Hyperoxia Mediates Acute Lung Injury and Increased Lethality in Murine *Legionella* Pneumonia: The Role of Apoptosis

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Hyperoxia Mediates Acute Lung Injury and Increased Lethality in Murine Legionella Pneumonia: The Role of Apoptosis

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Legionella pneumophila is a major cause of life-threatening pneumonia, which is characterized by a high incidence of acute lung injury and resultant severe hypoxemia. Mechanical ventilation using high oxygen concentrations is often required in the treatment of patients with L. pneumophila pneumonia. Unfortunately, oxygen itself may propagate various forms of tissue damage, including acute lung injury. The effect of hyperoxia as a cofactor in the course of L. pneumophila pneumonia is poorly understood. In this study, we show that exposure to hyperoxic conditions during the evolution of pneumonia results in a marked increase in lethality in mice with Legionella pneumonia. The enhanced lethality was associated with an increase in lung permeability, but not changes in either lung bacterial burden or leukocyte accumulation. Interestingly, accelerated apoptosis as evidenced by assessment of histone-DNA fragments and caspase-3 activity were noted in the infected lungs of mice exposed to hyperoxia. TUNEL staining of infected lung sections demonstrated increased apoptosis in hyperoxic mice, predominantly in macrophages and alveolar epithelial cells. In vitro exposure of primary murine alveolar epithelial cells to Legionella in conjunction with hyperoxia accelerated apoptosis and loss of barrier function. Fas-deficient mice demonstrated partial resistance to the lethal effects of Legionella infection induced by hyperoxia, which was associated with attenuated apoptosis in the lung. These results demonstrate that hyperoxia serves as an important cofactor for the development of acute lung injury and lethality in L. pneumophila pneumonia. Exaggerated apoptosis, in part through Fas-mediated signaling, may accelerate hyperoxia-induced acute lung injury in Legionella pneumonia. The Journal of Immunology, 2003, 170: 4209–4216.

Legionella pneumophila is a Gram-negative intracellular pathogen that often causes a serious and life-threatening pneumonia (1, 2). Potentially lethal complications, including acute lung injury and acute respiratory distress syndrome, are frequent consequences in these patients (3, 4), although the exact pathogenesis of lung injury in Legionella disease is still poorly understood. Despite aggressive supportive care, including antibiotic therapy and oxygen supplementation, high mortality rates reaching 50% have been reported, especially in immunocompromised patients (3, 5, 6).

In lung tissue, bacteria multiply in several types of host cells, including macrophages, monocytes, and alveolar epithelial cells (7–9). Cytopathogenicity of L. pneumophila to host cells has been well demonstrated (10–12), although incompletely understood. Accumulating data indicate that L. pneumophila can induce apoptosis in macrophages and alveolar epithelial cells in vitro (13–15). Apoptosis is a highly regulated process of cell death that is required for development and homeostasis of multicellular organisms in physiological condition (16, 17). The apoptosis-inducing receptors (death receptors), such as TNFR (p55 and p75) and Fas, bind TNF-α and Fas ligand (death factors), respectively, which result in activation of downstream signaling molecules, including caspases and phospholipases. Fas-mediated signal appears to exclusively drive apoptosis, whereas TNFRs are capable of both activating cells, and eliminating cells by initiating apoptosis (18, 19). Several investigators have reported critical roles of death receptor-mediated apoptosis for modulation of host responses in certain infectious diseases (20, 21). A wide variety of pathogens modulate the host cell-death pathway by direct interaction with key components of the apoptosis machinery of the host (22–24). However, how apoptosis is involved in the pathogenesis of Legionella disease is still unknown.

Oxygen supplementation is commonly given to the patients with severe pneumonia, including Legionella disease. This is a critical supportive therapy, especially for the patients demonstrating severe hypoxemia. However, prolonged administration or even transient supplementation of oxygen can promote lung damage (25–28). Cells at risk for hyperoxia-induced injury include alveolar epithelial cell and lung microvascular endothelial cells. Although mechanisms of oxygen toxicity to the lungs have not been carefully defined, it is likely that apoptosis plays a certain role in hyperoxia-associated lung injury (27, 29). Moreover, the effect of hyperoxia as a cofactor in the development of acute lung injury in bacterial pneumonia is unknown.

We examined the interaction between hyperoxia and L. pneumophila infection in a murine model of pneumonia. Our findings indicate that hyperoxia markedly increases mortality in mice with L. pneumophila pneumonia. The enhanced lethality is associated with acceleration of acute lung injury, but not increases in bacterial burden in the lungs. Interestingly, hyperoxia enhances apoptosis in the lungs of animals with Legionella pneumonia. It is likely that death receptor-mediated signaling is involved in the acceleration of apoptosis in hyperoxic conditions, because an increase in survival was observed in Fas-deficient mice with Legionella pneumonia in the setting of hyperoxia.
Materials and Methods

Animals

Female specific pathogen-free 4- to 6-wk-old A/J, C57BL/6, and B6.MRL-Fasl−/+ (Fas-deficient mice) were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in specific pathogen-free conditions within the animal care facility at the University of Michigan until the day of sacrifice.

Culture and inoculation of bacteria

We used a clinical isolate of *L. pneumophila* Suzuki strain (serogroup 1) for all experiments. N-(2-acetamido)-2-aminothanesulfonic acid (Sigma-Aldrich, St. Louis, MO)-buffered yeast extract (BYE) broth supplemented with l-cysteine (0.4 mg/ml) and ferric nitrate (0.135 mg/ml) was used as liquid medium (BYE-broth). To prepare solid medium, activated charcoal (2 mg/ml) and agar (15 mg/ml) were added to liquid medium (buffered charcoal yeast extract-agar). *L. pneumophila* was incubated on buffered charcoal yeast extract-agar for 4 days at 37°C. A single colony was transferred to 3 ml of BYE-broth, and was then incubated overnight at 37°C with constant shaking. Bacterial suspension was again transferred to fresh BYE-broth, and incubated overnight in the same condition. According to a standard of absorbencies based on known CFU, the bacterial suspension was diluted to the desired concentration in saline. Animals were anesthetized i.p. with 6 and 100 mg/kg xylazine and ketamine, respectively. The trachea was exposed, and 30 μl of inoculum or saline was administered via a sterile 26-gauge needle. The skin incision was closed with surgical staples.

Oxygen exposure

Two hours after intratracheal (i.t.) administration of bacteria, one group of mice was kept in hypoxic conditions in a 50 × 30 × 30 cm airtight chamber for 60 h, whereas another group was placed in room air conditions. For hypoxic exposure, the oxygen concentration in the chamber was kept between 90 and 94% by a constant flow of gas, which was monitored with an in-line oxygen analyzer (model D2; Beckman, Fullerton, CA). Carbon dioxide levels in the chamber were maintained at ~0.03–0.04% during the course of experiments. Both groups of mice were fed food and water ad lib and kept on a 12-h dark-light cycle at room temperature.

Lung harvesting for analysis

At designated time points, mice were sacrificed by CO2 asphyxia. Before lung removal, the pulmonary vasculature was perfused with 1 ml of PBS containing 5 mM EDTA, via the right ventricle. Whole lungs were then harvested for assessment of bacterial number, cytokine protein expression, and apoptosis. After removal, whole lungs were homogenized in 1.0 ml of PBS with protease inhibitor (Boehringer Mannheim, Indianapolis, IN) using a tissue homogenizer (Biospec Products, Bartlesville, OK) under a vented hood. Portions of homogenates (10 μl) were inoculated on agar after serial 1/10 dilutions with PBS. The remaining homogenates were incubated on ice for 30 min, then centrifuged at 2500 rpm for 10 min. Supernatants were collected, passed through a 0.45-μm filter (Gelman Sciences, Ann Arbor, MI), and then stored at −20°C for further analysis.

Bronchoalveolar lavage (BAL)

Mice were sacrificed 2 days after bacterial challenge, and BAL was performed. The trachea was exposed and intubated using a 1.7-mm outer diameter polyethylene catheter. BAL was performed by instilling PBS containing 5 mM EDTA, via the right ventricle. Whole lungs were then harvested for assessment of bacterial number, cytokine protein expression, and apoptosis. After removal, whole lungs were homogenized in 1.0 ml of PBS with protease inhibitor (Boehringer Mannheim, Indianapolis, IN) using a tissue homogenizer (Biospec Products, Bartlesville, OK) under a vented hood. Portions of homogenates (10 μl) were inoculated on agar after serial 1/10 dilutions with PBS. The remaining homogenates were incubated on ice for 30 min, then centrifuged at 2500 rpm for 10 min. Supernatants were collected, passed through a 0.45-μm filter (Gelman Sciences, Ann Arbor, MI), and then stored at −20°C for further analysis.

TUNEL staining

The TUNEL assay was performed on paraffin-embedded lung tissue sections using a commercially available kit (FragEL DNA fragmentation detection kit; Oncogene Research Products, Boston, MA).

Murine alveolar epithelial cell isolation and culture

Murine type II alveolar epithelial cells were isolated using the method developed by Corti et al. (30). Briefly, following pentobarbital anesthesia and heparin administration, C57Bl/6 mice were exsanguinated, and the pulmonwy vasculature was perfused via the right ventricle with 0.9% NaCl. The lungs were then filtered via the trachea with 1–2 ml of dispase (worthington, Lakewood, NJ). Subsequently, 0.45 ml of low melting point agarose was infused via the trachea, and the lungs were placed in ice for 2 min to harden the agarose. The lungs were then submerged in dispase for 45 min at 24°C before the lung tissue was teased from the airways and minced in DMEM with 0.01% DNase. After swirling for 10 min following exposure to a series of nylon filters, the cell suspension was collected by centrifugation and incubated with bionylated Abs (anti-CD32 and anti-CD45; BD Pharmingen, San Diego, CA). After incubation with streptavidin-coated magnetic particles, bone marrow-derived cells were removed with a magnetic tube separator. Mesenchymal cells were removed by overnight adherence in a petri dish. The nonadherent cells after this initial plating were plated at a density of 2 × 10⁵–2 × 10⁶/cm² on plastic dishes coated with fibronectin and were maintained in DMEM with penicillin/streptomycin and 10% FCS. Cells were washed with PBS 1 h after plating. This technique routinely generates 5–6 × 10⁶ cells/mouse, with a final adherent population that includes only 4% nonepithelial cells (by intermediate filament staining). Culture cells on day 3 were placed in a glass chamber and exposed to either normoxic or hyperoxic conditions (95% O₂) in the presence or absence of *L. pneumophila* (15 to 1 bacteria to cell ratio). After inoculation of ~10⁶ CFU of bacteria, no increase of bacterial number in each well was observed during the course of experiments. The 3 day time point was chosen because isolated alveolar epithelial cells in this time in culture are relatively pure (>90% alveolar epithelial cells) and have begun a transition to a more type I-like phenotype. At indicated time points, histone-associated DNA fragments and transepithelial voltage were determined as described in Materials and Methods.

Assessment of transepithelial voltage differences

To assess alveolar epithelial cell barrier function in vitro, type II cells were cultured in the upper chamber of Transwell culture dishes on tissue culture-treated polycarbonate membranes (0.4-μm pore size, 6.5 mm diameter; Corning Costar, Cambridge, MA). The resistance across the monolayer was determined at various time points using a Millicell device (Millipore, Bedford, MA) with one sterile electrode in the medium in the upper chamber and one sterile electrode in the medium in the lower chamber (31).

Determination of histone-associated DNA fragments and caspase-3 activity

To evaluate induction of apoptosis, levels of histone-associated DNA fragments and caspase-3 activity were determined in lung homogenates. DNA fragmentation was quantified by measuring histone-associated DNA fragments using an ELISA kit (Cell Death Detection ELISAplus; Roche Diagnostics, Mannheim, Germany). Because *L. pneumophila* is reported to induce apoptosis in vitro in a caspase-3-dependent manner (14), caspase-3 activity was determined by a colorimetric assay (R&D Systems, Minneapolis, MN), in which caspase-specific peptide conjugated to the color reporter molecule p-nitroanilide was used. The data are expressed as a fold increase, comparing to those of control mice (n = 5).

Murine TNF-α ELISA

Murine TNF-α was quantitated using a modification of a double ligand method as previously described (32). Standards were 2-fold dilutions of murine recombinant cytokine from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected murine TNF-α concentration >20–50 pg/ml. The ELISA did not cross-react with other cytokines, such as IL-1, IL-2, IL-6, IL-12, and IFN-γ. In addition, the ELISA did not cross-react with members of the murine chemokine family, including murine KC, macrophage-inflammatory protein-2 and -1α, monocyte chemotractant protein-1, or RANTES.

Neutralization of TNF-α by specific antiserum

To neutralize TNF-α biologic activity, rabbit anti-murine TNF-α antiserum was i.p. injected into mice 2 h (0.5 ml) before and 48 h (0.2 ml) after bacterial challenge. Normal rabbit preimmune serum was used as a control. This Ab has been shown to neutralize TNF-α both in vitro and in vivo (32).

Statistical analysis

Statistical significance was determined using the unpaired, two-tailed alternate Welch t test and the nonparametric Mann-Whitney test. Calculations were performed using InStat for Macintosh (GraphPad, San Diego, CA). Statistical analyses of survival curves were performed by the log rank test using the Prism software program (GraphPad).
in hyperoxic condition for 60 h, and then survival was observed 7–8 days after bacterial challenge (Fig. 1). After i.t. administration of bacteria, one group of animals was kept in hyperoxic conditions for 60 h, while another group was placed in room air during the observation period. Interestingly, in both strains of mice, drastic decreases in survival were observed in the group of animals exposed to hyperoxic conditions for 60 h, while another group was placed in room air during the observation period. Specifically, in C57BL/6 mice, only 10% of control mice died, whereas >90% of mice exposed to hyperoxia died by 7 days post-infectious challenge. A similar increase in lethality was observed in infected A/J mice under hyperoxic condition, as compared with infected animals breathing room air. Importantly, hyperoxia treatment alone (90–94% oxygen for 60 h), without infection, induced no death of mice in either strain (data not shown). To determine whether the hyperoxia-associated increase in lethality was specific for Legionella pulmonary infection, we assessed survival of mice with Klebsiella pneumoniae pneumonia in normal and hyperoxic conditions. In contrast to Legionella pneumonia, we did not observe any detrimental effects of hyperoxia on survival of animals with Klebsiella pneumonia (data not shown). These data clearly demonstrate that hyperoxia increases the lethality of L. pneumophila pneumonia, but not K. pneumoniae pneumonia. Moreover, increase of lethality was observed in both permissive A/J and nonpermissive C57BL/6 mice, suggesting that intracellular growth of Legionella is not required for hyperoxia-induced lethality.

Effect of hyperoxia on lung bacterial burden and inflammatory cell influx in mice with Legionella pneumonia

To determine the cause of increased lethality under hyperoxic conditions, we next examined bacterial number in the lungs of C57BL/6 mice on days 2 and 4 after challenge with L. pneumophila (6.7 × 10⁶ CFU). In the room air and hyperoxic groups, bacterial numbers on day 2 were 6.6 × 10⁵ and 7.0 × 10⁵ CFU/lungs, which decreased to 3.0 × 10⁴ and 5.2 × 10⁴ CFU/lungs on day 4, respectively. Importantly, we did not observe any differences in pulmonary bacterial number between these two groups. In addition, there was no evidence of dissemination of bacteria to extrapulmonary organs, including spleen and blood (data not shown). Similarly, exposure to hyperoxia did not alter bacterial counts in the lungs of A/J mice challenged with L. pneumophila (data not shown). Next, we examined cell numbers and differential in BAL fluid of mice on day 2 after Legionella challenge. Hyperoxia alone did not induce any changes in total BAL cell number. A >10-fold increase in total cell number was demonstrated in the BAL fluid of infected mice; however, no difference between mice exposed to hyperoxia or room air was observed. Cell differential of the infected mice indicated that ~80% of cells were neutrophils in both hyperoxia and room air groups, and again there was no difference between these two groups (data not shown). These data indicate that hyperoxia-induced lethality is not due to increased bacterial burden in the lungs nor bacterial dissemination to extrapulmonary organs. Moreover, inflammatory cell numbers and cell types recruited into the lung did not account for the increased lethal sensitivity to Legionella pneumonia after exposure to hyperoxia.

Effect of hyperoxia on the development of lung injury in mice with Legionella pneumonia

To evaluate the magnitude of lung injury in Legionella-infected mice exposed to room air and hyperoxic conditions, we compared total lung weights and albumin concentrations in BAL fluid on day 2 after Legionella challenge (Fig. 2). Hyperoxic conditions used in the present study (90–95% oxygen for 60 h) failed to induce...
changes in lung weights, as compared with that observed in control uninfected mice. In contrast, *Legionella* infection induced a clear increase in total lung weights, with a 1.5-fold increase in infected mice kept in room air and a nearly 2-fold increase of total lung weight in *Legionella*-infected animals exposed to hyperoxic conditions (*p* < 0.05). Albumin concentration in BAL fluid correlated well with changes in lung weights. Specifically, *Legionella* challenge resulted in significantly higher BAL albumin concentrations, as compared with uninfected animals. Importantly, a >2-fold increase in BAL albumin levels were noted in infected mice exposed to hyperoxia compared with those observed in infected mice kept in normoxic conditions (*p* < 0.05). These data demonstrate that *Legionella* infection significantly increases lung permeability and that lung leak is exacerbated by exposure to hyperoxia independent of changes in lung bacterial burden.

**Effects of hyperoxia on apoptosis in *Legionella pneumonia***

To investigate the cause of accelerated lung injury in hyperoxia, we examined quantitative markers of apoptosis, including histone-associated DNA fragments and caspase-3, in the lungs of mice 2 days after *Legionella* challenge (Fig. 3). Histone-associated DNA fragments are a marker for DNA fragmentation, one of the main characteristics of apoptosis, whereas caspase-3 is an essential protease mediating apoptosis. Hyperoxia alone did not induce evidence of apoptosis. However, *Legionella* infection induced a clear increase in the presence of both markers within the lungs. The increase in DNA fragments and caspase-3 activity was most marked in the lungs of infected animals exposed to hyperoxia, with 22- and 1.8-fold increases, respectively, compared with control uninfected animals (*p* < 0.05). In addition, evidence of DNA laddering was observed in agarose gel analysis after extraction of chromosomal DNA from infected lungs, which was more pronounced in the lungs of hyperoxic animals (data not shown). These studies indicate that hyperoxia treatment exaggerates apoptosis in the lungs of mice infected with *L. pneumophila*. In contrast, we did not observe an increase of histone-associated DNA levels in *K. pneumoniae*-infected lungs in the setting of hyperoxia (data not shown), which closely correlated with the lack of oxygen effects on survival in *Klebsiella*-infected mice.

**FIGURE 3.** Effect of hyperoxia on apoptosis markers, histone-associated DNA fragments, and caspase-3 in *Legionella pneumonia*. C57BL/6 mice were i.t. infected with 3.0 × 10^8 CFU/mouse. Then, one group was kept in room air, whereas another group was placed in hyperoxic condition. Histone-associated DNA fragments (A) and caspase-3 activity (B) in total lung homogenates were determined 2 days after bacterial challenge in mice kept in room air and hyperoxic conditions (*n* = 5). The data were expressed as fold increase, as compared with those of control uninfected mice (*n* = 5). *p* < 0.05, compared with those of infected mice kept in room air.

**FIGURE 4.** TUNEL staining of the lung sections. Cells with blue nuclei are normal, whereas nuclei stained with brown or black demonstrate cells undergoing apoptosis. In mice of control and hyperoxia treatment alone, there were few apoptotic cells in the lungs. In contrast, *Legionella* infection, especially infection plus hyperoxia, increased the number of apoptotic cells. The majority of apoptotic cells were considered to be macrophages (black arrow) and alveolar epithelial cells (dotted arrow).
To define which population of cells were undergoing apoptosis in vivo, TUNEL staining of lung sections from infected mice was performed (Fig. 4). In mice exposed to ambient air or hyperoxia alone, there were very few apoptotic cells in the lungs. In contrast, Legionella infection, especially infection plus hyperoxia, increased the number of apoptotic cells. Based on morphological criteria, the majority of apoptotic cells were alveolar and interstitial macrophages. In addition, we observed evidence of apoptosis of alveolar epithelial cells in the lungs.

**Effect of hyperoxia on Legionella-induced murine alveolar epithelial cell apoptosis and changes in barrier function**

To more directly define the effect of hyperoxia on Legionella-induced alveolar epithelial cell apoptosis and barrier dysfunction, we established an in vitro system to model in vivo oxygen exposure. As shown in Fig. 5A, exposure to normoxia for 24 h in the presence or absence of L. pneumophila resulted in minimal alveolar epithelial cell apoptosis. In contrast, exposure to hyperoxia alone resulted in a modest elevation of histone-DNA complexes, which was significantly increased in the presence of L. pneumophila (p < 0.01, comparing to room air plus Legionella infection).

![FIGURE 6. Survival of Fas-deficient mice in Legionella pneumonia under hyperoxia](image)

To assess the effects of hyperoxia on barrier function, transepithelial voltage differences were determined across the epithelial monolayer (Fig. 5B). No differences in initial transepithelial voltage were noted in infected or uninfected alveolar epithelial cells cultured for 24 h at various oxygen tensions. However, by 48 h of exposure to hyperoxia, there was a significant decrease in transepithelial voltage (indicative of increased lung permeability) in Legionella-infected cells, as compared with infected or uninfected cells cultured at room air, or uninfected cells cultured in hyperoxia. Taken together, these data indicate that hyperoxia can potentiate Legionella-induced alveolar epithelial cell apoptosis and epithelial permeability.

**Survival and evidence of apoptosis in Fas-deficient mice with Legionella pneumonia after exposure to hyperoxia**

To examine the role of apoptosis in the survival of mice with Legionella pneumonia exposed to hyperoxia, we infected Fas-deficient and control C57BL/6 mice with L. pneumophila, and then placed animals in hyperoxic conditions for 60 h (Fig. 6). This dose of bacteria was sublethal for control mice in normoxic conditions. However, C57BL/6 mice exposed to hyperoxia started to die by day 3 after bacterial challenge, with survival of only 33% by the end of observation. In contrast, Fas-deficient mice demonstrated partial resistance to the lethal effects of Legionella after hyperoxic exposure, with no death observed until day 5 after bacterial challenge, and 67% of Fas-deficient mice surviving to the end of observation (p < 0.05). Next, we examined histone-associated DNA fragments in the lungs of these control C57BL/6 and Fas-deficient mice (Fig. 7). Consistent with survival data, the hyperoxia-induced increase in histone-associated DNA fragments observed in infection was significantly attenuated in Fas-deficient mice (p < 0.05). These results strongly suggest that Fas-mediated apoptosis partially mediates hyperoxia-associated lethality in mice with Legionella pneumonia.

**Roles of TNF-α on hyperoxia-associated apoptosis**

To further define potential mechanisms of hyperoxia-associated apoptosis in Legionella pneumonia, we examined pulmonary TNF-α levels 2 days after bacterial challenge (Fig. 8). TNF-α was targeted because this cytokine has been shown to be up-regulated in the setting of oxidant stress (35, 36), and can induce apoptosis in certain settings (16, 19). Hyperoxia alone failed to induce TNF-α production in lungs. However, i.t. challenge with L. pneumophila substantially increased lung TNF-α levels. This was particularly evident in animals exposed to hyperoxia, with TNF-α levels...
levels being 2.2-fold greater than those observed in infected animals under room air conditions ($p < 0.05$). To examine the role of TNF-$\alpha$ in the induction of apoptosis, mice were treated with anti-TNF-$\alpha$ antisera, and then apoptosis in the lungs was examined on day 2 after bacterial challenge (Fig. 9). TNF-$\alpha$ blockade attenuated apoptosis, as indicated by a decrease in histone-associated DNA fragments and caspase-3 levels in both mice kept in hyperoxia and those kept in room air conditions. However, TNF-blockade did not provide survival benefit to hyperoxic mice (data not shown). These results suggest that TNF-$\alpha$ plays a role in the induction of apoptosis observed in Legionella-infected mice, although in contrast to Fas-mediated apoptosis, TNF-mediated apoptosis was not appreciably enhanced in the setting of hyperoxia.

**Discussion**

The current study demonstrates that transient oxygen supplementation after the onset of pneumonia markedly increases lethality of mice with *L. pneumophila*. Increased mortality was associated with a significant increase in lung permeability, but not bacterial burden in the lungs. Because there was no evidence of dissemination of bacteria to extrapulmonary organs, it is likely that lung injury contributed to the death of these animals in hyperoxic conditions. These results demonstrate, for the first time, that supplemental oxygen therapy may exacerbate acute lung injury in the setting of specific pulmonary infections, including *Legionella*, even in a situation that oxygen by itself does not cause excessive lung tissue injury.

Induction of apoptosis in the lungs of *L. pneumophila* pneumonia was demonstrated using multiple approaches, including quantification of histone-associated DNA fragments, caspase-3 activity, DNA laddering, and TUNEL staining. We observed acceleration of apoptosis in the infected lungs of mice exposed to hyperoxia, which was well correlated with enhanced lethal sensitivity. *L. pneumophila* has been reported to induce apoptosis in macrophages and alveolar epithelial cells in vitro in a caspase-3-dependent fashion within a few hours of infection (14). Our data are consistent with these previous results and further support a critical role of caspase-3-mediated apoptosis in the pathogenesis of *Legionella* pneumonia. Because *Legionella*-associated apoptosis was demonstrated in nonreplicative C57BL/6 mice, intracellular multiplication may not be a prerequisite for induction of apoptosis. Moreover, it is likely that exaggeration of apoptosis in the setting of hyperoxia is detrimental to outcome, in part through acceleration of acute lung injury.

TUNEL staining of the infected lung sections demonstrates that macrophages (alveolar and interstitial) and alveolar epithelial cells are the predominant cells undergoing apoptosis. In particular, alveolar epithelial cell apoptosis may be crucial for initiation of enhanced pulmonary permeability because these cells cover $>95\%$ of alveolar air space and are paramount in maintaining the integrity of the alveolar-capillary barrier (37). In addition, because *Legionella* has a strong predilection for invasion of alveolar epithelial cells (9, 13, 14), bacterial cytotoxicity, in combination with host factors (e.g., TNF-$\alpha$), may be pivotal to the development of epithelial injury, acute lung damage, and increased mortality in *Legionella* pneumonia. Our in vitro experiments mimicking in vivo hyperoxia support a direct effect of hyperoxia as a mediator of alveolar epithelial cell apoptosis. In contrast, the role of macrophage apoptosis in *Legionella* pneumonia is still poorly understood. It has been shown in vitro that *L. pneumophila* kills macrophages by activating the host apoptosis cascade upon contact with these cells (13, 14), although whether apoptosis of infected macrophages benefits the host or the bacteria is still unclear. For example, apoptosis, but not necrosis of human monocytes, is shown to limit the growth of other intracellular pathogens, including *Mycobacterium bovis* (38) and *Mycobacterium avium* (39). An area of interest in this regard is how macrophage apoptosis affects the viability of intracellular bacteria in replicative AJ mice model of pneumonia, which is an area of ongoing investigation.

Several investigators have reported crucial roles of death receptor-mediated apoptosis in certain infectious diseases. Kanaly et al. (21) have reported that the TNF-receptor is important for successful regression through induction of lymphocyte apoptosis in *Leishmania major* or *Rhodococcus equi* infections, once pathogens are eliminated. In murine CMV infection, Fas-mediated apoptosis is required for the down-modulation of the immune response to virus, which prevented detrimental chronic inflammatory responses (20). Our data suggest that apoptosis occurs in *Legionella* infection, especially in the setting of hyperoxia, and that both TNF and Fas are relevant mediators. Specifically, we observed a $>2$-fold increase in TNF-$\alpha$ in the infected lungs of mice exposed to hyperoxic conditions. In addition, TNF-$\alpha$ blockade resulted in attenuation of apoptosis in both infected mice kept in hyperoxia and those kept in room air. Furthermore, the observation obtained in Fas-deficient mice suggests that Fas mediates the acceleration of apoptosis in hyperoxic conditions, and that Fas-mediated apoptosis may be detrimental in *Legionella* pneumonia in the setting of hyperoxia. The fact that Fas deficiency only partially, not totally,
restored resistance to the lethal effects of *Legionella* after hyperoxic exposure suggests involvement of Fas-independent pathway(s) (e.g., TNFRI -II) in the pathogenesis of exaggerated lethal sensitivity of these mice. However, it should be noted that Fas knockout mice are not completely Fas-deficient, which may have resulted in an underestimation of Fas-dependent events (40).

TNF-α blockade failed to provide a survival benefit in hyperoxic mice infected with *Legionella*. It has been shown that TNF-α contributes not only to apoptosis but also to antimicrobial immunity against a variety of intracellular and extracellular pathogens, including *Legionella* (41, 42). Therefore, the lack of beneficial effects of TNF blockade on survival of mice in hyperoxic conditions may reflect multipotential activity of TNF-α on host biological and immunological systems.

How hyperoxia accelerates *Legionella*-associated apoptosis in the lung is not clearly defined, although death receptor-mediated signaling appears to play a certain role. We speculate on at least two possibilities in which hyperoxia affects apoptosis in *Legionella* pneumonia. First, hyperoxia or higher oxygen concentration may affect the expression of virulence factors produced by *L. pneumophila*, which may amplify host responses, including TNF-α production. Alternatively, these virulence factors may directly induce apoptosis through death receptor-dependent and/or -independent mechanisms. Although apoptosis-inducing factor(s) in *Legionella* pneumonia have not yet been defined, *dot/ic* (14) and pore-forming toxins (11) may be potential candidates. It is of great interest to investigate whether the virulence-deficient mutants are capable of accelerating apoptosis in the setting of hyperoxia. A second possibility is that hyperoxia sensitizes or primes host cells to apoptosis-triggering stimuli that are elaborated by *Legionella* infection. Hyperoxia may affect a variety of host biological systems, such as antioxidant enzymes (43) and cytokine production (28), through excessive production of reactive oxygen species. Several investigators have reported that hyperoxia up-regulates TNF-α gene and protein expression, especially after triggering stimuli (35, 36, 44, 45). In addition, hyperoxia has been reported to induce a marked increase in message and protein levels of Fas in lungs, although Fas-deficient mice are not resistant to hyperoxia-induced lung damage itself (29). These suggest that both Fas and bacterial/ bacterial products may cooperatively mediate *Legionella*-induced lethality in hyperoxic conditions. Modulation of host cell sensitivity to apoptosis-triggering stimuli, both host and bacterial factors, in addition to expression of death receptors on responsible cells, remained to be investigated in this model.

In conclusion, the present results suggest that hyperoxia serves as an important cofactor for the development of acute lung injury and lethality in *L. pneumophila* pneumonia. Exaggerated apoptosis, in part through the death receptor-mediated signals, accelerates hyperoxia-induced acute lung injury in *Legionella* pneumonia. Further investigations into the roles of death receptors and death factors in hyperoxia-associated apoptosis in *Legionella* pneumonia, and identification of related virulence factor(s), are warranted. Given the large number of patients with pneumonia exposed to high oxygen tensions, the observations made in this study are of greater clinical relevance and similar studies should be pursued in patient populations at risk.

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**References**


