial CFU counts recovered from the AM from the room air group, even though there were more bacteria initially taken up by the AM from the room air group. Therefore, bacterial growth, calculated based on PI and the recovered CFU, was significantly higher in cultures of AM from mice exposed to hyperoxia compared with room air controls. Even though control AM took up more bacteria these bacteria proved less viable than those taken up by AM from the hyperoxia group.
indicating impaired bacterial killing ex vivo by AM following in vivo exposure to hyperoxia (Fig. 6C).

**FIGURE 6.** Phagocytosis and killing of bacteria ex vivo. AM were incubated ex vivo with heat-killed BODIPY-labeled *E. coli* with rat serum as an opsonin. Phagocytosis of opsonized bacteria was expressed as the percentage of AM containing bacteria (A) as well as a PI (B). AM phagocytosis of bacteria was significantly impaired in the hyperoxia group (*, p < 0.01 for both percentage of cells containing bacteria and PI). AM were incubated ex vivo with *K. pneumoniae* with rat serum as an opsonin. More viable organisms were recovered when *K. pneumoniae* was coincubated with AM from the hyperoxia group (C). AM killing ability was expressed as a bacterial GI based on recovered CFU and PI for phagocytosis of bacteria (GI = ([bacterial colonies per milliliter AM cell lysate]/PI) × 100). *, p = 0.004 compared with the room air group.

**Ex vivo production of TNF-α and IL-6**

AM are responsible for the production of early response proinflammatory cytokines such as TNF-α and IL-6 during initiation of the inflammatory cascade. AM harvested from mice in room air or hyperoxia were stimulated with increasing concentrations of LPS, and protein concentrations of TNF-α and IL-6 in the culture supernatants were measured after 24 h. As expected, LPS induced significant cytokine production in AM from mice in the room air group. In contrast, AM production of TNF-α and IL-6 were profoundly suppressed following in vivo exposure to hyperoxia (Fig. 7). Thus, in vivo exposure to hyperoxia led to significant impairment in the ability of AMs to generate early inflammatory mediators.

**FIGURE 7.** Ex vivo production of proinflammatory cytokines. LPS-induced TNF-α production by AM was measured ex vivo following in vivo hyperoxia. Production of TNF-α by AM from the room air group increased appropriately in response to LPS but did not change in AM from the hyperoxia group (A). *, p < 0.0001 compared with room air group. Similarly LPS-induced production of IL-6 by AM was impaired in the hyperoxia group (B). *, p = 0.0009 compared with room air group. (Five mice per group, duplicate wells. Experiments repeated five times).

**Levels of TNF-α mRNA**

To determine whether impaired TNF-α protein secretion was a reflection of changes in the level of mRNA expression, we measured the levels of TNF-α mRNA produced by AM in response to LPS with real-time PCR. Levels of TNF-α mRNA in AM from mice in room air were significantly increased in response to LPS. However, TNF-α mRNA in AM from mice exposed to hyperoxia did not increase in response to LPS (Fig. 8). This suggested that the defect in AM was at the level of transcription or further upstream along the signaling pathway responsible for response to LPS.

**Expression of TLR-4**

LPS-induced production of the proinflammatory cytokines TNF-α and IL-6 by AM is initiated via the surface pattern recognition receptor TLR-4, heterodimerized with the nontransmembrane protein MD2. We measured expression of TLR-4/MD2 on the surface of AM from mice on room air and mice exposed to hyperoxia (representative data are shown in Fig. 9). The percentage of AM expressing TLR-4/MD2 at levels above threshold for positivity generated from isotype control Ab was significantly lower after in
vivo exposure to hyperoxia (55% ± 12 vs 12% ± 3 for three replicate experiments). This suggests that the decreased response to LPS by AM from mice exposed to hyperoxia was due to decreased expression of TLR-4 by these AM.

Discussion
We have demonstrated that exposure to sublethal hyperoxia impairs pulmonary innate immunity, rendering mice more susceptible to mortality from Gram-negative bacterial pneumonia. Mice exposed to hyperoxia had significantly increased mortality due to bacterial pneumonia with *K. pneumoniae*, with a higher lung burden of organisms and increased extrapulmonary septic dissemination of infection. Sublethal hyperoxia resulted in reduction in chemokine production and inflammatory cell accumulation in the lung. Ex vivo studies demonstrated moderately impaired bacterial killing and greatly impaired LPS-induced production of proinflammatory cytokines by AM following in vivo hyperoxia. The failure to respond to LPS was associated with decreased AM TLR-4/MD2 expression.

The pulmonary alveolar space is exposed to relatively high tensions of oxygen. Despite a variety of protective mechanisms, breathing high concentrations of oxygen for extended periods of time results in acute lung injury (31, 32). The sequence of events leading to acute lung injury after exposure to hyperoxia has been described in several different models and the focus of most studies has been on the endothelial cells and epithelial cells of the alveolar wall (7–9, 11, 12). A number of different growth factors and cytokines (IL-6, IL-11, IL-13, keratinocyte growth factor and vascular endothelial cell growth factor) can modify this injury response to hyperoxia (33–39). TNF-α has been found to have a complex role in the pathogenesis of acute lung injury due to hyperoxia (40–42). However, much less is known about the impact of in vivo hyperoxia on pulmonary innate immunity and AM function.

We have established a model of sublethal hyperoxia, with limited morbidity and mortality from hyperoxia-induced acute lung injury, but with reproducible effects on AM function. C57BL/6 mice maintained in >95% hyperoxia for 4 days have no mortality and recover without any significant permanent lung injury. We used this approach to model exposure to high oxygen tension in critically ill patients with respiratory failure not caused by pneumonia. This model allows for study of the effects of hyperoxia on pulmonary innate immunity but without inducing severe acute lung injury leading to morbidity or mortality.

*Klebsiella pneumoniae* was selected as the agent of experimental bacterial pneumonia because of its clinical relevance and well-established role as an infectious agent with macrophage-dependent clearance. *K. pneumoniae* is a causative agent of acute community-acquired pneumonia and is also a frequent etiologic agent of nosocomial pneumonia. Mice are very susceptible to pneumonia by *K. pneumoniae* and develop significant disease with a small inoculum. Finally, the role of AM is recognized in clearance of *K. pneumoniae*, making it an organism whose pathogenicity is clearly dependent on pulmonary cellular innate immunity (43–45).

Neutrophil accumulation was impaired in pneumonia following in vivo exposure to hyperoxia. CXC chemokines induce neutrophil chemotaxis and stimulate neutrophil activation in pulmonary inflammatory responses. MIP-2 and KC are ligands for CXCR2 in the mouse, and are the murine counterparts of human IL-8, growth-related oncogene-α, β, and epithelial cell-derived neutrophil attractant-78 (46). MIP-2 and KC can be produced by a variety of cells in the lungs including AM and recruited neutrophils. Neutralization of either chemokine alone can lead to significant reductions in neutrophil recruitment (16, 17). Prior exposure to in vivo hyperoxia was associated with a significant reduction in levels of
MIP-2 in the lungs of infected mice. Although chemotaxis mediated through CXC chemokine receptor CXCR2 is a redundant system, this reduction in levels of MIP-2 may help account for the decreased accumulation of inflammatory cells in response to pneumonia and their inability to contain the infectious process after exposure to hyperoxia.

We have focused on AM function in these studies because these cells play a pivotal role in the pulmonary innate immune response. The increase in mortality with bacterial Gram-negative pneumonia in our model was associated with impairment of both phagocytic and regulatory functions of AM. Following exposure to in vivo hyperoxia, AM were less effective in their ability to phagocytize and kill bacteria. Perhaps more importantly, AM from mice exposed to sublethal hyperoxia displayed a profound defect in production of early proinflammatory mediators responsible for the activation of local and systemic inflammatory responses. TNF-α is one of the earliest and most important cytokines produced in response to infection and has a well-established pivotal role in pulmonary immunity (47, 48). Decreased production or blockade of TNF-α lead to poor outcomes in bacterial pneumonia. IL-6 is also an important early cytokine produced by AM in response to infection, with kinetics that parallel those of TNF-α (49).

Prior exposure to hyperoxia led to a dramatic reduction in the ability of AM to generate TNF-α and IL-6 in response to LPS. We believe this defect is a main factor leading to the increase in mortality in our model. This reduction was regulated at the level of mRNA, and its mechanism appears to involve decreased cell surface expression of TLR-4/MD2 by AM after exposure to in vivo hyperoxia. TLR-4 expression is critical for normal host defense against Gram-negative organisms. Naturally occurring TLR-4 mutant mice and transgenic TLR-4 null mice have increased susceptibility to respiratory and systemic infections with Gram-negative bacteria (50–53). We now present the novel observation that AM expression of TLR-4/MD2 is modulated by in vivo hyperoxia. This decreased expression is likely to be an important mechanism explaining the ability of hyperoxia to increase susceptibility to lethal Gram-negative pneumonia.

The precise underlying mechanism for these defects of AM function remains unclear. In contrast to our work, previous studies have described increased proinflammatory responses and particularly in LPS-induced TNF-α production by AM following in vitro hyperoxia (19, 22, 54). These results are quite different from our finding of impaired LPS-induced cytokine production. Our model uses in vivo exposure to hyperoxia, which might have effects on the alveolar milieu beyond direct effects on AM. Furthermore, our model allows for a more prolonged exposure to hyperoxia, which may account for the different responses by AM. Although release of anti-inflammatory mediators could account for some of our findings, we found no significant change in the levels of IL-10 or TGF-β. Hyperoxia induces a broad spectrum of changes in AM function without impacting their viability. Harvested AM are still viable and metabolically active, so that cell death is not a likely explanation for our findings. Hyperoxia has direct effects on the alveolar epithelium, which nourishes and supports AM. We postulate that loss of supportive signals from alveolar epithelial cell may contribute to the impaired AM function.

The phenotype of the AM is clearly altered following in vivo hyperoxia, with greatly reduced expression of TLR-4. Many of the changes observed on AM function are similar to those seen in AM from transgenic mice deficient in GM-CSF, GM-CSF receptor, or PU.1. a GM-CSF-dependent transcription factor that is essential for AM maturation (29, 55, 56). Exposure to in vivo hyperoxia alone did not decrease protein levels of PU.1 in AM compared with AM harvested from mice in room air (data not shown). This suggests a more complex interaction between these mediators leading to the observed immunosuppressed phenotype.

High tensions of supplemental oxygen are commonly used in the supportive care of critically ill patients. Nosocomial pneumonia frequently affects these critically ill, vulnerable patients, prolonging intensive care unit stays, increasing the number of days on mechanical ventilation, and carrying significant associated morbidity and mortality. Pneumonia is the major cause of death due to infectious diseases in the U.S. and nosocomial pneumonia is the leading cause of mortality due to nosocomial infections (57, 58). Several risk factors for nosocomial pneumonia are recognized, with interest focused on aspects such as endotracheal tubes, ventilators and bacterial biofilm, as well as the effects of systemic diseases, such as sepsis on pulmonary defenses (59–62). However, high tension of oxygen alone is not a well-established risk factor for nosocomial pneumonia. Our findings suggest that high concentrations of supplemental oxygen may impair pulmonary innate immunity contributing to increased risk of nosocomial bacterial pneumonia in critically ill patients.

References


