Efficient resolution of *Pneumocystis murina* infection in surfactant protein A-deficient mice following withdrawal of corticosteroid-induced immunosuppression

Michael Linke,1,2 Alan Ashbaugh,2 Judith Koch,2 Reiko Tanaka2 and Peter Walzer1,2

1Department of Veterans Affairs Medical Center, Research Service, 3200 Vine St, Cincinnati, OH 45220, USA
2University of Cincinnati College of Medicine, Department of Internal Medicine, Division of Infectious Diseases, 231 Bethesda, Cincinnati, OH 45221, USA

Following withdrawal of immunosuppression, surfactant protein A (SP-A)-deficient and wild-type mice cleared *Pneumocystis murina* infection in a similar manner, but exhibited significant differences in lymphocyte populations, interleukin (IL)-6 levels and chemokine expression levels. A higher percentage of lymphocytes were detected in lung lavage fluid from SP-A-deficient mice, but more CD4+ T cells were isolated from lung tissue of wild-type mice. Higher concentrations of IL-6 were detected in lavage fluid and enhanced expression of lymphotactin and RANTES were detected in the lungs of wild-type mice. Equal levels of surfactant protein D were detected in SP-A-deficient and wild-type mice and no differences were detected in markers of lung injury between the two strains of mice. Thus, SP-A does not enhance organism clearance, but does modulate the host immune response during resolution of *P. murina* infection.

**INTRODUCTION**

*Pneumocystis* pneumonia (PcP) results in significant morbidity and mortality in immunocompromised patients (Morris et al., 2004; Thomas & Limper et al., 2004). Initiation of anti-PcP therapy can result in a deleterious hyperpulmonary inflammatory response and lead to severe lung damage (Barry et al., 2002; Koval et al., 2002; Limper et al., 1989; Nuesch et al., 1999; Sepkowitz, 2002).

Surfactant protein A (SP-A) is a component of the innate immune system and is implicated in the host response to numerous pulmonary pathogens (McCormack & Whitsett, 2002). The major antimicrobial activity of SP-A is mediated through interaction of the molecule with surface carbohydrates of microbes, resulting in enhanced recognition and phagocytosis by alveolar macrophages (McNeely & Coonrod, 1993; Van Iwaarden et al., 1994; Zimmerman et al., 1992). SP-A has also been shown to have direct microbiocidal activity against both bacteria and fungi (McCormack et al., 2003; Wu et al., 2003). In addition to its antimicrobial properties, SP-A modulates the host pulmonary inflammatory response. The development of SP-A-deficient (KO) mice by targeted gene disruption has provided a model to analyse the role of this molecule in the host response to pulmonary pathogens *in vivo* (Korfhagen et al., 1998). Several studies have demonstrated that KO mice are more susceptible to viral and bacterial infections and in general develop a more robust inflammatory response than wild-type (WT) mice (LeVine et al., 1997, 1998, 1999).

KO mice develop more intense *Pneumocystis murina* infections than WT mice following corticosteroid-induced immunosuppression or antibody-mediated CD4+ T-cell depletion (Linke et al., 2001, 2005; Atochina et al., 2004). The enhanced levels of *P. murina* infection in immunosuppressed KO mice demonstrate that this molecule limits the infection and is capable of modulating the host response during active infection in the immunocompromised host.

In the present study, the role of SP-A in the resolution of a fulminant *P. murina* infection was examined. Experiments were performed to test the hypothesis that SP-A enhances clearance of the infection, limits the inflammatory response and decreases lung damage during immune-mediated resolution of *P. murina* infection.

**METHODS**

Immunosuppression was induced in C3H/HeN KO or WT mice by the addition of dexamethasone (DEX) to their drinking water and they were infected with *P. murina* through intratracheal inoculation.
as described previously (Linke et al., 2001, 2005). Immunosuppression was continued for 4 weeks to allow development of fulminant *P. murina* infection and the DEX treatment was then stopped. Stopping DEX treatment restores host immune responses and initiates resolution of the infection (Walzer et al., 1984, 1987). On days 1, 7 and 14 after withdrawal of DEX, mice were weighed and sacrificed and the lungs were processed as described previously (Linke et al., 2005). Briefly, the lungs were lavaged and the lung tissue was flash-frozen in liquid nitrogen. Lavage fluid was stored on ice until further processing. The frozen lung tissue was ground into a fine powder, weighed and reconstituted in PBS. *P. murina* was purified from the homogenate and slides were prepared for microscopic quantification of *P. murina* cyst forms. Immune cells were recovered from the lavage fluid by centrifugation, slides were made for differential counting and enumerated as described previously (Linke et al., 2005). As described previously, mononuclear cells were purified from lung tissue homogenates, CD4+ and CD8+ T cells were identified by cell-surface antibody staining and enumerated by flow cytometry analysis on days 7 and 14 after withdrawal of DEX (Thullen et al., 2004). Cytokine concentrations were quantified in lavage fluid by ELISA (R&D Systems) as described previously (Linke et al., 2005). Surfactant protein D (SP-D) levels in lavage supernatants were analysed by SDS-PAGE and immunoblot as described previously (Elhalwagi et al., 1999). Chemokine expression was monitored by a ribonuclease protection assay (RPA). RPA analysis was performed on total RNA isolated from frozen lung tissue with the Trizol reagent using a BD RiboQuant mouse chemokine multi-probe template set as described previously (Thullen et al., 2004).

**RESULTS AND DISCUSSION**

Withdrawal of DEX treatment resulted in a significant steady decline in the number of *Pneumocystis* cyst forms detected in the lungs of KO and WT mice, with no significant differences between WT and KO mice infection levels at any of the time points (Fig. 1). These results indicate that restoration of the immune response allows other factors to compensate for the lack of SP-A in the KO mice, resulting in the same efficiency of clearance detected in the WT mice.

The percentage of lymphocytes detected in lavage fluid increased significantly in KO mice by day 7, but a significant increase was not detected until day 14 in the WT mice (Fig. 2). In addition, the percentage of lymphocytes detected in the KO mice was significantly higher than that detected in the WT mice at the day 7 time point. There was no significant difference in lymphocyte percentages between the groups at day 14. The detection of a higher percentage of lymphocytes in lavage fluid from the KO mice indicates that SP-A inhibits migration of these cells out of the lungs and into the airways. Increases in lymphocyte percentages in the lungs have been associated with development of hyperinflammatory response in patients with PCP following initiation of antiretroviral and anti-PcP therapy. There were no significant differences in the percentages of neutrophils, alveolar macrophages or eosinophils between WT and KO mice at any time point (data not shown). To investigate further the effects of SP-A on lymphocyte migration to the lungs during resolution of PcP, CD4+ and CD8+ T cells were enumerated in lung tissue. Surprisingly, at day 7, no significant differences were evident in CD4+ or CD8+ T-cell numbers between the WT and KO mice and, at day 14, significantly fewer CD4+ T cells were detected in the lungs of KO mice than in WT mice. There were no significant differences in the number of CD8+ T cells between the WT and KO mice at the day 14 time point (Fig. 3). These results suggest that the increased percentage of lymphocytes detected in the lavage fluid is not the result of enhanced migration of lymphocytes to the lung and suggest that, in this model, SP-A may enhance recruitment of CD4+ T cells.

**Fig. 1.** Clearance of *P. murina* from the lungs of wild-type (WT) mice and mice deficient in SP-A (KO). The limit of detection of *P. murina* is approximately \(2.5 \times 10^4\) cyst forms per lung (log_{10} 4.4). Data were expressed as \(\log_{10}\) cysts per lung. Horizontal lines indicate mean log cysts per group. Each point represents a single mouse. The results shown are representative of three independent experiments. Infection levels were compared between all of the WT and KO groups by ANOVA with a Tukey–Kramer multiple comparisons test. *, \(P < 0.001\) compared with day 1 of same background.

**Fig. 2.** Percentage of lymphocytes detected in lavage fluid from WT (open bars) and KO (shaded bars) mice by differential counting. Results were pooled from two experiments and each group contained at least five mice. *, \(P < 0.001\) compared with day 7 WT; #, \(P < 0.05\) compared with day 1 of same background as determined by ANOVA with a Tukey–Kramer multiple comparisons test.
to the lung during clearance of *P. murina*, but may inhibit migration of lymphocytes out of the lungs.

To identify factors that might be involved in the enhanced trafficking of lymphocytes to the lungs in WT mice, chemokine expression levels were analysed in lung tissue by RPA. Approximately twofold increases in expression of RANTES and lymphotactin (Ltn) were detected in WT mice at day 1 compared with KO mice (Fig. 4). Significantly higher relative expression levels were detected in the WT mice at this time point as determined by the Mann–Whitney test. RANTES and Ltn are both associated with recruitment of lymphocytes to areas of inflammation and enhanced RANTES expression has been described during clearance of *P. murina* infection following immunoreconstitution and the expression was localized to areas of infection (Wright *et al.*, 1999). The increased levels of RANTES and Ltn in the WT mice at the day 1 time point does not correlate with the cell enumeration data described above, but suggests that SP-A enhances expression of these chemokines during clearance of infection; however, the physiological effects of this increased expression are unclear.

Expression of many cytokines that have been implicated in the host response to *P. murina* has also been shown to be modulated by SP-A. Cytokine concentrations were measured in lavage fluid to characterize the effects of SP-A on cytokine expression during recovery from *P. murina* infection. There were no differences in tumour necrosis factor alpha (TNF-α), interferon gamma (IFN-γ) or interleukin (IL)-1 expression levels between WT and KO mice at any of the time points (data not shown). In both WT and KO mice, low levels of IL-6 were detected at day 1 followed by significant increases at day 7 and day 14; however, by day 14, WT mice had significantly higher levels of IL-6 than did KO mice (Fig. 5). IL-6 is usually classified as a proinflammatory cytokine, but in *P. murina*-infected mice it...
has been shown to dampen the inflammatory response (Chen et al., 1993). SP-A has been shown to have divergent effects on IL-6 expression. Increased levels of IL-6 were detected in SP-A-deficient mice infected with Pseudomonas or respiratory syncytial virus (LeVine et al., 1998, 1999). In Mycobacterium tuberculosis-infected macrophages, SP-A enhances expression of IL-6; however, in non-infected macrophages, SP-A was shown to suppress IL-6 expression (Gold et al., 2004). The decreased expression of IL-6 in KO mice detected in the current study suggests that SP-A enhances expression of IL-6 during clearance of P. murina following withdrawal of DEX and that IL-6 may have divergent effects during clearance of P. murina following withdrawal of DEX. At day 7, increased IL-6 levels were associated with increased percentages of lymphocytes detected in lavage fluid of KO mice, but increases in IL-6 concentration at day 14 correlated with increased numbers of CD4 + T cells in the lungs of WT mice.

SP-D levels were measured in lavage supernatant to determine whether the other innate pulmonary immune molecule was upregulated in the KO mice during clearance of P. murina infection. Immunoblot analysis demonstrated that SP-D levels increased during the course of the experiment in both strains of mice, and there were no apparent differences in expression levels between KO and WT mice at any of the time points (data not shown). These results suggest that SP-D is not upregulated in the KO mice to compensate for the lack of SP-A.

Lung weights and lung weight:body weight (LW:BW) ratios were used as a marker of lung injury during the inflammatory response. Both factors increased over time during clearance of the infection following withdrawal of the DEX treatment (data not shown). No significant differences were detected between the WT and KO mice in the lung weight or the LW:BW ratio at any time point. These results indicate that the lack of SP-A in the KO mice did not result in enhanced lung injury.

Characterization of molecules involved in the host response during resolution of PCP may provide insight into the causes of the hyperinflammatory conditions associated with clearance of the infections in humans and lead to better therapies to prevent its occurrence. The findings in this report suggest that SP-A does not enhance the clearance of P. murina, but can modulate the host response during resolution of PCP.

REFERENCES


