INFECTION AND IMMUNITY, Oct. 2004, p. 6002–6011 Vol. 72, No. 10
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.10.6002–6011.2004

Enhanced Lung Injury and Delayed Clearance of *Pneumocystis carinii* in Surfactant Protein A-Deficient Mice: Attenuation of Cytokine Responses and Reactive Oxygen-Nitrogen Species

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Received 28 April 2004/Returned for modification 2 June 2004/Accepted 13 July 2004

Surfactant protein A (SP-A), a member of the collectin family, selectively binds to *Pneumocystis carinii* and mediates interactions between pathogen and host alveolar macrophages in vitro. To test the hypothesis that mice lacking SP-A have delayed clearance of *Pneumocystis* organisms and enhanced lung injury, wild-type C57BL/6 (WT) and SP-A-deficient mice (SP-A$^{-/-}$) with or without selective CD4^{+}-T-cell depletion were intratracheally inoculated with *Pneumocystis* organisms. Four weeks later, CD4-depleted SP-A-deficient mice had developed a more severe *Pneumocystis* infection than CD4-depleted WT (*P. carinii* pneumonia [PCP] scores of 3 versus 2, respectively). Whereas all non-CD4-depleted WT mice were free of PCP, intact SP-A$^{-/-}$ mice also had evidence of increased organism burden. *Pneumocystis* infection in SP-A-deficient mice was associated histologically with enhanced peribronchial and/or perivascular cellularity (score of 4 versus 2, SP-A$^{-/-}$ versus C57BL/6 mice, respectively) and a corresponding increase in bronchoalveolar lavage (BAL) cell counts. Increases in SP-D content, gamma interferon, interleukin-4, interleukin-5, and tumor necrosis factor alpha in BAL fluid occurred but were attenuated in PCP-infected SP-A$^{-/-}$ mice compared to WT mice. There were increases in total BAL NO levels in both infected groups, but nitrite levels were higher in SP-A$^{-/-}$ mice, indicating a reduction in production of higher oxides of nitrogen that was also reflected in lower levels of 3-nitrotyrosine staining in the SP-A$^{-/-}$ group. We conclude that despite increases in inflammatory cells, SP-A-deficient mice infected with *P. carinii* exhibit an enhanced susceptibility to the organism and attenuated production of proinflammatory cytokines and reactive oxygen-nitrogen species. These data support the concept that SP-A is a local effector molecule in the lung host defense against *P. carinii* in vivo.

Despite advances in diagnosis, highly active antiretroviral therapy, and prophylactic regimens, *Pneumocystis carinii* pneumonia (PCP) remains a significant cause of morbidity and mortality in human immunodeficiency virus (HIV)-infected patients, as well as an important life-threatening opportunistic infection in other immunocompromised patients with defects in cell-mediated immunity (14, 52, 54, 59). From a large body of published studies, it is now apparent that complex interactions between host and pathogen develop during PCP. Once in the distal air spaces, *P. carinii* encounters a thin film covering the epithelia that contains inflammatory cells (macrophages), pulmonary surfactant, and water-soluble aqueous protein forms. From here the combination of organism and host triggers the involvement of both the adaptive (cellular and humoral) and the innate immune systems, leading to a directed inflammatory cascade characterized by initiation of macrophage-pathogen interactions, opsonization of organisms, macrophage activation with elaboration of nitric oxide and reactive oxygen species, recruitment of mononuclear cells, specific CD4 and CD8 lymphocyte responses with production of Th1-like (gamma interferon [IFN-γ]) and Th2-like (interleukin-4 [IL-4]) cytokines, and generation of other proinflammatory cytokines (tumor necrosis factor alpha [TNF-α]) important for clearance (9, 42, 56).

Because of the intimate association of *Pneumocystis* and the alveolar epithelia, recent experimental work has focused on the importance of the surfactant system in defense against this pathogen. Isolated from the cell-free bronchoalveolar lavage (BAL), pulmonary surfactant represents a surface-active mixture of phospholipid and protein secreted by the alveolar type 2 cells that reduces surface tension at the air-liquid interface and allows for maintenance of alveolar stability at low lung volumes (28). Biochemical analysis has identified four unique surfactant proteins (SPs) designated SP-A, SP-B, SP-C, and SP-D. The hydrophobic proteins, SP-B and SP-C, are involved in organization and adsorption at the air-liquid interface (61), whereas the more hydrophilic proteins, SP-A and SP-D, do not have a primary role in the reduction of surface tension but are important components of antibody-independent innate lung immunity (22). It is known that each of the individual compo-

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nents in pulmonary surfactant (phospholipids, SP-A, SP-B, SP-C, and SP-D) can, by varying degrees, modulate host inflammatory mechanisms (5, 21, 27, 61).

SP-A, the most abundant SP, is a multifunctional sialoglycoprotein of 28 to 36 kDa that contains a COOH-terminal C-type lectin motif, a triple helical collagen domain, and a carbohydrate recognition domain (64). Monomeric SP-A is assembled into a higher-order 18-mer from six identical trimeric subunits linked by interchain disulfide bonding. SP-A, along with SP-D, and several nonlung proteins (mannose-binding protein, bovine conglutinin, and CL-43) are part of a growing family of pattern recognition proteins known as collagen-like lectins or "collectins" that are important to innate immunity (22). Based upon its spatial localization in both the monolayer and the hypophase of the alveolar lining fluid, SP-A is uniquely positioned to provide a first line of defense against invading organisms. The importance of SP-A to host defense has been supported by the demonstration that SP-A-deficient mice are more susceptible to bacteria such as group B streptococci and *Pseudomonas aeruginosa* (38, 39). In vitro, SP-A has also been shown to have direct cytotoxic activity against microbes (68). Additional in vitro studies have suggested that SP-A plays an important role in the modulation of the inflammatory and immune responses of alveolar macrophages and lymphocytes. SP-A promotes the microbicidal activity of macrophages by stimulating macrophage chemotaxis (65), by enhancing the phagocytosis of microorganisms (26, 46), and by increasing the production of reactive oxygen-nitrogen species (RONS) (33, 62). The ability of the pulmonary collectin SP-A to modulate NO production by alveolar macrophages has been demonstrated with transformed cell lines (35) and primary rat alveolar macrophages (18). The importance of SP-A and NO for pulmonary bacterial clearance was shown in a mouse model of respiratory mycoplasmosis in which transgenic mice deficient in SP-A or lacking the inducible form of nitric oxide synthase (iNOS) were each shown to clear mycoplasma less efficiently (32).

SP-A has also been shown to participate in the host response to PCP. In mouse models, development of PCP is accompanied by an increase in total alveolar SP-A content (2). Immunohistochemistry of lung sections from *Pneumocystis*-infected mice demonstrates that upregulation of SP-A was localized to regions of atelectasis and cellular infiltrates containing organisms (3). In a corticosteroid-treated rat model of PCP the SP-A levels are also reported to be increased (50, 58) and, similarly, BAL samples from HIV-infected patients with PCP have an elevated SP-A content (49). SP-A has been shown to bind to isolated *Pneumocystis* organisms in a calcium- and mannose-dependent manner via its carbohydrate recognition domain to gpA (gp-120), the major glycoprotein found on cell surfaces of trophozoites and cysts (69). In vitro, SP-A augments binding and clearance of labeled rat *Pneumocystis* by cultured rat alveolar macrophages (63) and enhances the attachment of mouse-derived *Pneumocystis* to murine alveolar macrophages (44), but such interactions have not been shown to occur with human macrophages (37). Recently, Swiss Black mice deficient in SP-A demonstrated increased frequency and intensity of PCP when challenged either by intratracheal inoculation or direct exposure to infected animals (44). In that study, however, immunosuppression with corticosteroids was required to induce susceptibility to infection in the mice. The nonspecific anti-inflammatory effects of corticosteroids, as well as their confounding effects on SP expression, limit interpretation of the data. Furthermore, the mechanisms for the observed findings were not elaborated.

The goals of the present study were to further define the role of SP-A in host defense against *Pneumocystis* by using a well-characterized murine model of infection that does not depend upon corticosteroid immunosuppression. We hypothesized that transgenically engineered mice deficient in SP-A would demonstrate delayed clearance of *Pneumocystis* organisms and enhanced lung injury. Using direct intratracheal inoculation of the organism, we show that SP-A-deficient mice challenged with *Pneumocystis* organisms are less efficient at clearing the organism while also generating less RONS and producing fewer of the cytokines TNF-α and IFN-γ. Paradoxically, SP-A-deficient mice with PCP showed increased markers of lung injury, suggesting that toxicity was a direct effect of the organism burden or occurred via a cytokine/RONS independent pathway.

MATERIALS AND METHODS

Anti-CD4 antibody. Ascites production from the monoclonal hybridoma GK1.5 was performed commercially by Strategic Biosolutions, Inc., with NcR nude mice as previously described (56). An irrelevant immunoglobulin G1 was purchased from Sigma Chemical Co. (St. Louis, Mo.).

SP antisera. Monospecific, polyclonal SP antisera to SP-A and SP-B produced in rabbits have been described previously in detail (16, 60). A monospecific, polyclonal antibody against SP-D was produced commercially by Macromolecular Resources, Inc. (Fort Collins, Colo.), by using synthetic peptides corresponding to two homologous regions of the mouse/human SP-D sequences as the immunizing antigen in rabbits as previously published (20). The peptides were from the N-terminal region (peptide 1 [GRDGRDGREGPRGEKG]) and the C-terminal region (peptide 2 [KQAGGQLASPRSATENAA]). The resulting antiserum recognizes rat, murine, and human SP-D and does not cross-react with SP-A.

Anti-nitrotyrosine antiserum. A polyclonal antiserum against 3-nitrotyrosine (3-NT) was a generous gift of Harry Ischiropolous (Children's Hospital, Philadelphia, Pa.).

Mouse model of *Pneumocystis* infection. (i) SP-A-deficient mice. SP-A-deficient mice were produced by targeted ablation of the mouse SP-A locus on chromosome 8 as previously published (41). Mice were bred to homozygosity and backcrossed 10 generations onto the C57BL/6 background. The lungs of SP-A-deficient mice do not contain detectable SP-A mRNA or protein (data not shown). Age-matched wild-type C57BL/6 mice (WT) purchased from Jackson Laboratories, Inc., and cohoused with litters of SP-A-deficient mice prior to inoculation served as controls. Experiments were performed between 8 and 12 weeks of age on male and female mice.

All mice were housed in a barrier, isolation animal care facility at the University of Pennsylvania in filter-top cages while receiving sterile rodent chow and sterile drinking water. Normal sentinel mice were examined routinely for the presence of unintended pathogens by culture and serology. The Institutional Animal Care and Use Committee of the University of Pennsylvania approved all procedures.

(ii) Organisms. *Pneumocystis* organisms were obtained from the lungs of athymic mice (*nu*/*nu* on a BALB/c background; Taconic Laboratories, Germantown, N.Y.) in which *Pneumocystis* organisms had been propagated by serial passage as previously described (11, 56). Prior to the dispersal of *Pneumocystis* organisms by homogenization by using a Stomacher apparatus, bacterial contamination was excluded by the routine use of Gram staining of touch preparations of each harvested lung. After centrifugation, organisms collected in the resulting pellet were stained with modified Giemsa stain, counted, and diluted to a concentration of 2×10^6 organisms per ml. Isolated *Pneumocystis* organisms were then inoculated intratracheally into anesthetized mice. Utilizing direct visualization, 0.1 ml of the inoculum (2×10^5 *Pneumocystis* cysts) was injected at the level of the carina, immediately followed by an injection of 0.6 ml of air to ensure adequate dispersion of inoculum into the distal lung.

TABLE 1. Surfactant component expression during *Pneumocystis* infection

Mouse group and infection status	Mean amt (μg) ± SEM[a] of:				LA SP-B[b] (% of WT)
	Protein in:		Phospholipid in:		
	LA	SA	LA	SA	
WT mice					
Uninfected	86.2 ± 6.1	1,444 ± 91	192 ± 26	111 ± 14	100 ± 22
4 wk postinfection	80.3 ± 3.6	1,578 ± 162	136 ± 9*	190 ± 22*	52 ± 2*
SP-A KO mice					
Uninfected	67.6 ± 6.6	986 ± 132	173 ± 23	123 ± 10	50 ± 4*
4 wk postinfection	78.4 ± 3.3	2,075 ± 205*†	121 ± 16	169 ± 22	48 ± 1*

[a] All data are expressed as the means from $n = 3$ to 6 animals per group. *, significant difference from corresponding uninfected level ($P < 0.05$); †, significant difference from corresponding WT level ($P < 0.05$). Total amounts of protein or phospholipids in large-aggregate (LA) or small-aggregate (SA) surfactant fractions prepared from BAL are expressed as the amount per mouse lung. $n = 5$ in each group.

[b] The relative content of mature SP-B protein in each LA sample was determined by densitometric scanning of corresponding bands from multiple blots. Data are expressed as the percentage of uninfected WT levels ($n = 3$ to in each group).

(iii) Generation of *Pneumocystis* infection. WT and SP-A-deficient mice were selectively depleted of $CD4^+$ T cells by intraperitoneal (i.p.) injection twice a week with the monoclonal antibody GK1.5 as previously published (56). Nondepleted mice received i.p. injections of equal volumes of phosphate-buffered saline (PBS). Additional nondepleted controls consisting of i.p. injection of isotype-specific irrelevant antiserum failed to elicit changes in peripheral CD4 counts, BAL cellularity, or surfactant components levels compared to PBS (data not shown).

One week after initiation of CD4 depletion, experimental animals were inoculated under direct visualization with either 2×10^5 *Pneumocystis* cysts (100 μl) harvested from *P. carinii*-infected *nu*/*nu* mice or with 100 μl of lung homogenate from uninfected *nu*/*nu* donor mice.

Four weeks after inoculation, infected and uninfected mice were euthanized with a lethal dose of pentobarbital and exsanguinated by aortic transection. All lungs were lavaged with 0.5-ml aliquots of sterile saline to a total of 5 ml. After lavage, the left lung was inflated and fixed in paraformaldehyde (4% with 0.1 M sodium cacodylate [pH 7.3]) for histological analysis as previously described (11). Paraffin-embedded lung specimens were sectioned and stained with Gomori methenamine silver stain (to evaluate intensity of infection) and with hematoxylin-eosin stain (to evaluate intensity of inflammation). Sections were scored in a blinded fashion to grade the intensity of infection (0 [no organisms] to 4+[cysts and foamy exudate throughout alveoli in most regions]) and of inflammation (0 [no inflammation] to 5+[severe perivascular and periobronchiolar inflammation with effacement of alveolar parenchyma and small airways by sheets of inflammatory cells]) by using scoring systems previously described in detail and validated (53).

By any of three methods, latent *P. carinii* infection does not appear in experimental mice. Using real-time PCR, we have not detected *Pneumocystis* DNA in the lungs of mice that were not inoculated with organisms (J. M. Beck, unpublished observation). Histological examination of uninoculated mice (both WT and SP-A deficient) subjected to CD4 depletion for up to 6 weeks has failed failed to show development of PCP. Previous work from our group has not shown anti-*P. carinii* antibodies in the sera of mice that were not inoculated with the organism (8).

(iv) Preparation and analysis of BAL and surfactant components. Total cell counts from lavage samples were performed by using a Z1 particle counter (Beckman-Coulter, Inc., Miami, Fla.). BAL samples were centrifuged ($400 \times g$ for 10 min), and the cell pellet was gently resuspended in 1 ml of PBS (with Ca^{2+} and Mg^{2+}) for total cell counting. Cytospins prepared from an aliquot of cell suspension were stained with Dif-Quik, and differential cell counts were performed as described previously (1).

Levels of Th1 (IFN-γ), Th2 (IL-4 and IL-5), and TNF-α were determined from a 100-μl aliquot of cell-free supernatant of the BAL (5 ml [total volume]) by enzyme-linked immunosorbent assay (ELISA) with antibodies and recombinant cytokine standards from Pharmingen, Inc. (San Diego, Calif.), and performed as previously described (29).

Cell-free BAL supernatants were separated into two fractions by centrifugation ($20{,}000 \times g$ for 60 min at 4°C) as described previously (3), producing a biophysically active large-aggregate (LA) form in the pellet and a supernatant that contained soluble proteins, as well as the biophysically inactive surfactant forms containing unilamellar vesicles (termed the small-aggregate fraction [SA]).

Both SA fractions and LA pellets resuspended in saline were reserved for biochemical characterization. The total protein content of the samples from LA and SA fractions was determined by the method of Bradford, with bovine immunoglobulin G as a standard (19). The total phospholipid content of the samples from LA and SA fractions was determined by the method of Bartlett (6).

Polyacrylamide gel electrophoresis and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of LA aggregate surfactant samples for SP-B was performed in 16.5% polyacrylamide gels by using a Tris-Tricine buffer system. SA SP-D expression was determined by using NuPAGE–10% Bis-Tris gels (Novex, San Diego, Calif.) as previously described (3, 30). In both cases, electrophoresed proteins (5 μg of total protein per lane) separated under reducing conditions were transferred to 0.2-μm-pore-size nitrocellulose at 60 mA/cm^2 for 12 to 18 h for subsequent immunoblotting or autoradiography. Western blots were performed with monospecific, polyclonal SP antisera to SP-B and SP-D as previously described (3). Specific proteins were visualized by enhanced chemiluminescence by using the ECL Kit (Amersham, Inc., Arlington Heights, Ill.), and the band intensity of the exposed film was analyzed by densitometric scanning and quantitated by using Kodak 440 image analysis software.

Immunohistochemistry of lung tissue. Paraffin sections prepared from the lungs of WT and SP-A-deficient mice as described above were stained for NO metabolites by immunohistochemistry with a monoclonal antibody generated to nitrotyrosine as previously described (1). The specificity of staining was confirmed by using parallel sections treated with dithionite to remove nitrotyrosine prior to application of the primary antibody. Sections were also examined for protein carbonyl content by derivatizing with 2,4-dinitrophenyl hydrazine (DNPH) and then stained with an antibody generated against DNPH (40). Sections were scored for intensity of immunostaining as previously described (4).

Nitrogen oxide measurements. The analysis of NO metabolites was performed by using the Ionics/Sievers Nitric Oxide Analyzer 280 (NOA 280). Total NO was determined by treatment with an excess of vanadium chloride in HCl at 95°C, reducing nitrate, nitrite, and SNO to release NO. Nitrite was then measured independently and subtracted from the total to calculate nitrate content. Nitrite analysis was performed by using a potassium iodide and acetic acid mixture (23). Sodium nitrate and nitrite (Sigma) served as standards for the vanadium and iodide assays, respectively.

Data analysis. Data were analyzed by using a standard statistical package (Sigma Stat; Jandel Scientific, Inc.). Parametric data were expressed as the means ± the standard errors of the mean (SEM) and were compared by analysis of variance or Student *t* test. Nonparametric data were expressed as medians and were compared by the Wilcoxon-Kruskal-Wallis rank-sum test. In all cases, a *P* value of <0.05 was considered significant.

RESULTS

Surfactant component expression during *P. carinii* infection in WT and SP-A-deficient mice. The SP-A-deficient mice used in the present study were backcrossed more than 10 generations onto a C57BL/6 background. Consistent with data reported previously for other strains (36), these mice demonstrated that SP-A deficiency does not alter the total BAL phospholipid content compared to WT controls (Table 1).

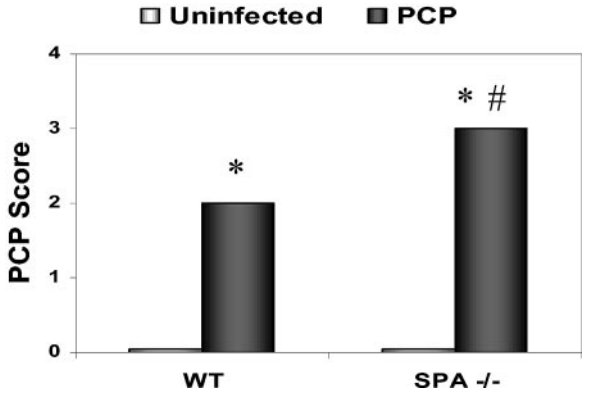

FIG. 1. CD4-depleted SP-A-deficient mice demonstrate delayed clearance of *Pneumocystis*. The intensity of the infection was determined after intratracheal inoculation of *P. carinii* in CD4 T-cell-depleted WT and SP-A-deficient mice. Histological sections of lung from each mouse were scored blindly for intensity of infection by using a scale previously described and validated for *Pneumocystis*. Bars: □, uninfected mice; ■, *P. carinii*-infected mice. Bars represent median values, with six samples in each group. ❋, Significant difference from the corresponding uninfected level ($P < 0.05$); #, significant difference from the corresponding WT level ($P < 0.05$ [Kruskal-Wallis test]).

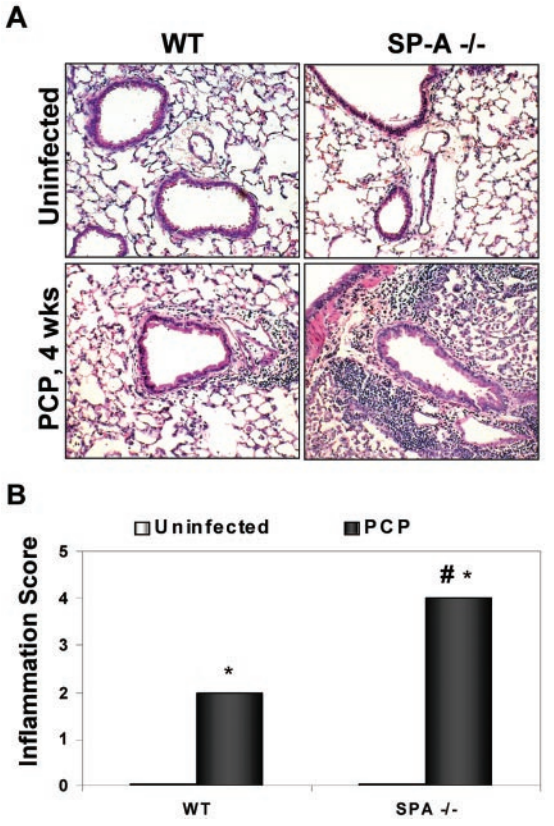

FIG. 2. *P. carinii*-infected SP-A-deficient mice demonstrate increased lung tissue inflammation. (A) Representative morphological changes in formalin-fixed, paraffin-embedded, hematoxylin-and-eosin-stained left lung sections of CD4-depleted WT and SP-A-deficient mice. Uninfected (upper panels) or *P. carinii*-infected (lower panels) WT and SP-A-deficient mice previously treated with GK1.5 were inoculated with sterile lung homogenate or 2×10^5 *Pneumocystis* organisms and sacrificed 4 weeks postinfection as labeled. Original magnification: × 200. (B) Intensity of inflammation in the lung tissue CD 4 depleted WT and SP-A-deficient mice inoculated with either uninfected lung homogenate or 2×10^5 *Pneumocystis* organisms. Histologic sections of lungs from each of the mice obtained 4 weeks postinoculation were scored blindly for the intensity of inflammation by using a scale previously described and validated (53). Bars: □, uninfected mice; ■, *P. carinii*-infected mice. Bars represent median values, with six samples in each group. ❋, Significant difference from the corresponding uninfected level ($P < 0.05$); #, significant difference from the corresponding WT level ($P < 0.05$ [Kruskal-Wallis test]).

Establishment of *Pneumocystis* infection is associated with a 70% reduction of large aggregate phospholipid levels in WT mice. *P. carinii*-infected SP-A-deficient mice demonstrated a similar trend but failed to reach statistical significant difference. As previously shown in a CD4-depleted mouse model, WT mice demonstrate a 50% reduction of SP-B in LA fraction 4 weeks after *P. carinii* inoculation (15). SP-A-deficient mice have a 50% reduction in baseline levels of SP-B versus WT controls and no further alterations in SP-B expression occurred during the development of *Pneumocystis* infection.

SP-A-deficient mice demonstrate delayed clearance of *P. carinii*. The intensity of infection was evaluated in SP-A-deficient and WT mice subjected to CD4 depletion 4 weeks after intratracheal inoculation with *Pneumocystis* organisms (Fig. 1). The 4-week interval was selected because CD4-depleted mice develop significant *Pneumocystis* infections but still survive for several weeks and because such an experimental design would allow for comparisons of our results with previously published studies. Under these conditions, CD4 depleted SP-A-deficient mice had increased intensity of infection (median PCP score of 3 versus 2 for WT mice [$P < 0.05$]). Interestingly, SP-A-deficient mice that were not CD4 depleted demonstrated mild infection (median PCP score of 1), whereas WT mice that were not depleted showed no evidence of infection (5 of 11 SP-A-deficient mice infected versus 0 of 11 WT mice [$P = 0.03$ by Kruskal-Wallis one-way analysis of variance on ranks]).

***P. carinii*-infected SP-A-deficient mice demonstrate significantly increased lung tissue inflammation.** Analysis of the histology from the lungs of uninfected SP-A-deficient mice revealed no abnormalities of lung structure and no evidence of inflammation (Fig. 2). However, the introduction of *Pneumocystis* infection into CD4 depleted SP-A-deficient mice produced a predominantly mononuclear inflammatory infiltrate localized to perivascular and peribronchiolar areas (Fig. 2A).

Scoring of the histological findings (Fig. 2B) confirmed the enhanced inflammatory response in the *P. carinii*-infected SP-A-deficient mice (median inflammation score of 4 versus 2 for WT mice [$P < 0.05$]). The inflammatory response noted in the lung tissue of SP-A-deficient mice was accompanied by an increase in total BAL cellularity compared to infected WT mice (Fig. 3A) which consisted a primarily of higher macrophage counts (Fig. 3B).

***P. carinii*-infected SP-A-deficient mice have increased lung injury.** CD4-depleted SP-A-deficient mice demonstrated a twofold increase in total BAL protein after intratracheal inoculation with *Pneumocystis* organisms (Fig. 4A). The enhanced protein leak recovered in the BAL at 4 weeks after introduction of PC organism was paralleled by an increase in SA protein content (Table 1) and in lung/body weight ratios in *P.*

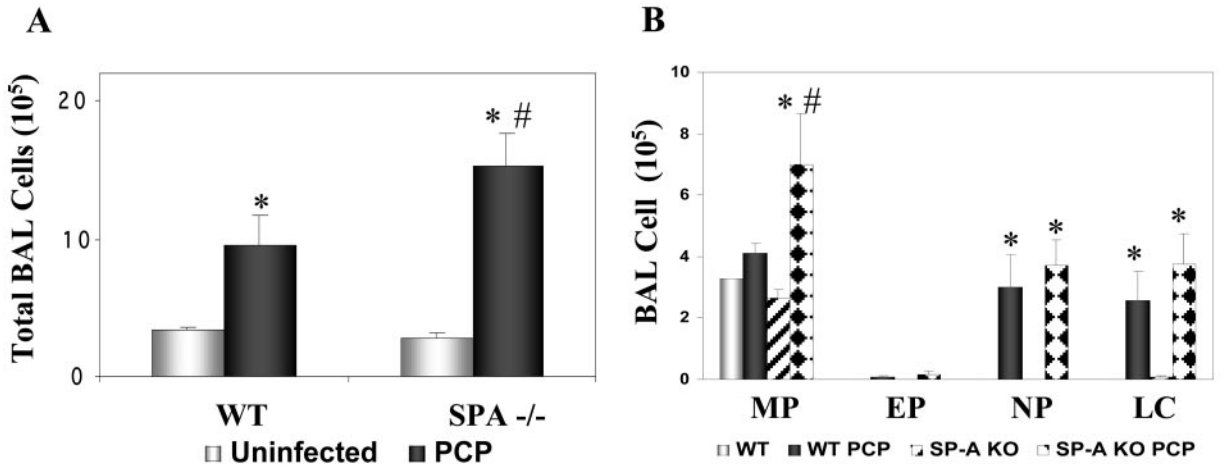

FIG. 3. *P. carinii*-infected SP-A-deficient mice develop increased BAL cellularity. WT and SP-A-deficient mice previously depleted of CD4 T cells by using GK1.5 were inoculated with sterile lung homogenate or 2×10^5 *Pneumocystis* organisms and then sacrificed 4 weeks postinfection. (A) Total numbers of BAL cells were derived from counts of stained cytospin preparations as described in Materials and Methods. Bars: □, uninfected mice; ■, *P. carinii*-infected mice. The data are expressed as means ± the SEM absolute numbers of BAL cells per mouse lung (n = 6 in each group). ✻, Significant difference from the corresponding uninfected level ($P < 0.05$); #, significant difference from the corresponding WT level ($P < 0.05$). (B) Differential cell counts (MP, macrophage; EP, eosinophil; NP, neutrophil; LC, lymphocyte) in each BAL sample was determined by Coulter counting as described in Materials and Methods. Bars: □, WT mice; ■, *P. carinii*-infected WT mice; ▨, SP-A-deficient mice; ▨, *P. carinii*-infected SP-A-deficient mice. The data expressed as the means ± the SEM absolute numbers of 10^5 BAL cells per mouse lung (n = 6 in each group). ✻, Significant difference from the corresponding uninfected level ($P < 0.05$); #, significant difference from the corresponding WT level ($P < 0.05$).

carinii-infected SP-A-deficient mice compared to WT mice (Fig. 4B).

SP-D protein levels are attenuated in *P. carinii*-infected SP-A-deficient mice. Previously, we have demonstrated that SP-D protein expression is increased in response to *P. carinii*-induced pulmonary inflammation, supporting the concept that this lung collectin may also play an active role in the inflammatory process. Figure 5 demonstrates that SP-D protein (43-kDa monomer) detectable in SA surfactant fraction of both WT and SP-A-deficient mice by Western blotting was increased after *P. carinii* inoculation. However, compared to corresponding WT mice, SP-D expression was attenuated in *P. carinii*-infected SP-A-deficient mice.

Cytokine levels in BAL fluid are attenuated in *P. carinii*-infected SP-A-deficient mice. Because IL-4, IFN-γ, and TNF-α have been previously shown to be important regulators of inflammation in response to *Pneumocystis* (25, 53), production of these cytokines was measured in BAL by ELISA. In the absence of *P. carinii* infection WT and SP-A-deficient mice contained almost undetectable levels of these cytokines. *Pneumocystis* infection significantly increased production of cytokines by BAL inflammatory cells in WT and SP-A-deficient mice, however, elaboration this response was significantly attenuated in SP-A-deficient mice in comparison to WT mice (Fig. 6).

NO metabolism is altered by PCP in SP-A-deficient mice. BAL samples of WT and SP-A-deficient mice were examined for changes in NO chemistry. Figure 7A demonstrates that basal production of total NO was similar in both uninfected WT and SP-A-deficient mice. *Pneumocystis* infection markedly increases BAL NO in both strains. However, BAL fluid from of uninfected SP-A-deficient mice contained ~40% less nitrite than wild-type counterparts (Fig. 7B). *P. carinii* infection sig-

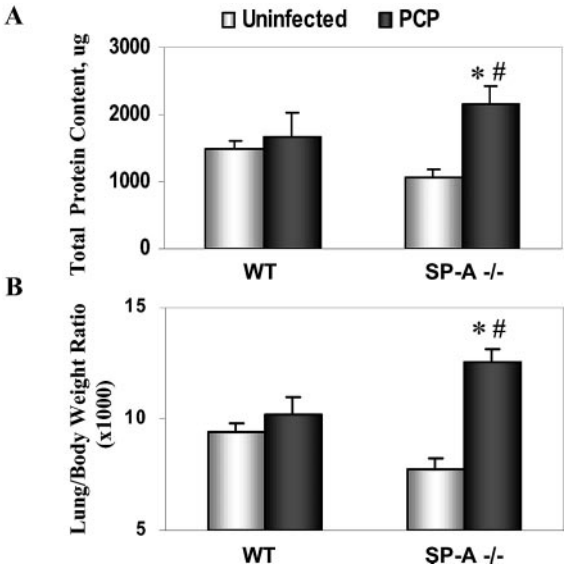

FIG. 4. Lung injury by *Pneumocystis* is enhanced in SP-A-deficient mice. (A) Total protein content of BAL fractions of uninfected or *P. carinii*-infected CD4-depleted WT and SP-A-deficient mice were determined by the Bradford method as described in the text. The data are expressed as mean amounts ± the SEM (in micrograms) of total protein per mouse lung (n = 3 to 6 in each group). Bars: □, uninfected mice; ■, *P. carinii*-infected mice. ✻, Significant difference from corresponding uninfected level ($P < 0.05$); #, significant difference from corresponding WT level ($P < 0.05$). (B) Wet weight of the right lung of each mouse was determined and normalized to total body weight with data expressed as mean ± the SEM of right lung wet weight to body weight ratio × 1,000 (n = 5 in each group). Bars: □, uninfected mice; ■, *P. carinii*-infected mice. ✻, Significant difference from the corresponding uninfected level ($P < 0.05$); #, significant difference from the corresponding WT level ($P < 0.05$).

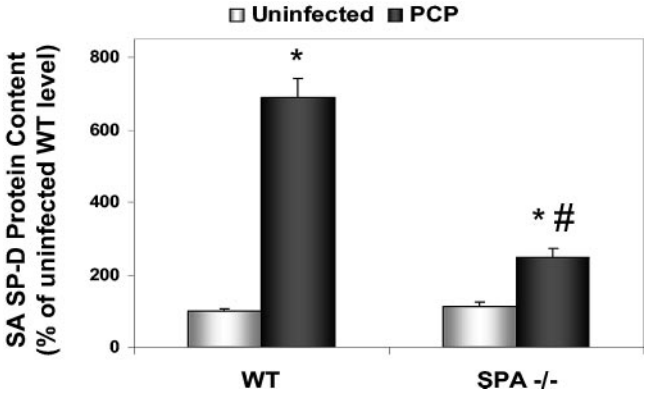

FIG. 5. SP-D protein levels are attenuated in *P. carinii*-infected SP-A-deficient mice. WT and SP-A-deficient mice previously depleted of CD4 T cells by using GK1.5 were inoculated with sterile lung homogenate or 2×10^5 *Pneumocystis* organisms and sacrificed at 4 weeks postinfection. Western blots normalized for total protein (10 μg of total protein/sample) of SA surfactants prepared from BAL of WT and SP-A-deficient mice were prepared with polyclonal antisera to SP-D and visualized by using enhanced chemiluminescence. Quantification of total SA SP-D content was performed by densitometric scanning of multiple blots as described in Materials and Methods. The data are expressed as a percentage of uninfected WT levels ($n = 3$ to 5 in each group). Bars: □, uninfected mice; ■, *P. carinii*-infected mice. ✻, Significant difference from the corresponding uninfected level ($P < 0.05$); #, significant difference from the corresponding WT level ($P < 0.05$).

nificantly decreased production of nitrite in WT mice, whereas the infected SP-A-deficient mice actually had increases in total nitrite. These observations suggest that, in contrast to WT mice with PCP, SP-A-deficient mice would not demonstrate a shift toward higher oxidation state NO intermediates via expression of RONS (peroxynitrite and nitrotyrosine). In agreement with this, uninfected SP-A-deficient mice had decreased 3-NT staining by immunocytochemical staining of whole lung tissue relative to uninfected WT mice (Fig. 8). Although PCP modestly increased production of 3-NT in both groups (Fig. 8, bottom panels), according to nonparametric semiquantitative scoring this did not reach statistical significance (WT mice, 3-NT score of 2 versus 3 [uninfected versus PCP]; SP-A-deficient mice, 3-NT score of 1 versus 2 [uninfected versus PCP]).

DISCUSSION

A convincing body of data is emerging to support the role of SP-A in the modulation of proinflammatory and anti-inflammatory responses required to effect the removal of pathogens and to limit lung injury (22, 64). Similarly, the local immune response within the lung appears to critically contribute to the pathogenesis of PCP. In a previous study, infection of immunocompromised mice with *Pneumocystis* led to an upregulation of SP-A protein levels (2). By using genetically engineered SP-A-deficient mice studied 4 weeks after direct intratracheal inoculation with the organism, we show a marked dependence of the lung upon SP-A both in the clearance of the organism and in the modulation of the proinflammatory response. Furthermore, despite attenuated levels of proinflammatory cytokines shown to be required for the clearance of PCP (53) and limitations in generation of RONS, enhanced lung damage was observed in the infected SP-A-deficient mice. These results both support previous studies obtained at similar time intervals showing a dependence of the lung on SP-A for proper clear-

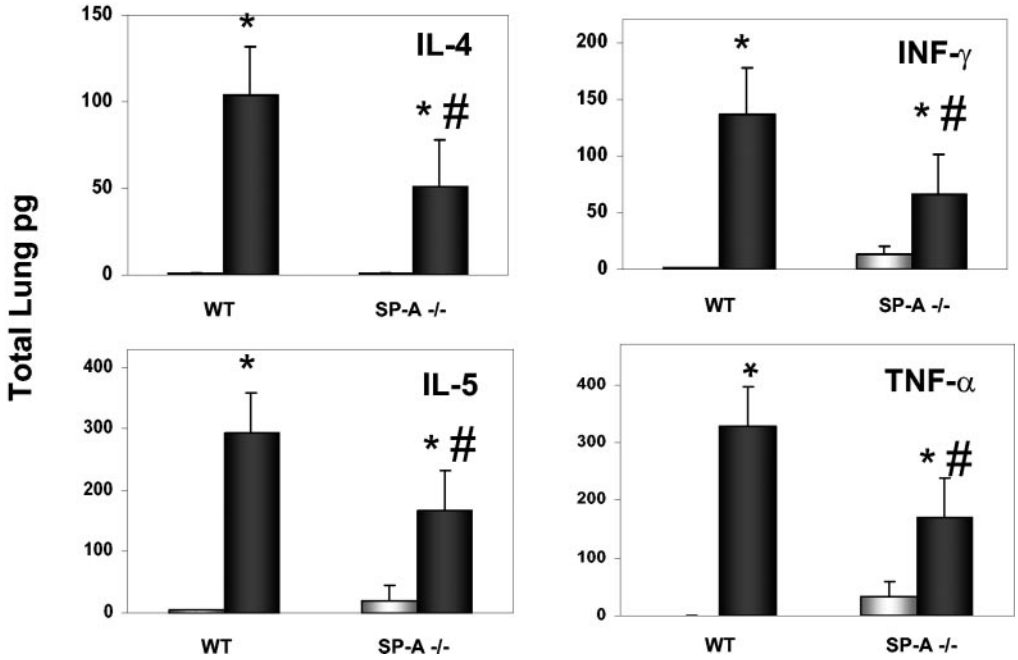

FIG. 6. Cytokine levels in BAL fluid are attenuated in *P. carinii*-infected SP-A-deficient mice. WT and SP-A-deficient mice previously depleted of CD4 T cells by using GK1.5 were inoculated with sterile lung homogenate or 2×10^5 *Pneumocystis* organisms and sacrificed 4 weeks postinfection. Cytokine levels in BAL fluid were determined by ELISA as described in Materials and Methods. The data are expressed as total picograms per mouse lung. Values are represented as means ± the SEM ($n = 6$ in each group). Bars: □, uninfected mice; ■, *P. carinii*-infected mice. ✻, Significant difference from corresponding uninfected level ($P < 0.05$); #, significant difference from corresponding WT level ($P < 0.05$).

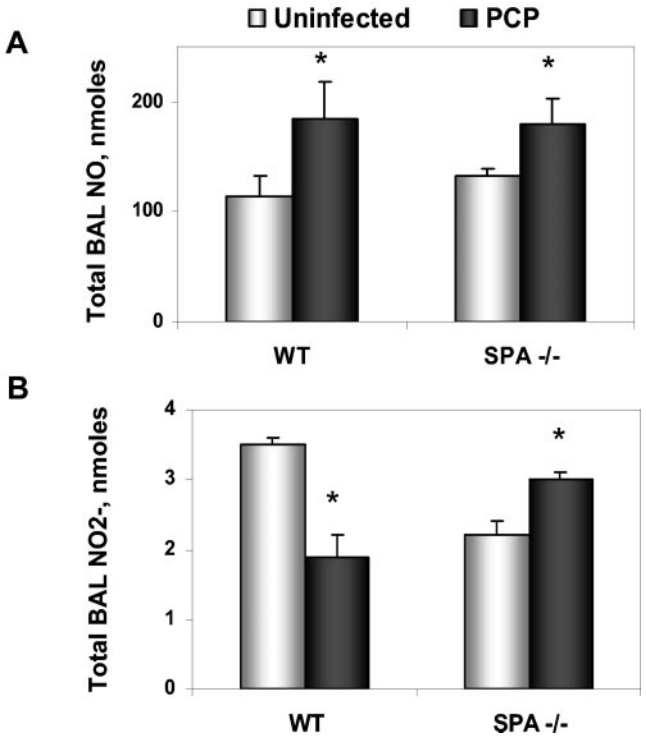

FIG. 7. The production of nitric oxide metabolites is increased in *P. carinii*-infected SP-A-deficient mice. WT and SP-A-deficient mice previously depleted of CD4 T cells by using GK1.5 were inoculated with sterile lung homogenate or 2×10^5 *Pneumocystis* organisms and sacrificed 4 weeks postinfection. BAL samples were analyzed by chemical reduction chemiluminescence for total nitrogen oxide (A) or nitrite (B) as described in Materials and Methods. The data are expressed as total nanomoles in BAL ($n = 5$ animals in each group). Values are means ± the SEM. Bars: □, uninfected mice; ■, *P. carinii*-infected mice. ✻, Significant difference from the corresponding uninfected level ($P < 0.05$); #, significant difference from the corresponding WT level ($P < 0.05$).

ance (44) of invading *Pneumocystis*, as well as extend our understanding of the complex interface of SP-A with both the innate and the acquired immune systems.

The enhanced susceptibility of the SP-A-deficient mice to PCP could result from disruption of normal host lung defense mechanisms at one of several levels. In our model, the development of *Pneumocystis* infection was associated with attenuation in the generation of proinflammatory cytokines TNF-α and IFN-γ. Previously, aerosolized IFN-γ has been shown to decrease the intensity of infection in vivo (10); however, in studies with IFN-γ and TNF-α receptor double-knockout mice (53), it appears that endogenous expression of and signaling by both cytokines are required for complete protection.

The presence of RONS is also important in innate host-defense in a variety of organisms. To characterize the essential mechanisms underlying SP-A-mediated defense, we studied the regulation of reactive oxygen-nitrogen intermediates in the SP-A-deficient mouse. The production of NO and its metabolites was characterized in the lungs of *P. carinii*-infected mice. When the BAL was examined for nitrate and nitrite content (Fig. 7), in contrast to what we previously observed in SP-D-deficient mice (1, 4), there was no major increase in overall baseline NO production in uninfected SP-A-deficient mice compared to WT controls. Both SP-A-deficient mice and WT controls with PCP had increases in total nitric oxide content (Fig. 7A), as has been observed for *P. carinii*-infected SP-D-deficient mice (1). However, with the rise in NO, WT mice were found to have a simultaneous drop in total BAL nitrite levels, whereas the SP-A-deficient mice actually had a significant increase in nitrite (Fig. 7B), indicating a failure to shift toward higher oxide chemistry. Consistent with this finding, SP-A-deficient mice with PCP had less 3-NT (Fig. 8). These changes cannot be attributed to altered oxidative stress since there was no significant change in the production of protein carbonyls in stained lung tissue sections subjected to semiquantitative scoring (WT mice, carbonyl score of 2 versus 2 [uninfected versus PCP, respectively]; SP-A-deficient mice, carbonyl score of 1 versus 2 [uninfected versus PCP, respectively]).

Activated macrophages are known to generate a number of reactive oxygen species, including superoxide anions ($O_2\cdot$), which can dismutate to form hydrogen peroxide (H_2O_2) (7) or interact with NO in the formation of highly reactive oxygen-nitrogen intermediates such as peroxynitrite (48). Hickman-Davis et al. demonstrated in vitro that activated alveolar macrophages from C57BL/6 mice produced significant amounts of nitric oxide and substantially decreased numbers of CFU of *Mycoplasma* in the presence but not in the absence of the SP-A, suggesting NO-mediated pathways are necessary for the killing of some respiratory pathogens (32, 33). SP-A has been shown to augment both NO production and reactive oxygen species by inflammatory cells in a variety of models of infection. Although the exact signal transduction mechanisms for these events are not completely defined, it has recently been shown that SP-A can stimulate a proinflammatory responses via binding by its collagenous tail to calreticulin/CD 91 (24). Although previous in vivo models of *Pneumocystis* infection have shown that the expression of iNOS (the enzyme responsible for generation of NO) was not altered by PCP (55), a failure of SP-A-deficient macrophages to activate could nonetheless result in a shift of NO metabolites to less-oxidized forms and suggest an alternative role for SP-A in the modulation of NO metabolism favoring generation of higher oxides independent of total NO production that could be utilized for killing.

Despite the marked attenuation in the generation of proinflammatory cytokines and RONS, SP-A-deficient mice infected with *Pneumocystis* had biochemical and physiological evidence of enhanced lung damage manifested by increases in total protein content in BAL (Fig. 4A) and SA surfactant (Table 1), elevated lung/body weight ratios (Fig. 4B), and greater BAL cell numbers (Fig. 3A). The mechanism for this is not entirely elucidated. Given that the SP-A-deficient mice had higher burdens of organisms, it is possible that the organism itself is mediating toxicity to the alveolar epithelia. However, while *Pneumocystis* organisms adhere to alveolar epithelia (47, 51), they do not directly injure cultured type II cells in vitro (12). Another potential mechanism could be epithelial damage from one of the resident inflammatory cells. Differential cell counts indicated that the predominant increase in BAL cells observed was due to increased numbers of alveolar macrophages; however, it appears from our data that in the absence of SP-A these cells are actually in a less-activated state. Recently, the

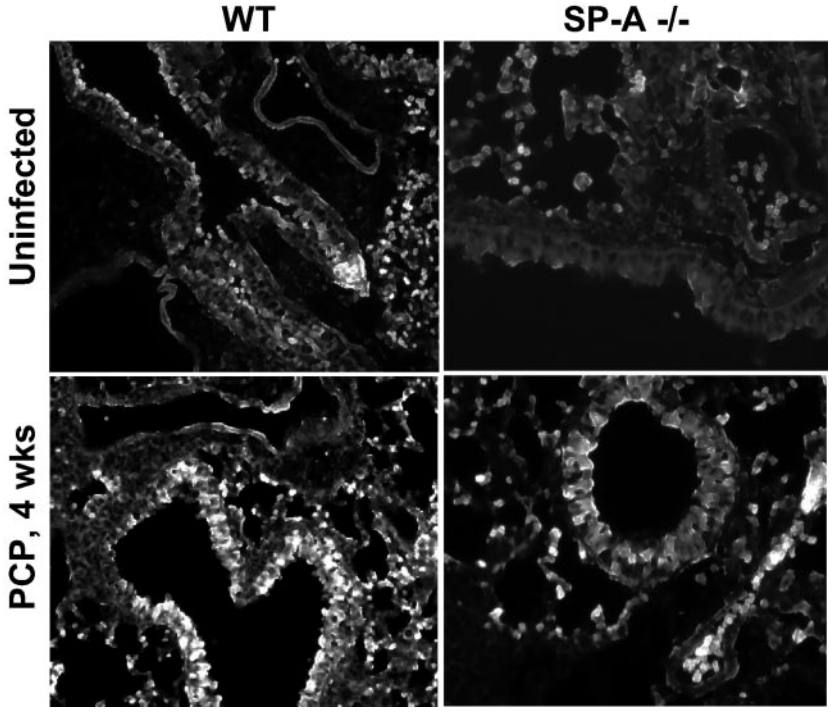

FIG. 8. Nitrotyrosine production is attenuated in the lung tissue of *P. carinii*-infected SP-A-deficient mice. WT and SP-A-deficient mice previously depleted of CD4 T cells by using GK1.5 were inoculated with sterile lung homogenate or 2×10^5 *Pneumocystis* organisms and sacrificed 4 weeks postinfection. Paraffin-embedded sections of lung tissue from uninfected (upper panels) and *P. carinii*-infected (lower panels) mice are shown. Staining with monoclonal anti-nitrotyrosine antibody was performed as described in Materials and Methods. The specificity of staining was confirmed by using parallel sections treated with dithionite to remove nitrotyrosine (not shown). Images shown are representative of five WT and five SP-A-deficient mice in each group. Magnification, ×200.

β-glucan wall of PC has been shown to mediate a proinflammatory response, including induction of MIP-2 from epithelial cells that could recruit neutrophils (31). Although neutrophilia has been associated clinically with enhanced lung damage, including the development of pneumothoraces (17), neutrophil counts in both SP-A-deficient mice and WT controls were similar in response to the development of PCP. In several models of *Pneumocystis*, lung damage has been shown to be a CD8-dependent mechanism (66, 67). Although the total BAL lymphocyte counts were elevated in all *P. carinii*-infected mice, no difference was observed between WT and SP-$A^{-/-}$ infected mice. However, McAllister et al. have recently shown that non-TC-1 $CD8^+$ T cells are mediators of lung injury in PCP (45). Thus, the derangement of lung barrier function observed in SP-A-deficient mice with PCP is likely multifactorial, which may involve indirect injury to the alveolar epithelial barrier through other inflammatory cells (macrophages or subsets of lymphocytes) or soluble factors.

The highest levels of *Pneumocystis* organisms were observed in SP-A-deficient mice simultaneously administered a monoclonal antibody against CD4 T cells. When GK1.5 was omitted, we also noted the presence of low levels of organisms in the lungs of some SP-A-deficient (but never wild-type) mice, suggesting that in the absence of suppression of adaptive (T-cell-mediated) immunity, SP-A-deficient mice also have an increased susceptibility to *Pneumocystis* but at much lower organism burdens. Previously, Linke et al. had demonstrated that SP-A-deficient mice had increased susceptibility to *Pneumocystis*, but infection required concomitant global immunosuppression with systemic corticosteroids (44). Although they were not able to detect overt infection in nonsteroid treated mice by cyst counting, the SP-A-deficient mice in their study did appear to have a detectable large subunit mitochondrial rRNA from *Pneumocystis*. The difficulties in definitive discernment of quantitative differences in infectivity in the nonimmunosuppressed population may lie in the relative insensitivity of current modes of detection (cyst counting or histopathological scoring) at low organism burdens. Recently, *Pneumocystis* specific reverse transcription-PCR-based methods to quantitate the presence of PCP organisms in whole lung tissue have emerged, and application of this methodology may be adaptable to enhance the ability to detect lower levels of infection in these models (13, 57).

In addition to SP-A, previous in vitro and in vivo studies have suggested an interaction between *Pneumocystis* and the other lung collectin present in the distal airspaces, SP-D. SP-D protein levels in alveolar lavage fluid from immunocompromised mice with *Pneumocystis* are markedly increased (2, 3, 43). As for SP-A-deficient mice, when inoculated with *Pneumocystis*, CD4-depleted, SP-D-deficient mice also develop increased intensity of infection compared to WT mice despite higher lung inflammation scores and increased amounts of alveolar inflammatory cells. However, in contrast to SP-A-deficient mice, the increased inflammation and lung damage seen in SP-D-deficient mice was associated with higher tissue levels of iNOS, total NO, and nitrotyrosine, implicating en-

hanced oxidative stress as a mediator of the injury (1). In the present study, SP-A-deficient mice had equivalent levels of SP-D versus WT mice at baseline and show increased but somewhat attenuated SP-D protein expression in response to *Pneumocystis* infection. Thus, it is unlikely that the results in the present study showing increases in intensity of infection and in lung damage with SP-A-deficient mice can be attributed to a commensurate acquired SP-D deficiency.

The blunted levels of SP-D in the BAL of *P. carinii*-infected SP-A-deficient mice are of interest and were associated with attenuated levels of the Th2 cytokines IL-4 and IL-5 (Fig. 6). These cytokines have been shown to be important regulatory molecules for the expression of SP-D. IL-4-overexpressing mice have marked increases in total SP-D in BAL and in tissue (34). IL-4 added to isolated rat type II cells in vitro stimulates SP-D production by these cells (20). The present results obtained in the SP-A-deficient mouse suggest an important link between SP-A, TNF-α expression, Th-2 cytokine (IL-4) production, Th-1 cytokine (IFN-γ) elaboration, and the regulation of innate immunity (SP-D).

In summary, we have shown that SP-A-deficient mice are more susceptible to *Pneumocystis* infection, with this effect being most evident with the concomitant use of selective CD4 depletion. The SP-A-deficient mice had a significantly higher incidence and intensity of *Pneumocystis* infection than did the SP-A intact mice. The absence of SP-A was also associated with an attenuated production of proinflammatory cytokines previously shown to be essential to effect the clearance of the organism in other model systems. In addition, macrophage activation, as indicated by both morphological changes and inhibition of RONS generation, suggests that SP-A modulates the clearance of the organism at a variety of levels, including agglutination, opsonization, and killing. Furthermore, despite deficits in the generation of toxic cytokines and RONS, we have also shown that the SP-A-deficient mouse infected with *Pneumocystis* is unable to coordinate and limit lung injury in response to the pathogen. We conclude that SP-A is an important mediator in the lung-host defense against *Pneumocystis* infection in vivo acting at multiple levels in the host defense paradigm. Further understanding of its mechanism of action may lead to improvements in directed approaches to therapy.

ACKNOWLEDGMENTS

This study was supported by NIH grants RO1 HL 59867 (M.F.B.), RO1 HL 64520 (M.F.B.), RO1 59823 (J.M.B.), HL-24075 (S.H.), and HL-58047 (S.H.) and a Department of Veterans Affairs Merit Review (J.M.B.).

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Editor: F. C. Fang