Granulocyte-Macrophage Colony-Stimulating Factor in the Innate Immune Response to *Pneumocystis carinii* Pneumonia in Mice

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Granulocyte-Macrophage Colony-Stimulating Factor in the Innate Immune Response to Pneumocystis carinii Pneumonia in Mice

Robert Paine III,2,* Angela M. Preston,* Steven Wilcoxen,* Hong Jin,* Brian B. Siu,* Susan B. Morris,* Jacquelyn A. Reed,‡ Gary Ross,‡ Jeffrey A. Whitsett,‡ and James M. Beck*†

Innate immunity plays an important role in pulmonary host defense against Pneumocystis carinii, an important pathogen in individuals with impaired cell-mediated immunity. We investigated the role of GM-CSF in host defense in a model of P. carinii pneumonia induced by intratracheal inoculation of CD4-depleted mice. Lung GM-CSF levels increased progressively during the infection and were significantly greater than those in uninfected controls 3, 4, and 5 wk after inoculation. When GM-CSF gene-targeted mice (GM−/−) depleted of CD4+ cells were inoculated with P. carinii, the intensities of infection and inflammation were increased significantly compared with those in CD4-depleted wild-type mice. In contrast, transgenic expression of GM-CSF directed solely in the lungs of GM−/− mice (using the surfactant protein C promoter) dramatically decreased the intensity of infection and inflammation 4 wk after inoculation. The concentrations of surfactant proteins A and D were greater in both uninfected and infected GM−/− mice compared with those in wild-type controls, suggesting that this component of the innate response was preserved in the GM−/− mice. However, alveolar macrophages (AM) from GM−/− mice demonstrated impaired phagocytosis of purified murine P. carinii organisms in vitro compared with AM from wild-type mice. Similarly, AM production of TNF-α in response to P. carinii in vitro was totally absent in AM from GM−/− mice, while GM-CSF-replete mice produced abundant TNF in this setting. Thus, GM-CSF plays a critical role in the inflammatory response to P. carinii in the setting of impaired cell-mediated immunity through effects on AM activation. The Journal of Immunology, 2000, 164: 2602–2609.

Pneumonia caused by Pneumocystis carinii remains an important cause of morbidity and mortality in patients infected with HIV and in other immunocompromised individuals. Despite the aggressive use of prophylactic antibiotics, individuals with AIDS often come to medical attention heavily infected with this organism. Extensive information from patients (1–3) and animal models (4, 5) has made it clear that abnormalities in cell-mediated immunity, especially diminished numbers of CD4+ T cells, are major factors increasing susceptibility to P. carinii pneumonia. However, it also is evident that innate immunity plays an important role in the host response to this infection. Alveolar macrophages (AM)3 bind and internalize P. carinii organisms (6, 7). AM also release inflammatory mediators in response to P. carinii, including TNF (8, 9), IL-8 (10, 11), and arachidonic acid metabolites (12). In a rat model of P. carinii pneumonia, depletion of AM results in impaired early clearance of organisms (6, 13), further supporting the importance of inflammatory cells and innate immunity within the alveolar space for defense against P. carinii.

A likely candidate molecule to modulate this innate immune response is GM-CSF, a cytokine expressed by a variety of pulmonary cells, including activated T cells, macrophages, fibroblasts, and epithelial cells (14). GM-CSF has potent effects on mononuclear cells. Specifically, GM-CSF is mitogenic (15, 16) and chemotactic (17) for alveolar macrophages and inhibits macrophage apoptosis (15). In addition to this ability to influence the number of macrophages at a site in the lung, GM-CSF activates macrophages for enhanced activity against bacterial and fungal pathogens (18–20). In the context of HIV infection, in vitro treatment of inflammatory cells with GM-CSF corrects the defect in Fe receptor-mediated phagocytosis by monocyte-derived macrophages (21) and restores the respiratory burst in response to P. carinii in neutrophils (22). Furthermore, GM-CSF is centrally involved in the regulation of levels of surfactant proteins A and D (SP-A and SP-D) in the lung. Both SP-A and SP-D can mediate binding of AM to P. carinii and influence the rate of phagocytosis of the organism (23–26). Taken together, these attributes have suggested that GM-CSF might be of therapeutic benefit for HIV-infected individuals with P. carinii pneumonia, improving pulmonary host defense against this opportunistic pathogen. In fact, systemic administration of recombinant GM-CSF to mice with established P. carinii pneumonia increases clearance of the organism, although the mechanisms remain unclear (27). However, the potential role of endogenous GM-CSF in the lung for host defense against P. carinii has not been investigated.
We hypothesized that GM-CSF would be expressed in the lung in a murine model of P. carinii pneumonia, and that endogenous pulmonary GM-CSF would play an important role in controlling the progression of this infection. We determined that pulmonary GM-CSF was induced during P. carinii pneumonia in CD4-deficient mice. Transgenic mice lacking GM-CSF (GM−/−) developed P. carinii pneumonia more rapidly and with more severe inflammation than wild-type control mice. Murine AM harvested from GM−/− mice were less efficient in the phagocytosis of P. carinii in vitro and produced far less TNF in response to P. carinii than AM from wild-type controls. When the GM-CSF gene (under control of the surfactant protein C (SP-C) promoter) was reinserted into the lungs of GM−/− mice, the mice were dramatically more resistant to P. carinii infection. Expression of GM-CSF in the alveolar space alone restored TNF production by AM exposed to P. carinii in vitro. Thus, these studies demonstrate a critical role for pulmonary GM-CSF in the innate defense against P. carinii in the lung and demonstrate two important mechanisms by which pulmonary GM-CSF controls host defense in the lung.

Materials and Methods

Animals

Athymic mice (nu/nu on a BALB/c background, used to passage P. carinii) and BALB/c mice were obtained from Taconic Laboratories (Germantown, NY). C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). GM−/− mice were generated by Dranoff et al. (28) by targeted interruption of the GM-CSF gene and express no detectable GM-CSF. These mice have been extensively back-crossed against C57BL/6 mice. SP-C-GM mice, in which GM-CSF is expressed only in the lung, were generated from GM−/− mice by transgenic expression of a chimeric gene containing GM-CSF under the SP-C promoter (29). All mice were housed in microisolator cages under laminar flow hoods in an isolation room of the animal care facilities at the University of Michigan and the Ann Arbor Veterans Affairs Medical Center. Mice were provided with autoclaved bedding, food and water, and handling of mice was performed in a biosafety cabinet. Sentinel mice cohoused with experimental mice are necropsied periodically for detection of P. carinii (by silver staining of lung sections) and other pathogens. All procedures were approved by the animal care committees at the University of Michigan and the Ann Arbor Veterans Affairs Medical Center.

Induction of P. carinii pneumonia

Mice were depleted of CD4+ T cells by weekly i.p. injections of rat anti-mouse mAb GK1.5 (4, 30). This hybridoma was obtained from American Type Culture Collection (Manassas, VA) and harvested as ascites from pristane-primed, uninfected scid mice. The adequacy of in vivo depletion was confirmed by flow cytometry in both BALB/c and C57BL/6 mice (4). Ab injections were continued for the duration of the experiments. Murine P. carinii organisms were obtained from the lungs of infected athymic mice in which P. carinii was passaged serially as previously described (4). To prepare the inoculum, infected athymic mice were euthanized and exsanguinated. Lungs were removed aseptically, placed in sterile PBS, and frozen at −20°C for 2 h. Touch preparations, stained with Gram’s stain, were used to exclude lungs with bacterial contamination. Frozen lungs were homogenized mechanically, filtered, and centrifuged at 500 × g for 10 min at 5°C. The pellet was resuspended in PBS, and smears were stained with modified Giemsa stain and Gomori methenamine silver stain to count organisms. This preparation was used to inoculate experimental mice with organisms (1 × 105 cysts) via the trachea under direct visualization. For each experiment, all recipients were inoculated with aliquots from the same preparation on the same day.

Determination of lung GM-CSF concentration

At appropriate experimental time points after intraotracheal inoculation with P. carinii, mice were euthanized with pentobarbital, and the lungs were perfused free of blood via a catheter placed in the right ventricle. The lungs were removed from the mice, dissected free of the central airways, and homogenized in PBS. The lung homogenate was stored at −70°C. The protein content of the lung homogenate was determined by a colorimetric assay (Bio-Rad, Hercules, CA). Subsequently, the concentration of GM-CSF in the lung homogenate was determined using an ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s recommendations using a Bio-Tek EL311 plate reader (Bio-Tek, Burlington, VT).

Histological determination of intensity of infection and inflammation

At serial time points after P. carinii inoculation, mice were euthanized with pentobarbital, and the lungs were perfused via the right ventricle until the effluent was free of blood. The lungs were removed and inflated first with air, then with neutral buffered formalin. Paraffin-embedded tissue blocks were sectioned and stained with hematoxylin-eosin and Gomori methenamine silver stains. The extent of P. carinii infection and of histologic inflammation was evaluated using scales previously described and validated (4, 31). Sections were scored by an observer blinded to the identity of the sections. P. carinii cyst scores were evaluated on sections stained with Gomori methenamine silver stain, with a range from 0 (no alveoli) to 4 (cysts throughout the alveolus in most regions with foamy extracellular alveolar exudate). Prior studies have demonstrated that grading of intensity of infection correlates strongly with organism counts performed on lung homogenates (32). The inflammatory infiltrate was scored on sections stained with the hematoxylin-eosin stain. Alveolar inflammation was scored with a range of 0 (no inflammatory cells or macrophages in the alveolar walls or parenchyma) to 5+ (confuent consolidation of the alveolar parenchyma).

Bronchoalveolar lavage (BAL) and differential cell counting

Mice were euthanized with pentobarbital, and the lungs were perfused via the right ventricle until the effluent was free of blood. The trachea was cannulated, and the lungs were lavaged with a total of 3 ml of PBS in 0.5-ml aliquots. The lavage aliquots for each animal were pooled, and the cell pellet was collected by centrifugation. Differential cell counts were performed on >200 cells/mouse stained with hematoxylin-eosin as described previously (4).

BAL fluid phospholipid and surfactant protein content

Mice were anesthetized and exsanguinated, and the trachea was cannulated. Following lung lavage, total BAL fluid for each animal was pooled, volume was recorded, and aliquots were prepared for analyses of saturated phosphatidylcholine or proteins. The concentration of saturated phosphatidylcholine was determined for each sample as described previously (33). Aliquots containing 1 µg of saturated phosphatidylcholine were subjected to SDS-PAGE in the presence of 2-ME for analysis of SP-A and SP-D. Proteins were separated on 8–16% acrylamide gel with Tris-glycine buffer and stained with amido black. The concentration of saturated phosphatidylcholine or proteins was determined by a colorimetric assay (Bio-Rad, Hercules, CA). Following electrophoresis, proteins were transferred to nitrocellulose (Schleicher & Schuell, Keene, NH). Immunoblot analysis was conducted with the following dilutions of antisera: SP-A, 1/25,000 guinea pig anti-rat SP-A; and SP-D, 1/10,000 rabbit anti-rat SP-D (34, 35). The rabbit anti-rat SP-D antiserum was a gift from Dr. Frances X. McCormack (University of Cincinnati, Cincinnati, OH). Appropriate peroxidase-conjugated secondary Abs were used at 1/10,000 dilutions. Immunoreactive bands were detected by chemiluminescence (ECL, Amersham, Arlington Heights, IL.). The relative intensity of the signal was measured using the National Institutes of Health Image 1.52 software (Bethesda, MD).

Isolation of P. carinii organisms for in vitro study

P. carinii organisms were isolated from the lungs of athymic mice using a modification of the method of Kaneshiro et al. (36). Athymic mice infected with P. carinii were killed during pentobarbital anesthesia, and the lungs were perfused via the right ventricle with NaCaHEPES buffer (150 mM NaCl, 1.8 mM CaCl2, and 25 mM HEPES, pH 7.4) containing 0.5% glucose and then were removed aseptically. Lungs were homogenized in a stomacher lab blender (Tekmar, Cincinnati, OH). The lung homogenate was passed through a sterile 60-mesh sieve and centrifuged at 925 × g for 10 min. The pellet was resuspended in NaCaHEPES, then centrifuged at 60 × g for 4°C for 10 min. The resulting pellet was treated with 0.85% NH4Cl, pH 6.8, at 37°C for 15 min to lyse host cells, then diluted 3-fold with NaCaHEPES. After centrifugation at 925 × g for 4°C for 10 min, the pellet was resuspended in NaCaHEPES, then centrifuged at 60 × g for 4°C for 10 min, and the supernatant was retained. After two additional cycles of low and high speed centrifugation, the preparation was passed through a 25-mm polycarbonate membrane with 8-µm pores (Poretics, Livermore, CA), followed by filtration through two membranes with 5-µm pores. The filtrate containing isolated centrifuged organisms was stored at −70°C until used. After microscopic confirmation of the purity of the preparation and enumeration of organisms using slides stained with modified Giemsa stain, the highly purified P. carinii organisms were labeled.
P. carinii organisms were labeled with FITC (Sigma) using a modification of a previously described technique (7). P. carinii organisms (3 × 10^5/ml in 800-μl volume) were labeled in 10 ml of FITC (0.1 mg/ml in PBS) at 37°C for 30 min. The labeled organisms were washed extensively with PBS, and an aliquot was removed to confirm labeling by fluorescence microscopy. The labeled organisms then were used immediately for in vitro macrophage assays.

**AM binding and phagocytosis of P. carinii in vitro**

The activity of AM for binding and phagocytosis of P. carinii in vitro was determined based on previously described methods (7, 37). Alveolar macrophages were obtained from wild-type C57BL/6 and GM^−/−^ mice by whole lung lavage. AM (10^5/well in DMEM without FCS) were adhered for 30 min in wells of eight-well tissue culture-treated plastic slides (Nunc, Naperville, IL) in the presence or the absence of recombinant murine GM-CSF (R & D Systems). The cells were washed, and FITC-labeled P. carinii (10^5 cysts/well) were added to wells. After 1-h incubation at 37°C, the wells were washed three times with PBS, then fixed with paraformaldehyde (0.5% in PBS) at room temperature for 2 min, and washed extensively. The cells were viewed by a blinded observer using a Nikon Labphot 2 microscope (Nikon, Melville, NY) equipped with epifluorescence. In each well, the fraction of cells containing labeled organisms was determined by microscopic counting of at least 200 cells in random high power fields. This approach does not distinguish between organisms contained within AM and those closely associated with the macrophage cell surface but not yet internalized. Six replicate wells were evaluated for each condition.

**TFN production by AM in vitro in response to P. carinii**

AM were obtained from the lungs of wild-type C57BL/6 and GM^−/−^ by whole lung lavage as described above and placed in culture in 96-well plates (10^5 cells/well). After the AM had adhered for 30 min the plates were washed, and P. carinii (5 × 10^5 cysts/well) were added to the wells. The cells were incubated for 18 h at 37°C, then the medium was harvested, and the concentration of TNF in the cell-free supernatant was determined using an ELISA kit (R & D Systems), following the manufacturer’s recommendations. In each instance supernatants from quadruplicate wells were measured.

**Statistical methods**

Scalar data were evaluated by t test (two groups) or by ANOVA with the Neuman-Keuls multiple range test (more than two groups). Ordinal data were evaluated by the Mann-Whitney test (two groups) or the Kruskal-Wallis test (more than two groups) (38). Analyses were performed using StatView 5.0 (SAS Institute, Cary, NC) or Instat 2.03 (GraphPad Software, San Diego, CA) software programs. p < 0.05 were accepted as indicating significance.

**Results**

**Increased pulmonary GM-CSF expression in P. carinii pneumonia**

To determine whether lung GM-CSF production is a component of innate defense against P. carinii we measured lung GM-CSF during experimental P. carinii pneumonia. BALB/c mice were depleted of CD4^+ T-cells by treatment with a mAb (GK1.5), then were inoculated intratracheally with P. carinii from the lungs of infected athymic mice. Previous work has shown that these immunosuppressed mice become heavily infected 4 wk after inoculation (4, 39). The expression of GM-CSF protein in lung homogenates increased progressively during 2, 3, and 4 wk of infection (Fig. 1). GM-CSF protein expression in the lungs of CD4-depleted mice was significantly greater at 3, 4, and 5 wk after inoculation with P. carinii than in the lungs of immunologically intact mice or CD4-depleted, uninfected mice. Thus, despite depletion of CD4^+ T cells, lung GM-CSF expression was induced during P. carinii pneumonia.

**Increased severity of P. carinii pneumonia in GM^−/−^ mice**

Having determined that GM-CSF expression was induced during P. carinii pneumonia, experiments were performed to determine whether this endogenous GM-CSF makes a biologically important contribution to host defense against this opportunistic pathogen. GM^−/−^ mice and wild-type (C57BL/6) controls that had been depleted of CD4^+ T-cells were inoculated intratracheally with murine P. carinii. Preliminary studies demonstrated that GM^−/−^ mice with intact CD4^+ T cells were not susceptible to P. carinii at this inoculum (data not shown). The burden of P. carinii organisms in the lungs was determined by scoring of Gomori methenamine silver-stained lung sections (Fig. 2A). GM^−/−^ mice were significantly more heavily infected than wild-type mice at both 2 and 4 wk after inoculation. Specifically, in the absence of GM-CSF, the intensity of infection at 2 wk resembled that in wild-type mice 4 wk after inoculation. By 6 wk both groups of mice had achieved maximal scores of 4+ in the histologic grading of infection.

Histological sections from the wild-type mice demonstrated only mild focal inflammation 2 wk after inoculation with P. carinii, with more extensive inflammation and alveolar exudates observed in GM^−/−^ mice after only 2 wk of infection. By 4 wk after inoculation, when more extensive inflammation had developed in the wild-type mice, the GM^−/−^ mice demonstrated severe pulmonary abnormalities with large numbers of inflammatory cells and extensive alveolar exudates (Fig. 3, right panels). Alveolar exudates were not found in lungs of uninfected GM^−/−^ mice. Quantitative assessment of the severity of lung inflammation using histological scoring demonstrated that 2, 4, and 6 wk after inoculation, inflammation was significantly more intense in the GM^−/−^ mice than in wild-type mice (Fig. 2B). Thus, in the absence of GM-CSF, pneumonia due to P. carinii in CD4-depleted mice is significantly more severe throughout the time course of infection investigated compared with that in CD4-depleted wild-type mice.

To determine whether the cellular composition of the inflammatory response differed between GM^−/−^ and GM-CSF^−/−^ mice in the first stages of infection with P. carinii, differential cell counts were performed on BAL fluid 2 wk after inoculation (Fig. 4). Significantly more inflammatory cells were found in the BAL fluid from the GM^−/−^ mice than in BAL fluid from wild-type controls, consistent with the histological grading of inflammation. In each group of mice, ~50% of the lavage cells were macrophages, with lesser numbers of neutrophils and lymphocytes. The absolute numbers of both AM and neutrophils were increased significantly in the P. carinii-infected GM^−/−^ mice compared with those in infected wild-type mice. Thus, following CD4 depletion,
GM−/− mice developed more severe pneumonia with P. carinii more rapidly than similarly depleted wild-type mice. This severe pneumonia involved both increased numbers of organisms in the lung and more intense inflammatory cell infiltration than in GM-CSF-replete mice.

Surfactant protein accumulation in GM−/− mice infected with P. carinii

In the absence of GM-CSF, surfactant phospholipid and protein accumulate due to impaired surfactant turnover (33). Because SP-A and SP-D bind to P. carinii and may influence phagocytosis of these organisms by AM, we examined the concentrations of SP-A and SP-D in BAL fluid from CD4-depleted GM−/− mice (Fig. 5). In wild-type mice infected with P. carinii SP-A was decreased (50% by densitometry), while SP-D was increased (80% by densitometry), compared with that in uninfected controls. Similarly, in GM−/− mice SP-A was decreased 15%, while SP-D was little changed following infection. Concentrations of SP-A and SP-D were markedly increased in uninfected GM−/− mice compared with those in uninfected wild-type mice (by 95% and 75%, respectively).

Binding and phagocytosis of P. carinii by GM−/− AM in vitro

A possible mechanism for the increased intensity of P. carinii infection in GM−/− mice compared with wild-type mice is impaired activity of AM from the GM−/− mice. To determine...
whether the binding and phagocytic activity of AM for \( P.\) carinii were impaired in the absence of GM-CSF. AM from uninfected wild-type and GM\(^{-/-}\) mice were exposed in vitro to purified murine \( P.\) carinii labeled with FITC. The percentage of AM containing or closely associated with \( P.\) carinii was increased significantly in wild-type mice compared with that in GM\(^{-/-}\) mice (Fig. 6). Short term exposure of AM to recombinant GM-CSF in vitro enhanced the binding/phagocytosis of \( P.\) carinii by AM from both control and GM\(^{-/-}\) mice. In fact, incubation with a sufficient concentration of recombinant GM-CSF (8 ng/ml) restored the binding/phagocytic capacity of AM from GM\(^{-/-}\) mice to levels comparable to those of AM from wild-type mice.

**Resistance to infection with \( P.\) carinii in SP-C-GM mice**

To determine whether the increased severity of \( P.\) carinii pneumonia in GM\(^{-/-}\) mice was a consequence of inadequate mononuclear phagocyte maturation in the bone marrow, we examined the severity of infection in SP-C-GM mice. These mice were generated by insertion of the GM-CSF gene under control of the SP-C promoter, against the background of mutant mice deficient in GM-CSF. SP-C-GM mice express GM-CSF at supernormal levels in the lungs, but do not express GM-CSF in other tissues (40). GM\(^{-/-}\) mice and SP-C-GM mice were depleted of CD4\(^+\) cells and inoculated with \( P.\) carinii. The concentration of GM-CSF in BALF from \( P.\) carinii-inoculated SP-C-GM mice was 114.2 ± 19.1 pg/ml, while GM-CSF was not detected in BAL fluid (in contrast to lung homogenates) of wild-type mice. The intensity of infection and inflammation were determined after 4 wk. GM\(^{-/-}\) mice were very heavily infected, consistent with previous experiments. In contrast, only rare organisms were present in the lungs of the SP-C-GM mice (Fig. 7A). In fact, organisms could be identified in only 25% of the SP-C-GM mice, but were readily identified in all GM\(^{-/-}\) mice. Similarly, the intensity of inflammation was greatly reduced in the SP-C-GM mice compared with that in the GM\(^{-/-}\) mice (Fig. 7B). Thus, overexpression of GM-CSF in the alveolar space alone, in the absence of GM-CSF at other sites, rendered the mice resistant to \( P.\) carinii.

**Decreased TNF release by GM\(^{-/-}\) AM in response to \( P.\) carinii in vitro**

Alveolar macrophages from wild-type, GM\(^{-/-}\), and SP-C-GM mice were placed in culture and exposed to purified murine \( P.\) carinii. After 18 h the culture supernatants were harvested, and TNF was measured by ELISA (Table I). AM from GM\(^{-/-}\) mice

![FIGURE 6](image-url)

**FIGURE 6.** AM from GM\(^{-/-}\) mice demonstrated impaired binding and phagocytosis of \( P.\) carinii in vitro. AM isolated by BAL from wild-type and GM\(^{-/-}\) mice were placed in culture for 1 h in medium alone or in medium supplemented with recombinant murine GM-CSF. \( P.\) carinii organisms, purified from murine lung and labeled with FITC, were then added to the cells. After 1 h, the cells were fixed, and the percentage of AM associated with labeled organisms was determined by microscopic counting on a fluorescence microscope (\( n = 4 \) mice in each group). *\( p < 0.05\) vs wild-type AM at each concentration of GM-CSF.

![FIGURE 7](image-url)

**FIGURE 7.** The severity of \( P.\) carinii pneumonia is reduced by directed expression of GM-CSF in the lung in GM\(^{-/-}\) mice. GM\(^{-/-}\) and SP-C-GM mice were depleted of CD4\(^+\) T cells and inoculated intratracheally with \( P.\) carinii. After 4 wk the lungs were stained with either Gomori methenamine silver (\( P.\) carinii score) or hematoxylin-eosin (inflammation score). The intensity of infection (A) and the intensity of inflammation (B) were determined by a blinded observer. Data are expressed as median values (\( n = 10 \) GM\(^{-/-}\) mice; \( n = 8 \) SP-C-GM mice). *\( p < 0.05\) vs GM\(^{-/-}\) mice.
failed to produce detectable TNF in response to \textit{P. carinii}. In contrast, AM from wild-type and SP-C-GM mice produced significant amounts of TNF in vitro following exposure to purified \textit{P. carinii}. Thus, in the absence of GM-CSF, macrophage production of TNF was greatly impaired, while expression of GM-CSF in the alveolar space restored TNF expression in response to \textit{P. carinii}.

**Discussion**

The current report provides the first information concerning the role of endogenous GM-CSF in host defense against \textit{P. carinii}. GM-CSF protein expression was induced in the lung during \textit{P. carinii} pneumonia. In experimental \textit{P. carinii} pneumonia in CD4-depleted mice, GM-CSF-deficient mice were susceptible to severe \textit{P. carinii} infection, developing more intense infection and increased inflammation compared with wild-type mice. Overexpression of GM-CSF within the lung greatly decreased the susceptibility of GM$^{-/-}$ mice to infection with \textit{P. carinii}. Phagocytic activity of AM from GM$^{-/-}$ mice for purified \textit{P. carinii} in vitro was impaired, but transient exposure of the AM to recombinant GM-CSF in vitro significantly enhanced the activity of AM from both groups of mice for the pathogen. In vitro expression of TNF in response to \textit{P. carinii} organisms was absent in AM from GM$^{-/-}$, but was restored in AM from deficient mice in which a GM-CSF transgene was expressed only in the lung. Taken together, these findings demonstrate an important role for GM-CSF in host defense against \textit{P. carinii} in the lung and in the activity of alveolar macrophages against \textit{P. carinii}.

We investigated three potential mechanisms by which endogenous GM-CSF might enhance host defense against \textit{P. carinii}. AM bind and internalize \textit{P. carinii} organisms (6, 7) and release TNF (8, 9) and other inflammatory mediators, such as IL-8 (10, 11) and arachadonic acid metabolites (12). In a rat model of \textit{P. carinii} pneumonia, depletion of AM before inoculation with \textit{P. carinii} resulted in impaired early clearance of organisms (6, 13). GM-CSF is a potent activator of AM. We found that AM obtained from GM$^{-/-}$ mice were less effective than AM from control mice in binding and phagocytosis of \textit{P. carinii} in vitro. Interestingly, this effect may be specific for this pathogen; in contrast to the findings with \textit{P. carinii}, macropathage phagocytosis of group B streptococci was not altered in the macrophages from the GM$^{-/-}$ mice compared with that in AM from control mice (20). Furthermore, we found that pulmonary GM-CSF protein expression was increased in \textit{P. carinii}-infected mice, and that the exposure of AM to GM-CSF in vitro resulted in enhanced binding/phagocytic activity against \textit{P. carinii}. These results support the hypothesis that pulmonary infection with \textit{P. carinii} results in increased local expression of GM-CSF, which, in turn, leads to enhanced AM activity against the pathogen.

A second potential mechanism to explain more severe \textit{P. carinii} infection in GM$^{-/-}$ mice is impaired TNF elaboration. AM from GM$^{-/-}$ mice failed to produce detectable TNF in response to \textit{P. carinii} in vitro. TNF is a necessary component of the host response to \textit{P. carinii} (8). When scid mice infected with \textit{P. carinii} are reconstituted with normal splenocytes, the infection is cleared (41). However, if the mice receive neutralizing anti-TNF Ab, the infection persists unrestrained. Similarly, in studies in which TNF activity has been blocked by adenovirus-induced overexpression of a TNF inhibitor, \textit{P. carinii} clearance by mice with intact CD4$^+$ cells is delayed, while CD4-depleted mice develop worsening infection (42). Taken together, our in vitro studies indicate that endogenous GM-CSF plays a critical role in promoting both direct AM activity against \textit{P. carinii} and the activity of AM as sentinel cells initiating the inflammatory cascade.

A third potential mechanism by which GM-CSF might influence host responses to \textit{P. carinii} is through effects on the expression of SP-A and SP-D (23, 43, 44). Alveolar concentrations of SP-A and SP-D are markedly increased in the lungs of GM$^{-/-}$ mice (33, 45). SP-A and SP-D bind to and enhance opsonization of a variety of pathogens (23). Transgenic mice genetically deficient in these surfactant proteins display increased susceptibility to a number of bacterial species. The data concerning the potential role of SP-A and SP-D in host defense against \textit{P. carinii} are complex. SP-A (26) and SP-D (24, 25) enhance binding of \textit{P. carinii} to AM in vitro. Transgenic mice genetically deficient in SP-A are more susceptible to \textit{P. carinii} than wild-type controls, supporting the contention that SP-A plays a protective role in host defense against this pathogen (46). However, it has also been argued that SP-A actually could inhibit phagocytosis of \textit{P. carinii} in the complex milieu in the lung (37). Not unexpectedly, we found that SP-A and SP-D levels were elevated in uninfected GM$^{-/-}$ mice compared with those in wild-type controls. During pulmonary infection with \textit{P. carinii}, the BAL fluid SP-A concentration decreased, while SP-D increased, in both GM$^{-/-}$ mice and wild-type mice. Thus, increased SP-A and SP-D, by themselves, were not sufficient for protection of the GM$^{-/-}$ mice against \textit{P. carinii} infection. However, these data leave open the possibility that excess SP-A could increase susceptibility to infection. Exogenous GM-CSF increased AM phagocytic activity for \textit{P. carinii}, indicating that GM-CSF enhances AM function in a manner independent of SP-A and SP-D.

Although GM-CSF alters AM function, the effects of GM-CSF on the vulnerability of the host to \textit{P. carinii} pneumonia also may be mediated by effects on alveolar epithelial cells. Rat type II epithelial cells express cell surface receptors for GM-CSF and proliferate in response to this growth factor (40). Furthermore, overexpression of GM-CSF in the peripheral lung in transgenic mice causes type II epithelial cell hyperplasia (40). Although there are no data available concerning the effects of GM-CSF during lung injury, in the skin, GM-CSF is mitogenic for keratinocytes. Injection of recombinant GM-CSF into the dermal lesions of patients with leprosy induces keratinocyte proliferation and regenerative differentiation (47). Type II alveolar epithelial cells function as stem cells for the alveolar epithelium, proliferating and subsequently differentiating to replace thin type I alveolar cells that are particularly susceptible to injury (48). It is plausible that the effects of GM-CSF on alveolar epithelial cells provide a measure of protection for the epithelium from acute injury.

There are several features of the model system used in these experiments that suggest that the results may be extrapolated to human disease. \textit{P. carinii} organisms derived from different host species are pathogenic specifically for that species (49, 50). The mechanism for this specificity has not yet been defined, but may

### Table I. AM from GM$^{-/-}$ mice do not secrete detectable TNF following exposure to \textit{P. carinii} in vitro

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<thead>
<tr>
<th>Group$^a$</th>
<th>TNF (pg/ml)$^b$</th>
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<td>Wild type</td>
<td>1768.1 ± 177.4</td>
</tr>
<tr>
<td>GM$^{-/-}$</td>
<td>None detected</td>
</tr>
<tr>
<td>SP-C-GM</td>
<td>1993.0 ± 515.7</td>
</tr>
</tbody>
</table>

$^a$ AM were isolated by BAL from uninfected wild-type, GM$^{-/-}$, and SP-C-GM mice. The AM were exposed to purified murine \textit{P. carinii} organisms (5 × 10$^5$ cysts/well) for 18 h.

$^b$ TNF protein in the culture supernatants was measured by ELISA. The sensitivity of this ELISA is 23.4 pg/ml. Data are expressed as mean ± SEM for quadruplicate wells.
intact immune responses, we chose to conduct these studies in our standard inoculum, even in mice depleted of CD4+ expression upon exposure to include impaired phagocytosis of organisms and diminished TNF.

P. carinii pneumonia supports the feasibility of these approaches (53, 54). Increased susceptibility of GM-CSF depleted mice infected with P. carinii leads to reduced infection/2 mice. However, it is important to note that the absence of GM-CSF alone in otherwise intact mice was not sufficient to render mice susceptible to establishment of P. carinii pneumonia using our standard inoculum of organisms. This observation makes it unlikely that the major contribution of CD4+ cells to resistance to P. carinii is production of GM-CSF.

The beneficial effect of endogenous GM-CSF induced in the lung during P. carinii pneumonia supports the concept that exogenous GM-CSF might be of therapeutic benefit in this infection. Indeed, systemic administration of recombinant GM-CSF to CD4-depleted mice infected with P. carinii leads to reduced infection scores and enhanced alveolar macrophage TNF production (27). However, there are potential difficulties with systemic administration of GM-CSF in individuals with AIDS, including systemic side effects and the possibility of increasing the burden of HIV (52).

Thus, local therapy with GM-CSF in the lung, either by aerosol administration or via gene transfer, has considerable appeal as an adjunct therapy in P. carinii pneumonia. Prior studies using these approaches to correct the surfactant abnormalities in GM-/- mice support the feasibility of these approaches (53, 54).

In conclusion, we have found that GM-CSF is induced in the lung during P. carinii pneumonia in CD4-depleted mice. The source of GM-CSF is likely to be intrinsic pulmonary epithelial cells and macrophages. Mutant mice deficient in GM-CSF develop more severe pneumonia with P. carinii than wild-type controls, while mice expressing GM-CSF only in the lungs develop far less severe P. carinii pneumonia. Potential mechanisms of the increased susceptibility of GM-/- mice to P. carinii pneumonia include impaired phagocytosis of organisms and diminished TNF expression upon exposure to P. carinii by AM from GM-/- mice. These studies indicate that endogenous pulmonary GM-CSF is an important constituent of the innate immune response to P. carinii in the lung and support the potential use of exogenous recombinant GM-CSF as adjunct therapy for this important infection.

References