Improved survival in a model of hyperoxia-induced acute lung injury in *Pneumocystis murina*-infected mice: Critical roles of inflammation and apoptosis

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Abstract

*Pneumocystis* infections increase host susceptibility to additional insults that would be tolerated in the absence of infection, such as hyperoxia. In an in vivo model using CD4-depleted mice, we previously demonstrated that *Pneumocystis murina* pneumonia causes significant mortality following an otherwise nonlethal hyperoxic insult. Infected mice demonstrated increased pulmonary inflammation and alveolar epithelial cell apoptosis compared to controls.

To test the mechanisms underlying these observations, we examined expression of components of the Fas-Fas ligand pathway in *P. murina*-infected mice exposed to hyperoxia. Hyperoxia alone increased expression of Fas on the surface of type II alveolar epithelial cells; conversely infection with *P. murina* led to increased lung expression of Fas ligand. We hypothesized that inhibition of inflammatory responses or direct inhibition of alveolar epithelial cell apoptosis would improve survival in *P. murina*-infected mice exposed to hyperoxia. Mice were depleted of CD4+ T cells and were infected with *P. murina*, then were exposed to > 95% oxygen for 4 days, followed by return to normoxia. Experimental groups received vehicle, dexamethasone, or granulocyte macrophage colony-stimulating factor (GM-CSF). Compared with vehicle, treatment with dexamethasone reduced Fas ligand expression and significantly improved survival. Similarly, treatment with GM-CSF, an agent we have shown to protect alveolar epithelial cells against apoptosis, decreased Fas ligand expression and also improved survival.

Our results suggest that the dual stress of *P. murina* infection and hyperoxia induces lung injury via activation of the Fas-Fas ligand pathway, and that corticosteroids and GM-CSF reduce mortality in *P. murina*-infected mice exposed to hyperoxic stress by inhibition of inflammation and apoptosis.
Introduction

*Pneumocystis jirovecii* infections remain a significant cause of morbidity and mortality in immunosuppressed individuals, including individuals with HIV infection and those receiving immunosuppressive therapy (27). Despite prompt institution of appropriate antibiotic therapy, a significant number of patients experience clinical deterioration and respiratory failure after hospital admission. The pathophysiologic mechanisms leading to deterioration in some patients have not been defined. Treatment with corticosteroids has become the standard of care for moderate to severe *P. jirovecii* pneumonia in patients with AIDS and has been found to decrease the rate of post-hospitalization progression to respiratory failure (2, 7, 13, 22). Presumably, the beneficial effects of corticosteroids are mediated by blunting patients’ pulmonary inflammatory responses, but the mechanisms by which this improvement occurs are not understood.

In previous work, we hypothesized that a secondary stress triggers respiratory failure in patients with *P. jirovecii* pneumonia (5). This secondary insult likely triggers an inflammatory response that results in detrimental effects to the host. Among potential triggers (acting alone or in combination) are drug effects, coincident bacterial infections, and supplemental oxygen therapy. We reasoned that oxidant stress might be a common mechanism precipitating progression to respiratory failure. To date, prospective or retrospective analysis of oxygen therapy as an independent predictor or respiratory failure has not been possible in patients with *P. jirovecii* pneumonia, with or without corticosteroid therapy. Therefore, we have used an animal model to test this concept. We previously developed a model in which mice were depleted of CD4+ T cells, were infected with *P. murina*, and were then exposed to a limited period of hyperoxia (5). This period of hyperoxia, by itself, caused minimal lung injury and no lethality. However, we found that *P. murina*-infected mice exposed to hyperoxia succumb to
this dual stress, whereas either individual insult alone did not result in death. We demonstrated that mice exposed to sublethal hyperoxia did not have an increased burden of organisms compared to mice maintained in normoxia. However, the dual stress of hyperoxia and *P. murina* infection resulted in significantly increased apoptosis of lung cells and in exuberant inflammatory responses. Histologically, we identified increased apoptosis in alveolar macrophages and alveolar epithelial cells, but did not examine the cells ex vivo or investigate the specific mechanisms by which this combined stress led to alveolar epithelial cell apoptosis.

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a product of normal alveolar epithelial cells that has important effects on pulmonary innate immunity and surfactant homeostasis. We previously demonstrated that GM-CSF is critical for host defense against *P. murina* (25). GM-CSF is also an anti-apoptotic factor for alveolar epithelial cells (24). In mice exposed to sustained hyperoxia, over-expression of GM-CSF conferred significant protection from alveolar epithelial cell injury and mortality (24). These effects were associated with reduced alveolar epithelial cell apoptosis, an effect that was reproduced with pharmacologic treatment with recombinant murine GM-CSF.

In the present experiments, we hypothesized that the combination of inflammation due to *P. murina* infection and hyperoxic stress results in increased vulnerability of alveolar epithelial cells to Fas-mediated apoptosis, leading to lung injury and death. We determined that the dual insults of *P. murina* infection and hyperoxic stress induce components of the Fas-Fas ligand apoptotic pathway and have found that treatment with GM-CSF improves survival in this immunologically relevant model of *Pneumocystis* pneumonia. Furthermore, treatment with corticosteroids improves survival in this model, recapitulating the benefit of treatment in human
disease and providing a possible explanation for the benefits humans derive from treatment with an anti-inflammatory medication in the setting of acute infection.
Materials and Methods

Mice

Wild-type C57BL/6 and C57BL/6 SCID mice were purchased from Jackson Laboratories and housed under specific pathogen-free conditions in isolator cages. Autoclaved water and food were given ad libitum. The animal care committees at the University of Michigan and at the Ann Arbor Veterans Affairs Medical Center approved all protocols.

Mice were depleted of CD4+ T cells as previously described (25). Mice received weekly intraperitoneal injections of rat anti-mouse monoclonal antibody GK1.5. The hybridoma (American Type Culture Collection) was harvested as ascites from pristane-primed SCID mice. The adequacy of in vivo depletion was confirmed by flow cytometry, and antibody injections were continued for the duration of the experiments.

Propagation of P. murina organisms

P. murina was propagated in SCID mice as previously described (26). Briefly, lungs harvested from SCID mice chronically infected with P. murina were removed and homogenized. Recovered P. murina organisms were inoculated intratracheally into subsequent generations of SCID mice.

Induction of P. murina pneumonia

Freshly prepared inoculum was always used for intratracheal inoculation to assure viability of organisms. Inoculation of mice was performed during pentobarbital anesthesia (6). After surgical exposure of the trachea, a blunted needle was passed through the mouth into the mid-trachea under direct vision. A polyethylene catheter was passed through this needle until the catheter tip was just distal to the needle, and 0.1 ml (1 x 10^5 cysts) of the inoculum was
injected. The inoculum was followed by injection of 0.4 ml of air, to insure adequate dispersion of the inoculum and clearance of the central airways. The neck incision was sutured and mice were placed prone for recovery. Using this protocol, progressive infection with extensive inflammation and heavy burden of organisms occurs by four weeks after inoculation. In contrast, we have not detected infections in CD4-depleted mice that were not inoculated with \textit{P. murina}. Thus, these CD4-depleted, uninfected mice served as controls.

To exclude the presence of unintended pathogens, sentinel mice co-housed with the experimental mice were examined by serologic surveillance and by culture of lung tissue. Portions of lungs from the experimental mice were examined to exclude the presence of pathogens other than \textit{P. murina}. Gram stains of touch preparations did not show other microorganisms, and bacterial and fungal cultures of lung tissue were always negative. In addition, histologic sections failed to show organisms when stained with a tissue Gram stain (Brown-Brenn stain) (4).

Exposure of mice to hyperoxia

Four weeks after \textit{P. murina} infection, mice were exposed to hyperoxia in shoebox-style cages within a 30”w x 20”d x 20”h Plexiglas chamber (5). This chamber was maintained at oxygen concentrations of >95% using a Proox Model 110 controller (Reming Bioinstruments). During the four day period of hyperoxia, mice remained unrestrained and had free access to water and food. We based the duration of hyperoxia on our previous studies (5), focusing on a period of hyperoxia that is not lethal to normal mice, but is lethal in \textit{P. murina}-infected mice exposed to hyperoxia. At the conclusion of the hyperoxia period, mice were returned to room air.
Isolation and purification of type II alveolar epithelial cells

After the four-day hyperoxia exposure period, mice were sacrificed and type II alveolar epithelial cells were isolated and purified using a modification of published methods (12, 24). Following pentobarbital anesthesia and heparinization, mice were exsanguinated and the pulmonary vasculature was perfused with saline. The trachea was cannulated and the lungs were filled with 1-2 ml dispase (BD Bioscience). Subsequently, low melting point agarose (1%, 0.45 ml) was infused via the trachea and the lungs were placed in iced PBS for 2 min to harden the agarose. The lungs were incubated in dispase, and parenchymal tissue was teased away from agarose-embedded airway tissue. Lung parenchymal tissues were minced in DMEM with 0.01% DNAse, and the resultant cellular suspension was filtered. The cells were incubated with biotinylated anti-CD32 and anti-CD45 (Pharmingen) followed by streptavidin-coated magnetic particles (Promega) for magnetic removal of leukocytes. Mesenchymal cells were removed by overnight adherence to tissue culture treated plastic. The non-adherent cells were plated in DMEM with penicillin/streptomycin and 10% fetal calf serum in wells coated with fibronectin. For each experiment, purity of the epithelial cell preparations was confirmed by vimentin staining with anti-vimentin (clone Vim-13.2, Sigma). Secondary staining was performed with FITC-conjugated anti-mouse IgM.

Immunohistochemical staining

Immunohistochemical staining for caspase-3 on alveolar epithelial cells was modified from a method we previously used to examine lung sections (5). Isolated type II alveolar epithelial cells were fixed to glass slides by cytospin, and were stained using purified polyclonal rabbit cleaved caspase-3 (Asp 175) antibody (Cell Signaling Technology), biotin-conjugated goat anti-rabbit IgG (Jackson Immunoresearch), and detection with the proliferating cell nuclear
antigen kit (Zymed). Scoring was performed in duplicate without knowledge of treatment with 300 cells scored per slide.

Expression of Fas (CD95) on type II alveolar epithelial cells was measured using a hamster anti-mouse antibody (Jo2, BD Pharmingen). Hamster IgG was used as a negative control. The secondary antibody was biotinylated goat anti-hamster antibody (Vector Laboratories). For in vivo analysis, lungs from mice were harvested, fixed with 4% freshly prepared paraformaldehyde instilled via the trachea, embedded in paraffin, and sectioned. Staining was performed using the same antibodies, and slides were analyzed at a final magnification of 125X.

**Measurement of lung Fas ligand**

Fas ligand was measured in lung homogenates using the mouse Fas Ligand/TNFSF6 Immunoassay (R&D Systems) according to the manufacturer’s directions. Protein concentrations were measured in lung homogenates (Pierce BCA kit) and were adjusted to 200 µg/ml. Samples were analyzed in triplicate and results expressed as pg/ml, interpreted against a recombinant standard.

To identify the cellular sources of Fas ligand in the lungs of infected mice, lung leukocytes were obtained by collagenase digestion, stained for cell-associated Fas ligand, and assessed by flow cytometry (4, 11). Briefly, lung portions were minced with a scalpel, followed by digestion in collagenase and DNase. The resultant cell suspension was centrifuged, resuspended, and washed extensively. Flow cytometry (FACScan, Becton-Dickinson Immunocytometry Systems) was performed using monoclonal antibodies directed against CD45 (30-F11) and CD178 Fas ligand (MFL3) that were conjugated to phycoerythrin (BD Pharmingen). In each case, duplicate aliquots of cells were examined after staining with
irrelevant, isotype-matched controls to exclude nonspecific antibody binding. Flow cytometry data are expressed as numbers of CD45-positive cells bearing CD178, gated on the lymphocyte, macrophage, and neutrophil populations by forward and right-angle scatter.

*Dexamethasone and GM-CSF treatments*

Groups of C57BL/6 mice were depleted of CD4+ T cells as described above, then were inoculated intratracheally with *P. murina*. After four weeks of infection, mice were randomized to receive dexamethasone (5 mg/kg intraperitoneally), GM-CSF (9 µg/kg, subcutaneously; R&D Systems) or an equivalent volume of vehicle (8). As in our other experiments, mice were subjected to hyperoxia or room air for four days. Injections were performed just prior to entry into hyperoxia, and after two days in hyperoxia. After four days of hyperoxia, mice were returned to room air and survival was monitored.

*Measurement of histone-associated DNA*

The extent of apoptosis was measured in lung homogenates as previously described (5), using the Cell Death Detection ELISA Plus apparatus (Roche). This test measures histone-associated DNA fragments, as an indication of apoptosis. Protein concentrations were measured in lung homogenates (Pierce BCA kit) and were adjusted to 200 µg/ml. Samples were then analyzed in triplicate as directed by the manufacturer, and data were expressed as relative ELISA absorbances.

*Statistical analysis*

Data are presented as means ± SEM. Group means were compared using a Student’s t-test for two-group comparisons, and ANOVA with Newman-Keuls follow-up testing for multiple comparisons, using the statistical software program Prism4 (GraphPad Software). For survival
experiments, groups were compared by Kaplan-Meier analysis. Comparisons were deemed statistically significant for P values < 0.05.
Results

Alveolar epithelial cells isolated from P. murina-infected mice exposed to hyperoxia show increased apoptosis.

We have shown previously that the dual stress of P. murina infection followed by transient exposure to hyperoxia resulted in increased lung cell apoptosis as measured in lung homogenates and by immunohistochemical staining for activated caspase 3. In order to confirm that this increase reflected changes in alveolar epithelial cell apoptosis, we isolated type II alveolar epithelial cells from mice after exposure to room air or hyperoxia, and with or without P. murina infection. After the exposure period, type II alveolar epithelial cells were isolated and the percentages of cells undergoing apoptosis were determined, as measured by staining for activated caspase 3. Both established P. murina infection and hyperoxia increased type II alveolar epithelial cell apoptosis significantly compared with uninfected mice maintained in air (Figure 1). However, after the combined insults of P. murina infection plus hyperoxia, type II alveolar epithelial cell apoptosis was significantly increased compared to either stress alone. These data demonstrate that the two insults in our model have additive effects in induction of type II alveolar epithelial cell apoptosis.

Hyperoxia induces Fas expression by alveolar epithelial cells.

To investigate the mechanisms by which the dual insults of P. murina infection and hyperoxia produce increased alveolar epithelial cell apoptosis, we next turned to examination of Fas-Fas ligand interactions. To determine whether Fas expression was increased on alveolar epithelial cells in this model, we examined Fas staining in freshly isolated type II alveolar epithelial cells from P. murina-infected or uninfected mice maintained in room air or exposed to hyperoxia for four days. Alveolar epithelial cells were isolated from all groups of mice at the
conclusion of the exposure period. The fraction of type II alveolar epithelial cells expressing cell surface Fas was increased significantly following exposure to hyperoxia (Figure 2). This effect appeared to be a direct consequence of hyperoxic stress alone, as type II alveolar epithelial cells from *P. murina*-infected mice maintained in room air were not different from uninfected controls. Furthermore, *P. murina* did not produce an additive effect when combined with exposure to hyperoxia. To confirm that these changes were a reflection of induction of Fas expression on alveolar wall cells in vivo, lungs taken from mice maintained in room air or exposed to hyperoxia for four days were stained for Fas expression. There was modest Fas expression on alveolar wall cells in lungs of mice maintained in normoxia, but expression was greatly increased following exposure to hyperoxia (Figure 3). Thus, alveolar epithelial Fas expression was induced in response hyperoxic stress.

*Fas ligand expression is induced in the lungs of *P. murina*-infected mice.*

Having determined that Fas was induced on alveolar epithelial cells by exposure to hyperoxia, we next determined whether its counter ligand, Fas ligand, was present and altered by exposure to hyperoxia or by *P. murina* infection. Four days of hyperoxia alone did not significantly increase soluble Fas ligand expression in lung homogenates, compared with homogenates obtained from mice maintained in normoxia (Figure 4). In contrast, *P. murina* infection significantly increased Fas ligand expression in the lung after four weeks of infection, compared with CD4-depleted control mice that were not infected (Figure 5).

To determine the cells expressing Fas ligand in *P. murina*-infected mice, we performed flow cytometry on single-cell suspensions prepared from minced, collagenase-digested lungs. We found that both lymphocytes and macrophages from the lungs of CD4-depleted mice infected with *P. murina* expressed Fas ligand in greater numbers than cells obtained from
uninfected, CD4-depleted mice. Lungs from infected mice contained 1.88 ± 0.69 \times 10^3 CD178-positive lymphocytes per mouse, compared with 0.01 ± 0.01 \times 10^3 CD178-positive lymphocytes per mouse from CD4-depleted, uninfected mice (n = 6, p < 0.037 by t-test). For macrophages, lungs from infected mice contained 175.30 ± 42.96 \times 10^3 CD178-positive cells per mouse, compared with 6.56 ± 6.41 \times 10^3 CD178-positive cells per mouse from CD4-depleted, uninfected mice (n = 6, p < 0.006 by t-test). Neutrophil accumulation was not prominent in the lungs of either group of mice, but numbers of CD178-positive neutrophils were also increased in the infected mice compared with CD4-depleted, uninfected controls (1.55 ± 0.56 \times 10^3 versus 0.01 ± 0.01 \times 10^3, respectively, n = 6, p < 0.034 by t-test). Thus, inflammation associated with *P. murina* infection resulted in significantly increased cell-associated Fas ligand expression in all three leukocyte populations.

*Dexamethasone reduces Fas ligand expression and improves survival of *P. murina*-infected mice exposed to hyperoxia.*

Treatment with corticosteroids significantly improves outcomes in HIV-infected humans with moderately severe *P. jirovecii* pneumonia by preventing progression to respiratory failure. We postulated that this benefit might be explained by anti-inflammatory effects leading to decreased Fas ligand expression. In order to explore this potential mechanism, we measured Fas ligand expression in the lungs of CD4-depleted mice infected with *P. murina* that were subjected to four days of hyperoxia. The experimental group was treated with dexamethasone (5 mg/kg intraperitoneally), given just before entry into hyperoxia and again after two days in hyperoxia, while the control group received vehicle. Treatment with dexamethasone significantly decreased Fas ligand expression compared with vehicle-treated mice (Figure 6). We next determined whether dexamethasone treatment would reduce mortality in *P. murina*-infected mice exposed to
hyperoxic stress. Groups of CD4-depleted mice that had been infected with *P. murina* for four weeks were randomized to receive dexamethasone or placebo. Mice received the injections at the time of entry into hyperoxia, and again after two days of hyperoxia. After four days of hyperoxia, all mice were maintained in normoxia and followed for survival. As in our previous experiments, the control mice demonstrated significant mortality after return to room air (Figure 7). In contrast, mice receiving dexamethasone showed significantly improved survival. These data are consistent with a model in which steroid treatment is protective in the context of *P. murina* pneumonia due to effects on pulmonary Fas ligand expression.

**GM-CSF reduces Fas ligand expression and improves survival of *P. murina*-infected mice exposed to hyperoxia.**

We have shown previously that GM-CSF is a mitogen and anti-apoptotic growth factor for alveolar epithelial cells. Specifically, we have shown that GM-CSF protects against alveolar epithelial cell apoptosis in a model of hyperoxic lung injury (24). To determine whether GM-CSF protects against apoptosis in the current model, CD4-depleted mice infected with *P. murina* were subjected to four days of hyperoxia. The experimental group was treated with GM-CSF (9 µg/kg, subcutaneously), given just before entry into hyperoxia and again after two days in hyperoxia, while the control group received vehicle. Interestingly, treatment with GM-CSF significantly reduced expression of Fas ligand in lung homogenates compared with vehicle treatment (Figure 6). The reduction in Fas ligand with GM-CSF was comparable to that achieved with dexamethasone treatment. To determine whether treatment with GM-CSF would also decrease mortality in the context of the dual stress of *P. murina* infection and hyperoxia, groups of mice were depleted of CD4+ T cells and then received intratracheal inoculation with *P. murina*. After four weeks of infection, the mice were randomized to receive GM-CSF (9 µg/kg,
subcutaneously) or vehicle at the time of entry into hyperoxia, and again after two days in 
hyperoxia. After four days in hyperoxia, all mice were returned to normoxia followed for 
survival. Treatment with GM-CSF significantly improved survival compared to the vehicle 
(Figure 8).

_Treatment with dexamethasone and GM-CSF modulates lung cell apoptosis during P. murina 
and hyperoxia._

Finally, we performed experiments to determine whether improved survival after 
treatments with dexamethasone or GM-CSF was mediated through effects on lung cell apoptosis. 
Groups of C57/BL6 mice were depleted of CD4+ T cells and infected with _P. murina_ for four 
weeks. Prior to exposure to hyperoxia, mice received vehicle, dexamethasone or GM-CSF and 
these treatments were repeated after 2 days of hyperoxia. After four days of hyperoxia, cell 
death was measured by ELISA in lung homogenates. As previously demonstrated, _P. murina_ 
infection plus hyperoxia resulted in lung cell apoptosis. However, dexamethasone and GM-CSF 
treatment both significantly decreased histone-associated DNA fragments in lung homogenates 
(Figure 9). Thus, these two interventions that improved survival in _P. murina_-infected mice 
exposed to hyperoxic stress were both associated with decreased lung cell apoptosis in this 
model.
Discussion

We have shown previously that mice heavily infected with P. murina are susceptible to acute lung injury and death when subjected to a hyperoxic insult that they would otherwise survive (5). We also found that this increased vulnerability was associated with increased apoptosis of lung cells. We have now extended these observations, providing a potential mechanism to explain increased alveolar epithelial cell apoptosis in the lungs of P. murina-infected mice exposed to a secondary stress. Specifically, P. murina infection results in increased expression of Fas ligand in the lung, while hyperoxia induces Fas expression by alveolar epithelial cells. Together, it is likely that these two stresses activate the Fas-Fas ligand apoptotic pathway, leading to alveolar cell apoptosis, lung injury and death. To confirm these in vitro observations, we performed survival experiments in mice exposed to both P. murina and hyperoxia. Treatment with dexamethasone, which decreases inflammation, decreases lung Fas ligand expression and improves survival. Additionally, treatment with GM-CSF, an inhibitor of alveolar epithelial cell apoptosis, improves survival of P. murina-infected mice exposed to hyperoxia and decreases Fas ligand expression in their lungs. Therefore, we provide in vitro and in vivo evidence implicating alveolar epithelial cell apoptosis as a critical mechanism in this model.

Pneumocystis species are known to have potent effects on alveolar epithelial cells. For example, P. murina induces alveolar epithelial cell release of monocyte chemotactic protein-1, a proinflammatory cytokine that contributes to lung injury (28) and alters alveolar epithelial cell gene expression through an NF-κB-dependent mechanism (29). Other investigators have demonstrated that the β-glucan isolated from the P. carinii cell wall increases macrophage inflammatory protein 2 production by alveolar epithelial cells (14). However, few data exist
addressing the importance of apoptosis in lung parenchymal cells during *Pneumocystis* infections. In our previous work, we did not examine the cells ex vivo or investigate the specific mechanisms by which this combined stress led to alveolar epithelial cell apoptosis. To our knowledge, however, ours was the only previous investigation examining alveolar epithelial cell apoptosis during *P. murina* infection. While the current study focused on alveolar epithelial cell apoptosis and its mechanisms, recent investigations demonstrate that alveolar macrophage apoptosis is also important in determining outcome of infection and host defense. In both rat and mouse models, *Pneumocystis* infections reduce alveolar macrophage numbers by inducing apoptosis, an effect mediated by polyamines (18). Furthermore, suppression of alveolar macrophage apoptosis prolongs survival of rats and mice with *Pneumocystis* pneumonia (17).

Combined with the data we present here, apoptosis of lung cells is clearly an important mechanism in determining the outcome of *Pneumocystis* infections.

Hyperoxia is known to have detrimental effects on the lung, including modulation of alveolar epithelial cell function (1). Continuous exposure to high concentrations of oxygen leads to lung injury and death in a variety of mammalian species, including mice. Based on these observations, hyperoxia has been used as a biologically relevant stimulus for acute lung injury in animal models. Using these models, the important contributions of inflammatory mediators and the protective effects of antioxidants have been defined (9, 16). A central feature of lung injury due to hyperoxia is death of alveolar epithelial cells and pulmonary capillary endothelial cells by both necrosis and apoptosis (23), leading directly to noncardiogenic pulmonary edema and respiratory failure.

In the present study we have identified a likely mechanism by which a combination of two sublethal insults, *P. murina* infection and transient hyperoxia, leads to lung injury and death.
Each stress induces a component of the Fas-Fas ligand apoptotic pathway. In particular, hyperoxia leads to increased cell surface Fas expression by alveolar epithelial cells, in effect priming the cells for death in the setting of an inflammatory stimulus inducing increased Fas ligand expression in the lung. We have shown that P. murina infection is just such a stimulus, inducing increased soluble Fas ligand in lung homogenates. Cell-associated Fas ligand on the surface of inflammatory cells recruited to the lung contributed to activation of this pathway. Indeed, we found that P. murina infection increased Fas ligand expression on macrophages, lymphocytes, and neutrophils in our model. In this context, it is noteworthy that CD8+ T-cells are increased in the lungs of CD4+ depleted mice exposed to P. murina (4, 6), and that CD8+ cells are an important source of Fas ligand (15).

Previous studies have found that crosslinking Fas in the alveolar airspace by intratracheal inoculation with antibodies or recombinant Fas ligand induces modest lung injury (19, 20). Repeated intratracheal inoculation causes a chronic injury leading to fibrosis (21). We have found that alveolar epithelial cells from normal (unstressed) mice express modest Fas on the cell surface, consistent with this result. However, following exposure to hyperoxia, expression of Fas is increased significantly, leading to widespread cell death following interaction with Fas ligand, with resultant mortality. These in vivo experiments, therefore, directly extend previous in vivo work demonstrating that Pneumocystis β-glucans activate dendritic cell cytokine elaboration through a Fas-Fas ligand-mediated mechanism (10). Taken together, the data indicate that modulation of the Fas-Fas ligand pathway is an important regulator of pulmonary inflammation in response to Pneumocystis.

Our previous work using lung homogenates demonstrated increased apoptosis without identifying the specific cells affected. In our current work, we have examined type II alveolar
epithelial cells isolated from *P. murina*-infected mice exposed to hyperoxia. We have confirmed that these type II alveolar epithelial cells have been induced to express cell-surface Fas and are apoptotic. Previously, we demonstrated staining for activated caspase 3 in the alveolar wall, and in the present work we found that Fas was induced by hyperoxia in cells along the alveolar wall. Based on these findings, we believe it likely that type I alveolar epithelial cell apoptosis also plays an important role in lung injury during hyperoxic stress in the setting of *P. murina* infection. We cannot exclude an additional contribution by capillary endothelial cells.

The dynamics of GM-CSF expression in the alveolar space are quite complex. In our previous work we have shown that lung GM-CSF expression is altered by both hyperoxia and by *P. murina* infection. GM-CSF is induced in the lung as part of the host response to *P. murina* (25). In support of the critical role of this endogenous GM-CSF in defense against this infection, mutant mice lacking GM-CSF are significantly more susceptible to severe infection than wild type controls, while mutant mice over expressing GM-CSF in the lung are less susceptible to *P. murina* infection, even in the setting of CD4+ cell depletion. Exposure of normal mice to hyperoxia leads to significantly reduced alveolar epithelial cell expression of GM-CSF and impaired pulmonary host defense (3). Mice over expressing GM-CSF in the alveolar space are significantly more tolerant of hyperoxia than wild-type controls, and treatment with recombinant GM-CSF decreases alveolar epithelial cell apoptosis in vivo and in vitro (24). Interestingly, hyperoxia alone leads to decreased pulmonary expression of GM-CSF (3).

We have now extended our previous observations to show that GM-CSF is protective in a more complex model involving the combination of infection and hyperoxic stress. The specific mechanisms by which GM-CSF exerts its beneficial effects are not clear. We found previously that GM-CSF exerts direct effects on type II alveolar epithelial cells in vitro. Conversely,
treatment with GM-CSF reduced soluble Fas ligand expression in the lungs of *P. murina-* infected mice exposed to hyperoxia. It is possible that GM-CSF also alters Fas expression in the hyperoxia-exposed lung. However, Western blot analysis for Fas in lung homogenates has not shown clear effects of GM-CSF on total lung Fas expression (data not shown). Additional effects of GM-CSF on epithelial cell barrier function or anti-oxidant defenses are the subjects of ongoing studies.

It has been recognized in humans that treatment with corticosteroids can significantly reduce progression to respiratory failure in HIV patients with moderately severe *P. jirovecii* pneumonia (2, 7, 13, 22). Although it has been presumed that this benefit is due to suppression of host immune and inflammatory responses, there has not been an animal model to allow more specific definition of the mechanisms involved. We now show for the first time that dexamethasone treatment improves survival in *P. murina* infected mice exposed to hyperoxia, and also decreases Fas ligand expression in the lung.

In considering the decreased mortality we observed with GM-CSF and dexamethasone treatment, we considered whether organism burden could be a factor. The infected mice entered hyperoxia four weeks after intratracheal inoculation with *P. murina*, assuring heavy organism burdens. Interestingly, in our previous work, the lungs of *P. murina*-infected mice that were exposed to sublethal hyperoxia actually contained fewer *P. murina* RNA copies than the lungs of similarly inoculated mice that were maintained in normoxia (5). Furthermore, alveolar macrophages obtained from the lungs of mice exposed to sublethal hyperoxia demonstrated enhanced phagocytosis of *P. murina* organisms. Therefore, we argued that increased organism burden was not a mechanism contributing to mortality in mice exposed to hyperoxia. Because the mice treated with vehicle, dexamethasone, and GM-CSF entered the survival experiments
with comparable organism burdens and intense pulmonary inflammation, it seems unlikely that
differences in organism burden after return to normoxia was the cause of improved survival.

In conclusion, we investigated the mechanisms leading to lung injury in *P. murina-*infected mice exposed to hyperoxic stress. We have found that *P. murina* infection induces Fas ligand expression in the lung, while hyperoxia leads to increased alveolar epithelial cell Fas expression. The dual induction of Fas and Fas ligand provides a potential mechanism to explain increased alveolar epithelial cell apoptosis, leading to lung injury and death. Treatment with dexamethasone reduces Fas ligand expression and improves survival, and provides a clinically relevant mechanism by which corticosteroids may improve survival in patients with *P. jirovecii* pneumonia. Additionally, treatment with GM-CSF, a growth factor previously shown to protect alveolar epithelial cells against apoptosis, reduces Fas ligand expression and improves survival in our model. Whether GM-CSF therapy has a role in the treatment of humans with *P. jirovecii* infection remains to be explored.
Acknowledgments

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FIGURE LEGENDS

Figure 1. P. murina infection and hyperoxia interact to cause increased apoptosis of alveolar epithelial cells. Mice were exposed to air or hyperoxia for 4 days, with and without exposure to P. murina, as described in Methods. Type II alveolar epithelial cells were isolated as described. The alveolar epithelial cells were stained for caspase 3 to quantify apoptosis. Data represent means ± SEM (n = 3 mice per group performed in triplicate; * P < 0.05 compared with all other groups by ANOVA).

Figure 2. Hyperoxia induces Fas expression in alveolar epithelial cells, examined in vitro, after exposure to hyperoxia. Mice were exposed to air or hyperoxia for 4 days, with and without exposure to P. murina as described in Methods. Type II alveolar epithelial cells were then isolated and were stained for JO-2 to detect Fas expression. Data represent means ± SEM (n = 3 mice per group performed in triplicate; * P < 0.05 for comparisons between groups as indicated by ANOVA).

Figure 3. Hyperoxia induces Fas expression in lung alveolar wall cells in vivo after exposure to hyperoxia. Mice were exposed to air or hyperoxia for 4 days, as described in Methods. Lung sections were then prepared and were stained for JO-2 to detect Fas expression. An irrelevant control antibody did not result in significant staining (Panel A), and there was only modest Fas expression in the lungs of mice exposed to air (Panel B). Exposure to hyperoxia greatly increased Fas expression (Panel C). Original magnification 125X.
Figure 4. Hyperoxia does not significantly increase Fas ligand expression in lung homogenates. Groups of C57BL/6 mice were maintained in normoxia or were exposed to hyperoxia for 4 days, as described in Methods. At the conclusion of the exposure period, lungs of mice were homogenized and Fas ligand expression was measured by ELISA. Data represent means ± SEM on measurements performed in triplicate for individual mice (n = 3 mice per group; * P < 0.05 by t-test).

Figure 5. P. murina infection increases Fas ligand expression in lung homogenates. Groups of C57BL/6 mice were depleted of CD4+ T cells, and were inoculated with P. murina or vehicle. After four weeks, lungs of mice were homogenized and Fas ligand expression was measured by ELISA. Data represent means ± SEM on measurements performed in triplicate for individual mice (n = 5 mice per group; * P < 0.05 by t-test).

Figure 6. Dexamethasone and GM-CSF reduce Fas ligand expression in lung homogenates of mice with P. murina infection and hyperoxia exposure. Groups of C57BL/6 mice were depleted of CD4+ T cells, and were inoculated with P. murina. After four weeks, groups of mice were exposed to hyperoxia for four days. At the conclusion of the exposure period, lungs of mice were homogenized and Fas ligand expression was measured by ELISA. Data represent means ± SEM on measurements performed in triplicate for individual mice (n = 5 mice per group; * P < 0.05 for comparisons between groups as indicated by ANOVA).

Figure 7. Dexamethasone treatment improves survival in mice with P. murina infection and sublethal hyperoxia exposure. Groups of C57BL/6 mice were depleted of CD4+ T cells and
inoculated with *P. murina*. After four weeks of infection, mice were randomized to receive
dexamethasone or vehicle as described in Methods. Mice were exposed to sublethal hyperoxia
and were then returned to normoxia. After return to normoxia, there was a significant
improvement in survival in mice treated with dexamethasone compared with control mice (n =
24 mice per group; P < 0.03 by Kaplan-Meier analysis).

**Figure 8.** *GM-CSF treatment improves survival in mice with P. murina infection and sublethal
hyperoxia exposure.* Groups of C57BL/6 mice were depleted of CD4+ T cells and inoculated
with *P. murina*. After four weeks of infection, mice were randomized to receive GM-CSF or
vehicle as described in Methods. Mice were exposed to sublethal hyperoxia and were then
returned to normoxia. After return to normoxia, there was a significant improvement in survival
in mice treated with GM-CSF compared with control mice (n = 15 mice per group; P < 0.05 by
Kaplan-Meier analysis).

**Figure 9.** *Dexamethasone and GM-CSF treatment reduce histone-associated DNA in lung
homogenates of mice with P. murina infection and hyperoxia exposure.* Groups of C57BL/6
mice were depleted of CD4+ T cells and were inoculated with *P. murina*. After four weeks of
infection, mice received vehicle, dexamethasone, or GM-CSF as described in Methods. After
four days of hyperoxia, lung homogenates were prepared. Compared with the vehicle control,
dexamethasone and GM-CSF significantly inhibited apoptosis, as measured by histone-
associated DNA (n = 8 mice per group; * P < 0.05 as indicated by ANOVA).
FIGURE 1

![Graph showing cleaved caspase-3 positive cells (%) for different conditions: Air, Air + P. murina, Hyperoxia, and Hyperoxia + P. murina. The graph indicates a significant difference (*) between Hyperoxia and Hyperoxia + P. murina.]
FIGURE 2

![Graph showing the percentage of JO-2 positive cells under different conditions: Air, Air + P. murina, Hyperoxia, and Hyperoxia + P. murina.](image-url)
FIGURE 3
FIGURE 4

![Bar graph showing Fas ligand levels in Normoxia and Hyperoxia](http://iai.asm.org/)

- **Y-axis**: Fas ligand (pg/ml homogenate)
- **X-axis**: Normoxia vs. Hyperoxia
FIGURE 5

![Bar graph showing Fas ligand levels with and without P. murina](http://iai.asm.org/)
FIGURE 6

![Bar chart showing Fas ligand levels in vehicle, dexamethasone, and GM-CSF conditions.](chart.png)

- Vehicle: High Fas ligand levels
- Dexamethasone: Moderate Fas ligand levels
- GM-CSF: Lower Fas ligand levels

Significant differences indicated by asterisks (*)
FIGURE 7

- Days after return to normoxia
- Survival (%)

Legend:
- Vehicle
- Dexamethasone
FIGURE 8

Days after return to normoxia

Survival (%)
FIGURE 9
Cleaved caspase-3 positive cells (%) for different conditions:

- **Air**
- **Air + P. murina**
- **Hyperoxia**
- **Hyperoxia + P. murina**

* indicates statistical significance.
Days after return to normoxia

Survival (%)