TNF Receptor Signaling Contributes to Chemokine Secretion, Inflammation, and Respiratory Deficits during Pneumocystis Pneumonia

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TNF Receptor Signaling Contributes to Chemokine Secretion, Inflammation, and Respiratory Deficits during Pneumocystis Pneumonia

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CD8⁺ T cells contribute to the pathophysiology of Pneumocystis pneumonia (PcP) in a murine model of AIDS-related disease. The present studies were undertaken to more precisely define the mechanisms by which these immune cells mediate the inflammatory response that leads to lung injury. Experimental mice were depleted of either CD4⁺ T cells or both CD4⁺ and CD8⁺ T cells and then infected with Pneumocystis. The CD4⁺-depleted mice had significantly greater pulmonary TNF-α levels than mice depleted of both CD4⁺ and CD8⁺ T cells. Elevated TNF-α levels were associated with increased lung concentrations of the chemokines RANTES, monocyte chemoattractant protein 1, macrophage-inflammatory protein 2, and cytokine-induced neutrophil chemoattractant. To determine whether TNFR signaling was involved in the CD8⁺ T cell-dependent chemokine response, TNFRI- and II-deficient mice were CD4⁺ depleted and infected with Pneumocystis. TNF-deficient mice had significantly reduced pulmonary RANTES, monocyte chemoattractant protein 1, macrophage-inflammatory protein 2, and cytokine-induced neutrophil chemoattractant responses, reduced inflammatory cell recruitment to the alveoli, and reduced histological evidence of PcP-related alveolitis as compared with infected wild-type mice. Diminished pulmonary inflammation correlated with improved surfactant activity and improved pulmonary function in the TNF-deficient mice. These data indicate that TNFR signaling is required for maximal CD8⁺ T cell-dependent pulmonary inflammation and lung injury during PcP and also demonstrate that CD8⁺ T cells can use TNFR signaling pathways to respond to an extracellular fungal pathogen.

Pneumocystis is an opportunistic pulmonary pathogen that produces a life-threatening pneumonia in patients suffering from a variety of immune deficiencies, including AIDS (1, 2). The clinical syndrome of Pneumocystis pneumonia (PcP)³ has been well described in terms of presentation, diagnosis, therapeutic interventions, and clinical outcome. However, much more is known about the specific injury mechanisms and pathways contributing to respiratory impairment. Although Pneumocystis organisms may have the potential to directly injure the lung, indirect mechanisms of lung injury mediated by the host’s immune system are becoming increasingly recognized. For example, the clinical syndrome of PcP-related immunorestitution disease can occur when CD4⁺ T cell-mediated immunity is restored following a period of immunosuppression in patients with asymptomatic or mildly symptomatic Pneumocystis infection (3, 4). The severity of PcP in this clinical setting is related to the intensity of the inflammatory response mounted against the preexisting infection. Similarly, the degree of pulmonary inflammation, but not organism burden, correlates with impaired lung function and a poor prognosis in AIDS patients with PcP (5–8). Although distinct cellular mediators are likely responsible for lung injury in these two clinical settings, the importance of inflammatory mechanisms in the pathophysiology of PcP is emphasized.

PcP-related accumulations of CD8⁺ T cells have been noted in the lungs of AIDS patients (9–11), CD4⁺ T cell-depleted mice (CD4⁺-depleted) (12, 13), steroid-treated rats (14), and SIV-infected macaques (15). Although it has been postulated that these cells are partially protective against Pneumocystis infection in the absence of fully functional CD4⁺ T cell-mediated immunity, a specific mechanism of protection has not been identified (12, 16). In contrast to these studies of host defense, we have been interested in the potential role of CD8⁺ T cells in lung injury during PcP. We recently demonstrated that the presence of CD8⁺ T cells causes significant pulmonary inflammation and contributes to PcP-related respiratory impairment in a CD4⁺ T cell-depleted mouse model of infection (17, 18). CD8⁺ T cell-mediated inflammation included a significant influx of lymphocytes and polymorphonuclear cells (PMNs), was associated with increased alveolar-capillary permeability, and decreased lung compliance, oxygenation, and pulmonary surfactant activity. However, the specific mechanisms involved in the generation of this inflammatory lung injury remain unknown. More defined studies of CD8⁺ T cell-mediated inflammation may help identify therapeutic targets to mitigate or prevent inflammatory-mediated lung injury during PcP.

TNF-α is a potent proinflammatory cytokine secreted by many cell types following tissue injury or infection. The numerous actions of TNF are mediated through two widely distributed and structurally related receptors, TNFRII (murine p55) and TNFRI

*Abbreviations used in this paper: PcP, Pneumocystis carinii pneumonia; BAL, bronchoalveolar lavage; PMN, polymorphonuclear cell; RPA, RNase protection assay; MCP-1, monocyte chemoattractant protein 1; MIP-2, macrophage-inflammatory protein 2; KC, cytokine-induced neutrophil chemoattractant; AEC, alveolar epithelial cell.
(murine p75) (19). It must be noted that these receptors also bind homotrimeric lymphotixin-α (TNF-β), but signaling is much less efficient than that induced by TNF (19, 20). TNF is released following exposure to Pneumocystis (21–23) and is involved in protection against initial Pneumocystis infection (24), clearance of organisms from the lung following recovery of immune function (25, 26), and control of Pneumocystis burden in CD4+ T cell-depleted mice (24). In addition, TNF is involved in the IFN-γ-stimulated killing of Pneumocystis by alveolar macrophages (27), and knockout of both TNFRs in combination with IFN-γ renders otherwise immunocompetent mice susceptible to Pneumocystis infection (28). Although these findings implicate TNF in several aspects of host defense against Pneumocystis, a role for TNF in PcP-related lung injury has not been directly proven. Previous studies have reported a temporal correlation between pulmonary TNF levels and chemokine expression, inflammatory cell recruitment, and pulmonary function deficits in a mouse model of PcP-related immunorestitution disease (17, 18, 29). Elevated TNF levels were also reported in the lungs of CD4+ T cell-depleted mice following inoculation with Pneumocystis (21, 24). However, it remains unclear whether CD8+ T cells are involved in the generation of this TNF response or whether TNF is involved in the CD8+ T cell-mediated damage observed in this model of PcP.

The multitude of functions ascribed to TNF include, but are not limited to, the modulation of inflammatory cell recruitment and activation by stimulating the secretion of a broad range of chemokines (30–33). Chemokines are a superfamily of chemotactic peptides that play a role in the recruitment of specific leukocyte populations to sites of infection or tissue damage (34, 35). They have been implicated in the initiation and amplification of a variety of pulmonary inflammatory responses, including those associated with Pneumocystis (29, 36, 37). Importantly, TNF-dependent chemokine secretion contributes to inflammation and lung injury in a murine model of CD8+ T cell-mediated bystander lung injury (32, 38). The association of persistent CD8+ T cell and PMN recruitment with respiratory impairment and a poor prognosis in both human and animal models of PcP suggests that chemokines specific for these cell populations may be involved in CD8+ T cell-mediated lung injury during PcP.

Although CD8+ T cells alone cannot protect against Pneumocystis infection in the absence of CD4+ T cells, they do generate an ineffective inflammatory response that contributes to PcP-related lung injury. The role of inflammatory mediators such as TNF and/or specific chemokines in this damaging host response has not been determined. Therefore, studies were performed to determine whether the presence of CD8+ T cells alters the secretion of TNF and/or chemokines during PcP and to determine whether chemokine secretion, inflammatory cell recruitment, and respiratory impairment are dependent upon functional TNFR signaling.

Materials and Methods

Pneumocystis source animals

Female SCID mice (C.B-17.H-129S6/SvEvTac–Prkdcsnd) were purchased from Taconic Farms (Germantown, NY). The mice were housed in microisolator cages and fed sterilized food and water. To induce infection, SCID mice were cohoused with Pneumocystis-infected SCID mice for 6 wk. At 10–12 wk after the initiation of cohousing, the mice were sacrificed and the lungs were used as a source of Pneumocystis organisms (17, 39). Slides of the Pneumocystis suspension were prepared and Pneumocystis cysts were enumerated by a standard ammoniacal silver staining method.

Mouse models of PcP

For initial studies of the CD8+ T cell-mediated inflammatory response during PcP, female C57BL/6 mice were depleted of CD4+ T cells or both CD4+ and CD8+ T cells and then infected with Pneumocystis as previously described (17). For studies of TNF signaling, 6- to 8-wk-old female mice genetically deficient in both TNFRI and TNFRII (B6;129S-Tnfrsf1atm20thx Tnfrsf1btm1Imx) were purchased from The Jackson Laboratory (Bar Harbor, ME). TNFR-deficient mice lack both receptors for TNF and therefore cannot respond to TNF or homotrimerized lymphotixin-α. The initial characterization of TNFR-deficient mice found that thymopoesis, lymphoid cellularity, and gross histology of spleen and lymph nodes showed no obvious abnormalities (40). However, these mice do demonstrate aberrant germinal center formation in the secondary lymphoid tissue. Age-matched female wild-type control mice (B6;129S2/Fj) were also purchased from The Jackson Laboratory. Both groups of mice were depleted of both CD4+ T cells with two weekly i.p. injections of anti-CD4 mAb (clone TIB 207; American Type Culture Collection, Manassas, VA). Injections of mAbs were begun at least 4 days before inoculation and were continued for the entire duration of the experiment. This anti-CD4 mAb treatment regimen resulted in >98% CD4+ T cell depletion from the lymphocyte population of the spleen and bronchoalveolar lavage (BAL) compartments.

Respiratory rate determination.

Respiratory rates of experimental mice were measured using a whole body plethysmograph (Buxco Electronics, Sharon, CT). A constant air flow of 1 L/min was maintained using a four-channel bias flow regulator (Buxco Electronics) connected to the plethysmograph. A single mouse was placed in a plastic tube with openings at both ends to restrict activity and give a more accurate measure of respiratory rate. The plastic tube containing the mouse was then placed into the plethysmograph chamber and the mouse was allowed to acclimate for ~5 min. Changes in air flow through the chamber corresponded to the animal’s breathing pattern and were measured by a flow transducer. Data were collected and analyzed using the Biosystems XA software package (Buxco Electronics), and respiratory rates were calculated as breaths per minute from the flow data. Breathing rejection criteria for the Biosystems XA software were set as follows to increase the probability that only real breaths contribute to the actual respiratory rate: maximum inspiratory time of 10 s; minimum inspiratory time of 0.04 s; and minimum tidal volume of 0.04 ml.

Physiologic assessment of pulmonary compliance and resistance in live, ventilated mice.

Dynamic lung compliance and resistance was measured in live mice using a previously described method with modifications (17). Mice were anesthetized by i.p. injection of 0.13 mg of sodium pentobarbital/g body weight. A tracheostomy was performed and a 20-gauge cannula was inserted 3 mm into an anterior nick in the exposed trachea. The thorax was then opened to equalize airway and transpulmonary pressure. To assure that the mice tolerated the procedure, they were examined for spontaneous respirations before proceeding further. Mice were immediately placed into a plethysmograph designed for anesthetized mice (Buxco Electronics) and connected to a Harvard rodent ventilator (Harvard Apparatus, Southnatick, MA). Mice were ventilated with a tidal volume of 0.01 ml/g body weight at a rate of 150 breaths per minute. Respiratory flow and pressure were measured using transducers attached to the plethysmograph chamber. Data were collected and analyzed using the Biosystems XA software package (Buxco Electronics). Dynamic lung compliance was calculated in milliliters per centimeter cm H2 O from the flow and pressure signals and then normalized for body weight. Resistance values were calculated in centimeters H2 O per milliliter per second from the same signals.

BAL and lung tissue preparation.

BAL and lung tissue samples were obtained following dynamic compliance measurements. The chest cavity was surgically opened to expose the lungs and trachea, and the left lung lobe was tied off securely at the bronchus with surgical silk and removed with sterile scissors. The isolated lung was immediately snap frozen in liquid nitrogen and stored at −80°C for RNA isolation. The remaining lung lobes were lavaged with four 1-ml aliquots of 1× HBSS via the tracheal cannula. Recovered lavage fluid (~3.5 ml/mouse) was centrifuged at 250 × g for 5 min to obtain the cellular fraction, and the supernatant was removed and frozen at −80°C for subsequent ELISA analyses of cytokine/chemokine content. The cells were resuspended in fresh HBSS, enumerated, centrifuged onto glass slides, and stained with Diff-Quick (Dade, Dudinghen, Switzerland) for differential
counting. In addition, multiparameter flow cytometric analysis was performed on BAL cells following staining with fluorochrome-conjugated Abs. Anti-CD4-fluorescein (clone RM4-4), anti-CD8a-peridinin chlorophyll-a protein (53-6-7), and anti-CD49/pan-NK-PE (clone DX5) were purchased from BD PharMingen (San Diego, CA). The anti-CD4 clone RM4-4 was used at a final concentration of 10 μg/ml because it is not blocked by the anti-CD4-depleting Ab. Stained BAL cells were analyzed on a FACSCalibur cell sorter (BD Biosciences, San Jose, CA). At least 10,000 events were routinely analyzed from the BAL of each Pneumocystis-infected experimental mouse. At least 5,000 events were analyzed from uninfected control mice.

In a subset of studies analyzing surfactant phospholipid content and surface activity, lavage was done on the whole intact lungs (without removal of the left lobe) with eight 1-ml aliquots of sterile 0.15 M NaCl. Recovered BAL fluid of 6.5–7 ml/mouse was centrifuged at 150 × g for 5 min to remove cells without affecting large surface active surfactant aggregates in the cell-free supernatant. Cell-free lavage was stored at −80°C. For lung tissue fixation, the left lobe was first removed for RNA isolation as described above. The remaining lung lobes were inflated with 15 cm H2O pressure using 10% Formalin fixative (Sigma-Aldrich, St. Louis, MO). The lungs were fixed for 10 min under gravity flow pressure and then carefully removed from the animal and placed in fixative for 1 h at 4°C. The lungs were rinsed and stored at 4°C in 70% ethanol. Before embedding, the right lung lobe of each animal was removed and placed in a tissue cassette. The lobe was embedded in paraffin, and 4-μm sections were cut from the tissue blocks. Slides were stained with a modified Giemsa stain to visualize lung architecture and inflammatory infiltrates.

**Enumeration of Pneumocystis**

Recent reports have described the use of real-time PCR techniques to quantify the *Pneumocystis* burden in lung specimens from both human patients and experimental animals (41–43). In the present study, a real-time PCR method was also used to enumerate *Pneumocystis* cyst DNA in the lungs of experimental mice. Following BAL, the lavaged right lung lobes were minced and frozen at −80°C. The frozen lungs were homogenized in 1 ml of sterile PBS/150 mg of lung tissue using a tissue homogenizer. The homogenizer was washed three times between samples with 10% sodium hypochlorite and rinsed three times with water to prevent cross-contamination. The homogenates were then boiled for 15 min, vigorously vortexed for 2–3 min, and then centrifuged for 5 min at 12,000 × g. The supernatant was carefully removed and stored at −80°C for real-time PCR analysis. Boiled lung DNA preparations from individual mice were assayed by quantitative PCR using TaqMan primer/fluorogenic probe chemistry and an Applied Biosystems Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). A primer/probe set specific for a 96-nt region of the mouse *Pneumocystis* keixin gene was designed using the Primer Express Software (Applied Biosystems) (44). The sequences of the primers and probe were as follows: forward primer, 5′-GACGCAGTTTATACCGGCTGTGTTG-3′; reverse primer, 5′-GAGCTATACGCGTTGCTG-3′; and fluorogenic probe, 5′-CGACACTGATCATCTCGGAGTCTTGTCCTC-3′. The quantitative PCR consisted of TaqMan Universal Master Mix (Applied Biosystems), 900 nM of each forward and reverse primer, 100 nM TaqMan probe, and 2.5 μl of DNA template in a total reaction volume of 25 μl. The thermocycler profile used was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Quantitation was determined by extrapolation against standard curves constructed from serial dilutions of known copy numbers of plasmid DNA containing the target keixin sequence. Data were analyzed using the Applied Biosystems Prism 7000 SDS version 1.0 software (Applied Biosystems) and is reported as total keixin DNA copies per right lung lobes.

**Surfactant content and surface activity measurements**

Cell-free BAL supernatants stored at −80°C were thawed and total phospholipid content was assessed by the colorimetric phosphate assay of Ames (47). Lavage supernatants were then centrifuged at 12,500 × g for 30 min to obtain large surfactant aggregates. The percentage of phospholipid pelleting as large aggregates was determined by a second phosphatase assay, and aggregates were then resuspended at a uniform concentration of 1 mg/ml 0.15 M NaCl for surface tension measurements on a pulsating bubble surfactometer (General Transco, Largo, FL, formerly Electronetics, Amherst, NY). This apparatus, based on the design of Enhorning (48), determines a physiologically relevant combination of overall surface tension lowering ability that includes the effects of both adsortion and dynamic film compression. A small air bubble, communicating with ambient air, was formed and pulsated between minimum and maximum radii of 0.4 and 0.55 mm by a precision pulsator moving liquid in and out of the sample chamber at 37°C. Bubble size was monitored through a microscope during continuous cycling at a rate of 20 cycles/min. The pressure drop across the air-liquid interface in the bubble (∆P) was measured with a pressure transducer, and surface tension (γ) was calculated from the Laplace equation for a sphere: ∆P = 2γ/radius. The accuracy of this data analysis procedure has been verified for air bubbles of the small size studied in this apparatus despite nonspherical shape deformations that occur at low surface tension (49). Surface activity data are reported here as surface tension at minimum bubble radius (minimum surface tension, γ_{min}) as a function of time from the initiation of bubble pulsation.

**Statistical analyses**

All values reported for each experimental group are mean ± 1 SE measurement. For each experiment, p values were determined by performing a one-way ANOVA with the SigmaStat software package (Jandel Scientific, San Rafael, CA). The Student-Newman-Keuls method was used for pairwise multiple comparisons of experimental groups.

**Results**

TNF production is associated with CD8+ T cell-dependent inflammation during PcP

To begin to understand the mechanisms by which CD8+ T cells contribute to inflammatory lung injury during PcP, TNF production was examined in the lungs of CD4+ T cell-depleted mice in the presence or absence of CD8+ T cells. Mice were depleted of either CD4+ T cells alone, or depleted of both CD4+ and CD8+ T cells, and then inoculated with *Pneumocystis*. By wk postoinoculation, mice depleted of only CD4+ T cells (i.e., with CD8+ T cells) had significantly greater pulmonary TNF mRNA and protein...
levels than mice additionally depleted of CD8+ T cells (Fig. 1). By 5 wk postinoculation, mice with CD8+ T cells demonstrated a nearly 3-fold increase in lung TNF mRNA and a nearly 8-fold increase in TNF protein in mice depleted of both CD4+ and CD8+ T cells (Fig. 1). Interestingly, the double-depleted mice did have higher TNF mRNA and protein levels than uninfected mice, suggesting that TNF production during PcP is not exclusively dependent upon CD8+ T cells (Fig. 1). These data showed that elevated TNF production was associated with the presence of CD8+ T cell-mediated inflammation during PcP.

To further characterize TNF signaling during PcP, steady-state mRNA levels of TNFRI and TNFRII were measured in the lungs of Pneumocystis-infected, CD4+ T cell-depleted mice, and in uninfected controls. TNFRI mRNA was constitutively expressed at similar levels in the lungs of both uninfected and infected mice (Fig. 2). In contrast, steady-state levels of TNFRII mRNA were nearly undetectable in uninfected mice, but elevated 2.4-fold in the lungs of mice infected with Pneumocystis (Fig. 2). Together, these data demonstrated that TNF and both TNFRs are expressed in the lung during PcP and that TNFRII mRNA levels are elevated in response to Pneumocystis infection. These data also suggested that TNF signaling pathways may be involved in CD8+ T cell-mediated inflammatory lung injury during PcP.

**Chemokine production during PcP is CD8+ T cell dependent**

CD8+ T cell-mediated inflammation during PcP was characterized by lymphocyte and PMN recruitment to the lung (17). Therefore, to determine the effect that CD8+ T cells have on pulmonary chemokine secretion during PcP, RANTES, MCP-1, MIP-2, and KC protein levels were measured in the BAL of mice depleted of either CD4+ T cells alone or depleted of both CD4+ and CD8+ T cells. By 5 wk postinoculation, chemokine levels in the lavage fluid were markedly increased by the presence of CD8+ T cells (Fig. 3). Mice depleted of only CD4+ T cells had a 5.5-fold greater concentration of RANTES, a 12-fold greater concentration of MCP-1, a 2.3-fold greater concentration of MIP-2, and a 16.8-fold greater concentration of KC in the lavage fluid than mice depleted of both CD4+ and CD8+ T cells (Fig. 3). Similar results were also observed when the steady-state mRNA levels of these chemokines were compared.

**Maximal CD8+ T cell-mediated inflammation during PcP is TNF dependent**

The CD8+ T cell-dependent chemokine production, inflammatory cell recruitment, and pulmonary function deficits observed during PcP were associated with increased production of TNF in the lung. Therefore, to determine whether TNF signaling through TNFRI
and/or TNFRII was directly involved in CD8\(^+\) T cell-mediated pulmonary inflammation, mice genetically deficient in both TNFRI and TNFRII (TNFR-deficient) were studied. Wild-type and TNFR-deficient mice were CD4\(^+\) T cell-depleted, infected with Pneumocystis, and then RANTES, MCP-1, MIP-2, and KC protein levels were measured in the BAL fluid at 3 and 5 wk postinoculation. At 3 wk postinoculation, MCP-1 and MIP-2 levels were slightly, but significantly, elevated in the lavage fluid of wild-type mice as compared with TNFR-deficient mice (Fig. 4, B and C). In contrast, RANTES and KC levels were not significantly altered by TNFR deficiency at this time. However, by 5 wk postinoculation, wild-type mice demonstrated a 5.7-fold greater concentration of RANTES, a 9.3-fold greater concentration of MCP-1, a 3-fold greater concentration of MIP-2, and a 2-fold greater concentration of KC in the BAL than TNFR-deficient mice (Fig. 4). All of these changes were statistically significant and suggested that maximal CD8\(^+\) T cell-mediated chemokine secretion during PcP was dependent on functional TNFR signaling pathways.

Since TNFR deficiency dramatically diminished the secretion of chemokines during PcP, the extent and nature of inflammatory cell recruitment in CD4\(^+\) T cell-depleted wild-type and TNFR-deficient mice were also analyzed. Uninfected wild-type and TNFR-deficient mice had comparable numbers of total cells recovered from the BAL fluid (Table I), of which >95% were alveolar macrophages. However, at 3 wk postinoculation with Pneumocystis, a slightly greater number of BAL cells was recovered from wild-type mice than from deficient mice (Table I). Furthermore, differential analysis demonstrated that significantly more lymphocytes (3.8-fold) and PMNs (14-fold) were recovered from the wild-type mice at this time (Table I). These differences were even more

![Graph](image1.png)

**FIGURE 3.** BAL chemokine levels in CD4\(^+\)- and CD4\(^+\)/CD8\(^+\) T cell-depleted mice with PcP. KC, MIP-2, RANTES, and MCP-1 levels were measured in the BAL of CD4\(^+\)- and CD4\(^+\)/CD8\(^+\) T cell-depleted mice at 5 wk postinoculation with Pneumocystis. BAL from uninfected immunocompetent mice was used as baseline controls for chemokine measurements. Values are means ± 1 SE measurement (n = 5), *, p < 0.05 as compared with uninfected mice. ***, p < 0.05 as compared with both uninfected mice and CD4\(^+\)/CD8\(^+\)-depleted mice.

![Graph](image2.png)

**FIGURE 4.** Chemokine levels in the BAL of CD4\(^+\) depletesed wild-type and TNFR-deficient mice with PcP. RANTES (A), MCP-1 (B), MIP-2 (C) and KC (D) levels were measured in the BAL of wild-type and TNFR-deficient mice at 0, 3, and 5 wk postinoculation with Pneumocystis. Values are means ± 1 SE measurement (n = 5). ***, p < 0.05 as compared with both uninfected mice (0 wk) and TNFR-deficient mice at the same time point. ***, p < 0.05 as compared with uninfectected mice (0 wk).
pronounced by 5 wk postinoculation, with a 3.4-fold greater number of BAL cells recovered from wild-type than TNFR-deficient mice (Table I). Again, the most striking difference was observed in the number of lymphocytes and PMNs recovered. A 10-fold greater number of lymphocytes and a 30-fold greater number of PMNs were recovered from wild-type mice than from deficient mice at this time (Table I). Flow cytometry demonstrated that 84 ± 2% of the BAL lymphocyte population from infected wild-type mice were CD8+ cells, while 3.8 ± 0.3% stained positive for a pan-NK cell marker. In contrast, the lymphocytes recovered from infected TNFR-deficient mice consisted of ~54 ± 2% CD8+ cells and 23 ± 2% pan-NK-positive cells.

To visualize the lung architecture of Pneumocystis-infected wild-type and TNFR-deficient mice, inflation-fixed lung sections were stained with a modified Giemsa stain and photographed under low power to provide a broad representation of airway and alveolar structures. Lung tissue from uninfected wild-type and deficient mice were used as controls and appeared to be healthy (Fig. 5, A and B). As expected, wild-type mice exhibited obvious inflammatory infiltrates obstructing the alveolar spaces at 5 wk postinoculation (Fig. 5C). In contrast, the TNFR-deficient mice showed significantly less of alveolar involvement, but did exhibit signs of peribronchiolar cellular infiltrates (Fig. 5D). Thus, histological evidence of alveolar inflammation correlated with the elevated chemokine levels noted in wild-type mice with PcP. Together, these data demonstrated that CD8+ T cell-mediated chemokine production and inflammatory cell recruitment during PcP were TNFR-dependent processes.

**TNFR deficiency attenuates lung injury and respiratory impairment during PcP**

TNF signaling was found to be critical for CD8+ T cell-dependent chemokine production and inflammatory cell recruitment during PcP. Therefore, to determine whether TNF signaling directly contributed to PcP-related respiratory deficits, pulmonary function measurements were performed on Pneumocystis-infected wild-type and TNFR-deficient mice. To assess pulmonary function in experimental mice, respiratory rates were monitored weekly and dynamic lung compliance and lung resistance were measured at 0, 3, and 5 wk postinoculation. Uninfected wild-type and TNFR-deficient mice were used as normal controls for baseline measurement of functional parameters. Wild-type and TNFR-deficient mice exhibited slight, progressive, and comparable elevations in respiratory rate at 1, 2, and 3 wk postinoculation (Fig. 6A). However, this progression increased dramatically in the wild-type mice to an average of 477 ± 11 and 526 ± 19 respirations/min at weeks 4 and 5 postinoculation, respectively. In contrast, the respiratory rates of the TNFR-deficient mice remained relatively constant at 368 ± 8.0 and 373 ± 14 respirations/min over this same time span (Fig. 6A).

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**FIGURE 5.** Histopathology of PcP in CD4+-depleted wild-type and TNFR-deficient mice. Lungs from uninfected wild-type (A) and TNFR-deficient (B) mice and CD4+-depleted wild-type (C) and TNFR-deficient (D) mice with PcP were inflation fixed. Sections were then stained with a modified Giemsa stain and photographed at ×100. PcP-related alveolitis is evident in the infected wild-type mice (C). Arrows denote peribronchiolar inflammation observed in TNFR-deficient mice with PcP (D).
Baseline dynamic lung compliance measurements of uninfected wild-type and TNFR-deficient mice were not statistically different (Fig. 6B). However, by 3 wk postinoculation, TNFR-deficient mice had lower lung compliance measurements than wild-type mice. Over the next 2 wk, wild-type mice exhibited a drastic deterioration of pulmonary function, while lung function in the TNFR-deficient mice remained relatively constant. From 3 to 5 wk postinoculation, wild-type mice demonstrated a striking 46% decrease in average lung compliance (Fig. 6B). In contrast, the TNFR-deficient mice only demonstrated a 7% decrease in compliance over this same time period. In direct comparison, the mean dynamic lung compliance of wild-type mice was 30% lower than that of TNFR-deficient mice at 5 wk postinfection. As an additional measure of pulmonary function, lung resistance was also measured in wild-type and knockout mice. Similar to the lung compliance data, resistance values for the infected TNFR-deficient and wild-type mice were comparable at 0 and 3 wk postinoculation, while a dramatic difference was observed at 5 wk postinoculation (Fig. 6C). At this time point, the TNFR-deficient mice exhibited significantly lower lung resistance measurements than the wild-type mice (1.66 ± 0.04 cm H₂O/ml/s vs 2.07 ± 0.06 cm H₂O/ml/s), supporting the conclusion that TNFR deficiency improves PcP-related pulmonary function deficits. The respiratory rate, lung compliance, and resistance data are a compilation of three separate experiments consisting of a total of 24 infected wild-type and 24 infected TNFR-deficient mice.

As a measure of tissue injury, alveolar-capillary permeability was assessed by measuring albumin concentration in the BAL fluid of experimental mice (Fig. 7). Albumin concentrations in both wild-type and TNFR-deficient mice at 3 wk postinoculation were slightly higher (2- to 3-fold) than in uninfected mice. However, by 5 wk postinoculation, the infected wild-type mice had an ~52-fold higher BAL albumin concentration than uninfected wild-type controls and a 9-fold greater albumin concentration than infected TNFR-deficient mice (Fig. 7). It is also important to note, however, that albumin concentrations in BAL from infected TNFR-deficient mice at 5 wk postinoculation were nearly 5-fold greater than that of uninfected mice, confirming that some PcP-related injury occurred despite the absence of TNFR signaling.

FIGURE 6. Pulmonary function measurements in CD4<sup>+</sup>-depleted wild-type and TNFR-deficient mice with PcP. Respiratory rates (A) of infected wild-type and TNFR-deficient mice were monitored weekly over a 5-wk period postinoculation. Baseline controls were uninfected wild-type and TNFR-deficient mice. Specific dynamic lung compliance (B) and lung resistance (C) measurements were also taken on wild-type and TNFR-deficient mice at 0, 3, and 5 wk postinoculation with Pneumocystis. Values are means ± 1 SE measurement (n ≥5). *, p < 0.05 as compared with Pneumocystis-infected wild-type mice at the same time point.

FIGURE 7. Albumin concentrations in the BAL of CD4<sup>+</sup>-depleted wild-type and TNFR-deficient mice with PcP. Albumin content was measured in the BAL of wild-type and TNFR-deficient mice at 0, 3, and 5 wk postinoculation with Pneumocystis. Values are means ± 1 SE measurement (n ≥5). *, p < 0.05 as compared with uninfected wild-type or TNFR-deficient mice. ***, p < 0.05 as compared with both uninfected mice and TNFR-deficient mice at the same time point.
To assess *Pneumocystis* burden in the experimental mice, a real-time PCR method was used to quantify the abundance of the *Pneumocystis* kexin gene in the lung. Real-time PCR analyses demonstrated that TNFR-deficient mice had lower *Pneumocystis* burdens than wild-type mice at 3 wk postinoculation. TNFR-deficient mice had an average of 4.8 ± 0.46 × 10⁶ total copies of the kexin DNA sequence in the right lung, while wild-type mice had an average of 6.1 ± 0.45 × 10⁶ copies. Conversely, TNFR-deficient mice had slightly higher *Pneumocystis* burdens at 5 wk postinoculation. TNFR-deficient mice had an average of 3.6 ± 0.64 × 10⁶ total copies of the kexin DNA sequence in the right lung, while wild-type mice had an average of 3.1 ± 0.47 × 10⁶ copies. However, the differences in *Pneumocystis* burden observed between TNFR-deficient and wild-type mice were not statistically significant at either time point examined.

**TNFR deficiency attenuates surfactant abnormalities during PcP**

To determine whether TNFR deficiency improves the surfactant dysfunction associated with PcP, a separate experiment was performed. Wild-type and TNFR-deficient mice were again CD4⁺ T cell-depleted and inoculated with *Pneumocystis*. At 5 wk postinoculation, dynamic lung compliance was measured, followed by exhaustive lavage of the lungs to collect surfactant. As expected, CD8⁺ T cell-mediated inflammation significantly altered the content and activity of lung surfactant aggregates recovered in the cell-free lavage from wild-type mice with PcP (Table II and Fig. 8). The phospholipid and protein concentrations of whole cell-free BAL, as well as the protein:phospholipid ratio, were significantly elevated in *Pneumocystis*-infected wild-type mice compared with uninfected controls. In addition, BAL from *Pneumocystis*-infected wild-type mice had a much lower content of large aggregates pelleted by centrifugation at 12,500 × g (Table II), and the remaining large aggregates had significantly impaired surface activity (Fig. 8).

The composition and surface activity of lavage from TNFR-deficient mice was also abnormal at 5 wk postinoculation with *Pneumocystis*, but to a lesser extent than in wild-type mice. Lavage fluid from TNFR-deficient mice with PcP had an average protein:phospholipid ratio of 107 ± 24 compared with 193 ± 45 in *Pneumocystis*-infected wild-type mice (Table II). The percentage of phospholipid pelleting as large aggregates in centrifuged BAL was significantly lower in both *Pneumocystis*-infected wild-type mice and TNFR-deficient mice relative to control mice (Table II). However, the surface activity of the remaining large aggregates was impaired to a lesser degree in the *Pneumocystis*-infected TNFR-deficient mice. As shown in Fig. 8, when resuspended at a low phospholipid concentration of 1 mg/ml, lavaged aggregates from wild-type mice with PcP had average minimum surface tensions that decreased from 36.8 ± 3.2 mN/m at 0.25 min of pulsation on the bubble surfactometer to 19.5 ± 1.8 mN/m after 20 min. In contrast, lavaged aggregates from TNFR-deficient mice had average minimum surface tensions that decreased from 23.9 ± 1.6 mN/m at 0.25 min to 15.6 ± 1.1 mN/m at 20 min. Lavaged aggregates from uninfected wild-type and TNFR-deficient mice demonstrated similar activity. (Fig. 8).

**Discussion**

Previous studies have demonstrated that CD8⁺ T cells contribute to inflammatory-mediated lung injury and respiratory impairment during PcP (17, 18). When CD4⁺-depleted mice are inoculated with *Pneumocystis*, they develop a persistent inflammatory condition that is characterized by the accumulation of CD8⁺ T lymphocytes and PMNs in the lung. This inflammatory response cannot completely control the *Pneumocystis* infection, but does cause deficits in pulmonary function and lung surfactant activity. Interestingly, when CD8⁺ T cells are additionally depleted, PMN recruitment is reduced and respiratory deficits are significantly improved.

The studies presented here expand upon these previous observations by demonstrating that TNF and chemokine levels are significantly elevated in the lungs of CD4⁺-depleted mice during PcP by a CD8⁺ T cell-dependent mechanism. In addition, studies using TNFR-deficient mice demonstrated that maximal chemokine secretion, lymphocyte and PMN recruitment, lung injury, and pulmonary function deficits during PcP are all TNFR dependent. At the same time, the remaining lung injury present in TNFR-deficient mice with PcP indicates that additional effects not mediated.

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**Table II. Biochemical composition of BAL fluid from TNFR-deficient and wild-type mice infected with *Pneumocystis***

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>n</th>
<th>C_DYN</th>
<th>Total Phospholipid (µg/ml)</th>
<th>Total Protein (µg/ml)</th>
<th>Protein/Phospholipid (%)</th>
<th>Phospholipid Recovery in Large Aggregate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (uninfected)</td>
<td>4</td>
<td>1.45 ± 0.12</td>
<td>73 ± 21</td>
<td>47 ± 15</td>
<td>65 ± 9.0</td>
<td>52 ± 13.3</td>
</tr>
<tr>
<td>Wild type (PcP infected)</td>
<td>4</td>
<td>0.76 ± 0.08</td>
<td>145 ± 28</td>
<td>267 ± 24</td>
<td>193 ± 45</td>
<td>28 ± 2.6</td>
</tr>
<tr>
<td>TNFR deficient (PcP infected)</td>
<td>4</td>
<td>1.14 ± 0.08</td>
<td>83 ± 19</td>
<td>92 ± 36</td>
<td>107 ± 24</td>
<td>32 ± 3.0</td>
</tr>
</tbody>
</table>

* Percentage of total phospholipid recovered with large aggregate surfactant.
* Wild-type and TNFR-deficient mice at 5 wk postinoculation with *Pneumocystis*.
* p < 0.05 as compared to uninfected wild-type control mice.
* p < 0.05 as compared to *Pneumocystis*-infected TNFR-deficient mice.
* p < 0.05 as compared to *Pneumocystis*-infected wild-type mice.

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**FIGURE 8.** Surface activity of lavaged surfactant from *Pneumocystis*-infected wild-type and TNFR-deficient mice. Minimum surface tensions are shown as a function of time of pulsation for resuspended surfactant aggregates from uninfected wild-type (▼), uninfected TNFR-deficient (▼), infected CD4⁺-depleted wild-type (●), and infected CD4⁺-depleted TNFR deficient (○) mice at 5 wk postinoculation with *Pneumocystis*. Surface tension measurements were made with a pulsating bubble surfactometer (37°C, 20 cycles/min) at a uniform low phospholipid concentration of 1 mg/ml. Values are means ± 1 SE measurement (n ≥4).
by TNF also contribute significantly to the pathophysiology of this condition.

Although it has been demonstrated that the CD8⁺ T cells recruited to the lung during Pp are *Pneumocystis* specific (12), the precise mechanism by which they generate lung injury has not been defined. Both direct mechanisms of cytotoxic lung injury and indirect mechanisms involving the recruitment and activation of other cell populations exist. CD8⁺ T cells may directly damage the alveolar epithelium by perforin/granzyme (50) mediated-, Fas ligand (51) mediated-, or transmembrane TNF-mediated mechanisms (52, 53). Alternatively, these cells can indirectly contribute to lung injury by producing cytokines and/or chemokines that recruit and activate other cell populations which subsequently contribute to injury (54, 55). For example, PMNs in the BAL are associated with a poor prognosis in patients with Pp, and studies of a mouse model of Pp suggest that CD8⁺ T cells can stimulate PMN recruitment to the lung, possibly through secretion of CXCR chemokines (17). These PMNs may either directly contribute to lung damage or merely represent a host response to the damage caused by CD8⁺ T cells. Further studies into the specific phenotype and activities of the CD8⁺ T lymphocytes in the lung during Pp need to be undertaken. In addition, the role that PMNs play in lung injury during Pp requires more precise definition.

The pulmonary recruitment of CD8⁺ T cells and PMNs is the most prominent feature of Pp in CD4⁺-depleted mice. Since chemokines are intimately involved in the differential recruitment and targeting of specific cell populations to sites of tissue injury and infection, the role of chemokine production during CD8⁺-mediated inflammation was evaluated. The presence of CD8⁺ T cells appears to amplify the inflammatory response associated with Pp by releasing and/or stimulating the release of chemokines, and this amplification may lead to the injury and pulmonary function deficits associated with Pp. The CC chemokines MCP-1 and RANTES and the CXC chemokines MIP-2 and KC were all significantly elevated through a CD8⁺ T cell-dependent mechanism. CD8⁺ T cells at sites of *Pneumocystis* infection may contribute to elevated chemokine production by: 1) secreting chemokines themselves (RANTES); 2) activating macrophages to secrete chemokines (RANTES, MCP-1, MIP-2, KC); 3) secreting IFN-γ to activate macrophages to secrete IL-1 and TNF, which in turn stimulate alveolar epithelial cells (AECs) to secrete chemokines (RANTES, MCP-1, MIP-2, KC); 4) secreting or expressing surface-bound TNF to activate AECs to secrete chemokines (RANTES, MCP-1, MIP-2, KC); or 5) all of the above. Together, these mechanisms of chemokine induction and amplification may cooperate for the CD8⁺ T cell-mediated recruitment of inflammatory cells to the lungs during Pp.

Interestingly, the CC chemokines MCP-1 and RANTES were present at the highest levels in the lung during CD8⁺ T cell-mediated inflammation and likely contribute to the persistent recruitment and activation of these cells during Pp (56–58). In addition, the CC chemokines have distinct, but partially overlapping roles in the generation of Ag-specific T cell responses by exhibiting direct effects on T lymphocytes, APCs, and macrophage effector functions (59–61). MCP-1 and RANTES play roles in T cell proliferation, activation and costimulation, and enhance class I-restricted CD8⁺ T cell-dependent CTL responses (60–62). In addition, RANTES and MCP-1 can induce the directional migration of monocyte/macrophages (34, 63, 64). RANTES can also activate macrophages for the uptake and intracellular destruction of parasitic organisms (59), and MCP-1 induces proinflammatory cytokine production by macrophages (63). Therefore, in addition to their role as chemoattractants for CD8⁺ T lymphocytes and macrophages during Pp, CC chemokines may also facilitate Ag-specific T cell responses and macrophage effector functions against *Pneumocystis*.

The data presented here demonstrate that abolishing TNF signaling using TNFR-deficient mice attenuates CD8⁺ T cell-mediated inflammatory lung injury during Pp. TNF has been implicated in other inflammatory conditions that are harmful to the host, including sepsis (65, 66), autoimmune diseases (67), and toxicant-induced inflammatory injuries (68). Although TNF may function on different levels to initiate and/or amplify inflammatory injury in this model, the ultimate effect of TNFR deficiency is the decreased production of chemokines, impaired lymphocyte and PMN recruitment to the lung, and improved pulmonary function. The effects of blocking TNFR signaling include, but are likely not limited to, reduced chemokine expression in the *Pneumocystis*-infected lung. It has been well documented that *Pneumocystis*-stimulated macrophages release TNF in vitro and that TNF is released in vivo immediately after *Pneumocystis* inoculation (21–23). During the initial stages of infection, impairment of TNF-mediated alveolar macrophage and AEC chemokine production may prevent or diminish CD8⁺ T cell recruitment to the lung and effectively prevent the initiation of the injurious inflammatory cascade. In addition, TNF signaling blockade may also prevent or diminish amplification of the chemokine response following the initial CD8⁺ T cell recruitment. Activated CD8⁺ T lymphocytes secrete RANTES, which can then serve to amplify or aid in the persistence of the inflammatory response by recruitment of more lymphocytes and potentially PMNs to the lung. It has also been demonstrated that CD8⁺ T cells can induce AEC MCP-1 and MIP-2 production through a TNFR-dependent mechanism and that this contributes to inflammatory infiltration and lung injury in a mouse model of CD8⁺ T cell-mediated lung injury (32). Thus, further studies are needed to elucidate the contribution of these inflammatory cytokines to lung injury during Pp.

It must be mentioned that there are also chemokine-independent mechanisms by which TNFR signaling blockade can attenuate CD8⁺ T cell-mediated inflammation and injury. AECs are sensitive to TNF-mediated apoptosis triggered by CD8⁺ T cells, and blockade of TNF signaling may prevent this mechanism of lung injury. TNF also induces adhesion molecule expression on the pulmonary endothelium (69, 70), and TNFR deficiency could significantly reduce transendothelial chemotaxis of inflammatory cells into the lung. This mechanism could be particularly relevant to our studies, as we observed altered distribution of inflammatory cells in the lungs of TNFR-deficient mice with Pp (Fig. 5). Lack of TNF signaling could also impact the ability of the host to generate CD8⁺ T cells to mediate injury. TNF plays a role in the proliferation of T lymphocytes and TNFR deficiency may impair the ability of the host to generate sufficient numbers of CD8⁺ T cells. Furthermore, although the cellular composition of the spleen, thymus, and lymph nodes are normal in TNFR-deficient mice (40), there are defects in the microarchitecture of the germinal centers of the secondary lymphoid tissue (33, 71). Although it might be expected that abnormal germinal center formation could interfere with humoral immune responses, we do not expect this defect would preclude the generation of the functional T cell responses we are studying. In fact, normal T cell responses have been reported in TNF- and TNFR-deficient mice (72, 73). However, it must be noted that this defect could potentially contribute to our findings through some as yet undetermined mechanism. Although we did find that *Pneumocystis* infection caused an increase in the number of CD8⁺ T cells in the BAL of TNFR-deficient mice with Pp, we did not perform definitive analysis of the CD8⁺ T cell response in this study.
Although it has been reported that inhibition of pulmonary TNF activity increases the Pneumocystis burden in CD4+ T cell-depleted mice (24), the data presented here suggest that organism burdens are comparable in CD4+ T cell-depleted wild-type and TNFR-deficient mice. One possibility for this discrepancy is the method of Pneumocystis enumeration in the two studies. The previous study only accounted for the cyst forms of Pneumocystis, which account for 10% or less of the total number of organisms. In contrast, we quantified the amount of Pneumocystis DNA in the lungs of experimental mice, which should represent all life stages of the organism. This is particularly relevant since it has been demonstrated that large variations in trophozoite numbers can occur in mice with similar cyst burdens when experimental manipulations are made to the model (17). Alternatively, the fact that we used knockout mice which develop in the total absence of TNFR signaling could contribute to the differences. In the absence of TNF, other redundant cytokines could compensate and help to control the Pneumocystis infection.

Ablation of TNFR I and TNFR II signaling has clear effects in models of chemical-induced inflammatory injury (68). However, determination of experimental differences is more complicated when an ongoing infection is involved. Although TNFR deficiency clearly attenuates lung inflammation and improves pulmonary function during PcP, it is apparent that other mechanisms of lung injury are also operating. None of the lung parameters assessed in TNFR-deficient mice with PcP were equal to those of normal mice. Thus, Pneumocystis itself may be producing some degree of lung injury in the absence of inflammation or other mechanisms of TNF-independent inflammatory lung injury exist. Therefore, careful consideration must be given to the use of adjunctive anti-TNF therapy. This is particularly important when targeting TNF, since it has been reported to play a protective role against Pneumocystis infection in CD4+ T cell-depleted mice and also plays a critical role in host-mediated clearance of the organism.

In summary, we have demonstrated that the CD8+ T cell-mediated inflammation associated with PcP is at least partly dependent upon TNF signaling pathways. Although redundancy in the cytokine network means that targeting one specific molecule does not always prove useful in therapeutic settings, the requirement of TNF for the genesis of many types of immune and inflammatory responses makes this molecule a popular candidate for beneficial therapy (74–76). Although our data suggest that anti-TNF therapy may prove useful for treating patients with PcP, further studies are required before this adjunctive therapy can be recommended. In particular, it must be noted that these mice lack TNF signaling from the beginning of the infection, and studies are required to determine how anti-TNF therapy will affect a host who is already infected and thus will already have elevated TNF levels when they present with signs of respiratory deficit. These studies will be particularly important because both pro- and anti-inflammatory roles have been reported for TNF.

References


