Role of Granulocyte Macrophage Colony-Stimulating Factor in Host Defense Against Pulmonary *Cryptococcus neoformans* Infection during Murine Allergic Bronchopulmonary Mycosis

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We investigated the role of granulocyte macrophage colony-stimulating factor (GM-CSF) in host defense in a murine model of pulmonary cryptococcosis induced by intratracheal inoculation of *Cryptococcus neoformans*. Pulmonary *C. neoformans* infection of C57BL/6 mice is an established model of an allergic bronchopulmonary mycosis. Our objective was to determine whether GM-CSF regulates the pulmonary Th2 immune response in *C. neoformans*-infected C57BL/6 mice. Long-term pulmonary fungistasis was lost in GM-CSF knockout (GM^{-/-}) mice, resulting in increased pulmonary burden of fungi between weeks 3 and 5. GM-CSF was required for the early influx of macrophages and CD4 and CD8 T cells into the lungs but was not required later in the infection. Lack of GM-CSF also resulted in reduced eosinophil recruitment and delayed recruitment of mononuclear cells into the airspace. Macrophages from GM^{+/+} mice showed numerous hallmarks of alternatively activated macrophages: higher numbers of intracellular cryptococci, YM1 crystals, and induction of CCL17. These hallmarks are absent in macrophages from GM^{-/-} mice. Mucus-producing goblet cells were abundantly present within the bronchial epithelial layer in GM^{+/+} mice but not in GM^{-/-} mice at week 5 after infection. Production of both Th1 and Th2 cytokines was impaired in the absence of GM-CSF, consistent with both reduced *C. neoformans* clearance and

absence of allergic lung pathology. (Am J Pathol 2007, 170:1028–1040; DOI: 10.2353/ajpath.2007.060595)

Cryptococcus neoformans, an opportunistic pathogenic fungal pathogen, enters the host through inhalation and causes lethal mycosis in individuals with compromised T-cell-mediated immunity, especially those with acquired immune deficiency syndrome (AIDS).¹ Studies using inbred mouse models of pulmonary *C. neoformans* infection indicate the genetic background of the host is a key determinant of the immune response. Inbred immunocompetent mouse strains such as CBA/J, C.B-17, and BALB/c clear *C. neoformans* infection from the lungs. In contrast, C57BL/6 mice develop a chronic cryptococcal pulmonary infection.^{2–5} Development of Th1-cell-mediated immunity is required for the clearance of *C. neoformans* in resistant hosts.^{6–8} Interferon- γ (IFN- γ), tumor necrosis factor (TNF)- α , interleukin (IL)-2, IL-12, IL-15, IL-18, monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1 α , and nitric oxide have been shown to play important roles in protective cell-mediated immunity in resistant mouse strains.^{8–16}

Chronic fungal infection can result from a Th1/Th2 imbalance when cellular immunity is shifted toward Th2 from Th1 immune responses.¹⁷ C57BL/6 mice are inherently susceptible to *C. neoformans* infection and develop a nonresolving pulmonary fungal infection characterized by the development of chronic infection and strong Th2 inflammatory responses.^{2,18–21} Chronic *C. neoformans* in-

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fections result in active fungal growth in the lungs with very low dissemination to the spleen and brain. Cultured lung leukocytes from infected C57BL/6 mice produce higher levels of IL-5 and lower levels of IFN- γ or IL-2 compared with lung leukocytes from infected CBA/J or C.B-17.^{2,4,22} Elevated production of IL-5 in the lungs of C57BL/6 mice promotes the recruitment and activation of eosinophils, resulting in eosinophil-mediated tissue destruction in the lungs. The level of susceptibility in cryptococcal infection correlates with the numbers of eosinophils infiltrating the lungs. Eosinophils are a main pulmonary cellular component of the inflammatory infiltrate in *C. neoformans* strain 24067-infected C57BL/6 mice.⁴ Taken together, *C. neoformans*-infected C57BL/6 mice display the characteristics of an allergic bronchopulmonary mycosis (ABPM).

Granulocyte macrophage-colony-stimulating factor (GM-CSF) is a cytokine expressed by a variety of pulmonary cells, including activated T cells, macrophages, fibroblasts, and epithelial cells.²³ GM-CSF activates macrophages for enhanced activity against bacterial and fungal pathogens.^{24–27} However, the potential role of endogenous GM-CSF in the lung for host immune defense against *C. neoformans* has not been investigated. The objective of this study was to determine whether GM-CSF plays a role in regulating the pulmonary Th2 immune response in *C. neoformans*-infected C57BL/6 mice during the progression of ABPM. Our data show that 1) GM-CSF-deficient mice developed progressive *C. neoformans* infection in their lungs, 2) GM-CSF supported early recruitment of leukocytes into the interstitial space of the lung and subsequently was required for leukocyte recruitment into the alveolar space, 3) GM-CSF was required for the production of Th1 and Th2 cytokines, and 4) the presence of GM-CSF was associated with the development of ABPM pathology in the *C. neoformans*-infected lungs and formation of alternatively activated macrophages. Taken together, in the absence of GM-CSF, mice were protected from Th2-driven pathology, but they also did not develop a protective immune response against *C. neoformans*.

Materials and Methods

C. neoformans

C. neoformans strain 24067 was obtained from the American Type Culture Collection (Manassas, VA). For infection, yeast cells were grown to stationary phase at 35°C for 48 to 72 hours in Sabouraud dextrose broth (1% neopeptone and 2% dextrose; Difco, Detroit, MI) on a shaker. The yeasts were then washed in sterile nonpyrogenic saline, counted on a hemocytometer, and diluted to 3.3×10^5 colony-forming units (CFU)/ml in saline.

Mice

C57BL/6 (GM^{+/+}) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). GM-CSF mutant mice (GM^{-/-}) were kindly provided by Dr. J. Whitsett (Chil-

dren's Hospital, Cincinnati, OH) by targeted interruption of the GM-CSF gene and express no detectable GM-CSF.²⁸ These mice have been extensively backcrossed against C57BL/6 mice. All mice were housed under specific pathogen-free conditions in closed filter-top cages in the animal facility of the University of Michigan medical center.

Mice were supplied with autoclaved bedding, food, and water, and handling of mice was performed in a biosafety cabinet. Bedding from the mice was transferred weekly to cages of uninfected sentinel mice that were periodically bled and found to be negative for antibodies to mouse hepatitis virus, Sendai virus, and *Mycoplasma pulmonis*.

Intratracheal Inoculations

Mice were anesthetized by intraperitoneal injection of pentobarbital (0.074 mg/g weight of mice) and were restrained on a small surgical board. A small incision was made through the skin over the trachea, and the underlying tissue was separated. A 30-gauge needle was bent and attached to a tuberculin syringe filled with the *C. neoformans* culture. The needle was inserted into the trachea, and 30 μ l of inoculum was dispensed into the lungs (10^4 CFU). The skin was closed with a cyanoacrylate adhesive. The mice recovered with minimal visible trauma.

CFU Assay

For lung CFU, small aliquots were collected from lung digests. For spleen and brain CFU, the organs were excised, placed in 2 ml of sterile water, and homogenized. Ten-microliter aliquots of the lungs, spleen, and brain were plated on Sabouraud dextrose agar plates in duplicate serial 10-fold dilutions and incubated at room temperature. *C. neoformans* colonies were counted 3 days later, and the number of CFUs was determined on a per organ basis.

Preparation of Lung Leukocytes

The lungs were excised, minced, and enzymatically digested for 30 minutes in 15 ml of digest solution [RPMI with 5% fetal calf serum, antibiotics, 1 mg/ml collagenase (Boehringer Mannheim Biochemical, Chicago, IL), and 30 μ g/ml DNase (Sigma Chemical Co., St. Louis, MO)]. The cell suspension and undigested fragments were further dispersed by drawing up and down 20 times through the bore of a 10-ml syringe. The total cell suspension was then pelleted, and the erythrocytes were lysed with ice-cold NH₄Cl buffer (0.83% NH₄Cl, 0.1% KHCO₃, and 0.037% Na₂EDTA, pH 7.4). Excess Hanks' balanced salt solution (Gibco, Carlsbad, CA) was added to bring the solution to isotonicity, and the cells were pelleted and resuspended in complete medium. Total lung cells were enumerated by hemocytometer counting in the presence of trypan blue. Subsets of isolated leukocytes (neutrophils, eosinophils, macrophages, and total lymphocytes)

were determined by Wright-Giemsa staining of samples cytospun onto slides.

Flow Cytometry of Lymphocyte Subsets

Lung cells (5×10^5 /sample) were incubated for 30 minutes on ice in a total volume of 120 μ l of staining buffer [fluorescent antibody buffer (Difco) with 0.1% sodium azide and 1% fetal calf serum]. Each sample was incubated with 0.12 μ g of phycoerythrin-labeled anti-CD45 (30-F11) and with 0.25 μ g of one of the following fluorescein isothiocyanate-labeled monoclonal antibodies: RM4-5 (anti-CD4), 53-6.7 (anti-CD8), RA3-6B2 (anti-B220), or isotype-matched rat IgG. The samples were washed in staining buffer and fixed with 1% paraformaldehyde (Sigma) in buffered saline. Stained samples were stored in the dark at 4°C until analyzed on a flow cytometer (Coulter Elite ESP, Palo Alto, CA). All monoclonal antibody reagents were purchased from BD PharMingen (San Diego, CA). Samples were gated for CD45-positive cells and then analyzed for staining by the specific fluorescein isothiocyanate-labeled antilymphocyte markers. The absolute number of each lymphocyte subset in the sample was obtained by the percentage of that type of lymphocyte subset multiplied by the total number of leukocytes.

Histology

Lungs were fixed by inflation with 10% neutral buffered formalin. After paraffin embedding, 5- μ m sections were cut and stained with hematoxylin and eosin for routine histology. In addition, periodic acid-Schiff staining (PAS) was performed to identify mucus-secreting cells (goblet cells) and examined by light microscopy.

Lung Leukocyte Culture and Cytokine Production

Single-cell suspensions of lung leukocytes were cultured at 5×10^6 cells/ml in six-well plates with 3 ml of complete medium without additional stimulation at 37°C and 5% CO₂. Culture supernatants were harvested at 24 hours and assayed for cytokines IL-4, IL-5, and IFN- γ (OptEIA kit; PharMingen) and chemokines monocyte chemoattractant protein 1 (MCP-1/CCL2), thymus and activation-regulated chemokine (TARC/CCL17), and monocyte-derived chemokine (MDC) by sandwich enzyme-linked immunosorbent assay (ELISA) following the manufacturer's protocols.

Bronchoalveolar Lavage and Alveolar Macrophage Isolation

Alveolar macrophages (AMs) were obtained from the lungs of GM^{+/+} and GM^{-/-} mice by whole-lung lavage with a total of 8 ml of phosphate-buffered saline in 0.8-ml aliquots. The lavage fluids from each group (five mice in each group per experiment) were pooled, and the cell

pellet was collected by centrifugation and placed in culture in 48-well plates (3×10^5 cells/well). After AMs had adhered for 30 minutes, the plates were washed, and heat-killed *C. neoformans* (6×10^5 /well) and/or GM-CSF (20 ng/ml) were added to the wells. Culture supernatants were harvested after 48 hours, and the levels of chemokines were determined using ELISA.

Total Serum IgE

Blood was obtained from the tail vein of the mice, and the blood was spun to obtain the serum. Serum samples were then assayed using an IgE-specific sandwich ELISA (BD PharMingen).

Statistical Analysis

Means, SEM, and unpaired Student's *t*-test results were used to analyze the data. In comparing groups, when *P* values of less than 0.05 were obtained, the groups were considered statistically different.

Results

GM-CSF Deficiency Results in Increased Pulmonary Cryptococcal Burden

Our first objective was to determine whether GM-CSF deficiency affected the host defense against this opportunistic pathogen. GM^{+/+} and GM^{-/-} were inoculated intratracheally with *C. neoformans*. The burden of *C. neoformans* organisms in the lungs was determined by CFU assay as described. GM^{-/-} mice had more CFUs at week 2 but were significantly more heavily infected than control mice at both 3 and 5 weeks after infection (Figure 1). Thus, GM-CSF deficiency results in a continuously increasing cryptococcal burden in the lungs of GM^{-/-} mice throughout the course of infection.

Pulmonary Inflammatory Response to *C. neoformans* Infection Is Delayed in the Absence of GM-CSF

We next investigated whether pulmonary leukocyte recruitment was affected in the absence of GM-CSF, thereby contributing to the increased burden of *C. neoformans*. GM^{+/+} and GM^{-/-} mice were inoculated intratracheally with *C. neoformans* (10^4 CFU), and the total number of lung leukocytes (CD45⁺) present in whole-lung digests was determined at weeks 0, 1, 2, 3, and 5 after inoculation (Figure 2). By week 1 after infection, a significant influx of leukocytes was observed in GM^{+/+} mice but was absent in GM^{-/-} mice. There was a dramatic increase in lung leukocytes in both GM^{+/+} and GM^{-/-} mice at week 2. By week 3, total lung leukocyte numbers had declined in GM^{+/+} mice, whereas lung leukocyte numbers continued to increase in GM^{-/-} mice.

Figure 1. Effect of GM-CSF deficiency on pulmonary growth of *C. neoformans* in infected GM^{+/+} and GM^{-/-} mice. Mice were inoculated intratracheally with 10⁴ CFU *C. neoformans* 52D and analyzed at weeks 1, 2, 3, and 5 after inoculation. Aliquots from lung digests were plated on Sabouraud dextrose agar in 10-fold serial dilutions, and total CFU per organ was determined. CFU at week 0 is actual CFU delivered intratracheally. **P* < 0.01, compared with GM^{+/+} mice at the same time point; *n* = 12 for each group, pooled from four separate experiments; values are means ± SEM.

Thus, the lack of GM-CSF causes a lag period before early stage recruitment of inflammatory cells but does not result in defects in lung total cell recruitment in response to pulmonary *C. neoformans* infection at late time points.

GM-CSF Is Required for Recruitment of Eosinophils

Because both GM^{+/+} and GM^{-/-} mice developed vigorous inflammatory cell recruitment in response to *C. neoformans* infection, we determined which leukocyte subsets were recruited into the lungs (Figure 3). Leukocytes were isolated from whole lungs by enzymatic

Figure 2. Total leukocyte recruitment into the lungs of *C. neoformans*-infected GM^{+/+} and GM^{-/-} mice. Total lung leukocytes were isolated from individual lungs at weeks 1, 2, 3, and 5 after infection, as described in Materials and Methods. Week 0 data are obtained from uninfected mice. **P* < 0.05, compared with GM^{+/+} mice at the same time point; *n* = 12 per data point, pooled from four separate experiments; values are means ± SEM.

Figure 3. Leukocyte subset recruitment after pulmonary *C. neoformans* infection in GM^{+/+} and GM^{-/-} mice. Total lung leukocyte suspensions were prepared as described in Materials and Methods. Samples of leukocyte suspensions from infected mice were cytospun onto slides and stained with Wright-Giemsa stain for visual quantification of neutrophils, eosinophils, macrophages, and lymphocytes. **P* < 0.05, #*P* < 0.005, compared with GM^{+/+} mice at the same time point; *n* = 12 per data point, pooled from four separate experiments; values are means ± SEM.

dispersion, and the leukocyte subsets were analyzed as described in Materials and Methods. At week 1, there were significantly fewer eosinophils, macrophages, and lymphocytes in the lungs of GM^{-/-} mice. GM^{-/-} mice had very minimal recruitment of eosinophils; there were significantly fewer eosinophils in GM^{-/-} mice than in GM^{+/+} mice throughout the entire period of infection (Figure 3). Both GM^{+/+} and GM^{-/-} mice showed an increase in pulmonary macrophages and lymphocytes from weeks 2 to 5. Macrophage numbers were significantly higher in GM^{-/-} mice at week 3. No differences were noted in lymphocyte recruitment between weeks 2 to 5 (Figure 3). Both infected GM^{+/+} and GM^{-/-} mice showed a dramatic increase in pulmonary neutrophil numbers over the first 2 weeks of infection. Neutrophil numbers markedly decreased in the lungs of GM^{+/+} mice at week 3, whereas neutrophil numbers remained elevated in GM^{-/-} mice. By week 5, neutrophil numbers had declined to similar numbers in both GM^{+/+} and GM^{-/-} mice (Figure 3). Thus, lack of GM-CSF prevents pulmonary eosinophilia and results in the delayed recruitment of macrophages and lymphocytes into the lung after *C. neoformans* infection.

Lack of GM-CSF Results in Delayed CD4⁺ and CD8⁺ T-Cell Recruitment

Recruitment of lymphocyte subsets into infected lungs was analyzed by flow cytometry. At week 1, GM^{-/-} mice had lower numbers of CD4⁺ T cells than wild-type mice. There was no difference at week 2, but by week 3, GM^{-/-} mice had twice the numbers of CD4⁺ T cells in the lung compared with control mice. Pulmonary CD8⁺ T cells were reduced in GM^{-/-} mice at weeks 1 and 2 compared with GM^{+/+} mice, and there was no

Figure 4. Lymphocyte subset recruitment after pulmonary *C. neoformans* infection in GM^{+/+} and GM^{-/-} mice. Samples of leukocyte suspensions from infected mice were stained with fluorochrome-labeled antibodies specific for CD4⁺, CD8⁺, and B220⁺ lymphocytes and analyzed by flow cytometry as described in Materials and Methods. **P* < 0.05, **P* < 0.01, compared with GM^{+/+} mice at the same time point; *n* = 12 per data point, pooled from four separate experiments; values are means ± SEM.

difference between two strains thereafter (Figure 4). Thus, the significant difference in total lymphocyte numbers in GM^{+/+} and GM^{-/-} mice at week 1 (Figure 4) could be attributed to reduced CD4⁺ and CD8⁺ T-cell recruitment exhibited by GM^{-/-} mice. There was no difference in pulmonary B220⁺ B cells throughout the course of infection. Taken together, the results demonstrate that GM-CSF is required for the early recruitment of CD4⁺ and CD8⁺ T lymphocytes into the lung in response to pulmonary *C. neoformans* infection.

GM-CSF Deficiency Alters the Inflammatory Response/Pathology Within *C. neoformans*-Infected Lungs

To determine the effects of GM-CSF on the development of lung pathology in *C. neoformans*-infected lungs, histological examination of lung sections at weeks 2, 4, and 5 was performed. Differences in histological appearance between infected lungs of GM^{+/+} and GM^{-/-} mice were apparent as early as week 2 after infection. Infected lungs in GM^{+/+} mice had inflammatory cells that formed tight inflammatory foci in both the alveolar and interstitial compartments (Figure 5A). Virtually all *C. neoformans* were contained within these tight inflammatory foci, and the infected areas of lungs were clearly separated from the uninfected lung tissue. In contrast, inflammatory foci were not well defined in the infected lungs of GM^{-/-} mice (Figure 5B). Leukocytes were present predominantly within the interstitial area surrounding airways and blood vessels. Markedly fewer leukocytes were present within the alveolar compartment, and they were loosely scattered throughout the infected areas; the infected and uninfected lung areas were not well separated from each other (Figure 5B). The differences in lung pathology between GM^{+/+} and GM^{-/-} mice were even more pronounced at week 4 after infection. In the lungs of GM^{+/+} mice, *C. neoformans* remained contained within dense inflammatory foci (Figure 5C). Leukocytes tightly infiltrated the alveolar compartment, entirely sequestering the infected areas. Consistent with the development of ABPM, the leukocyte infiltrates were composed of granulocytes with recognizable eosinophilic granules, large activated macrophages, and multinucleated giant cells. The majority of *C. neoformans* organisms in the lungs were contained within the macrophages, indicating efficient phagocytosis. In the lungs of GM^{-/-} mice, progressive growth of *C. neoformans* occurred in the inflammation-free alveolar compartments (Figure 5D). Leukocytes were absent in the infected alveoli but accumulated within the interstitium, forming tight cuffs around small airways and blood vessels. These leukocyte infiltrates were mainly composed of small mononuclear cells, suggestive of small inactive monocytes/macrophages and/or lymphocytes. Cryptococcal organisms remained extracellular within the alveoli. By week 5 after infection, lungs of GM^{+/+} demonstrated the evidence of severe ABPM pathology (Figure 5E) as described previously.^{29,30} Alveolar structures were no longer recognizable in the infected areas and had been replaced by an inflammatory infiltrate composed of macrophages/giant cells and eosinophils (Figure 5E). Macrophages contained YM1 crystals (Figure 5F), and occasional extracellular crystal deposits were present. This pathology was not present in the lungs of infected GM^{-/-} mice (Figure 5, G and H). Although almost all lung tissue appears infected, the alveolar architecture was preserved, and the hallmarks of ABPM (eosinophils, YM1 crystals) were absent (Figure 5H). In portions of GM^{-/-} lung, leukocytes (macrophages and neutrophils) were visible within the alveoli with mononuclear infiltrates in interstitial areas. Some

macrophages contained ingested cryptococci; however, large areas of lungs were filled with free-floating yeast cells (Figure 5D).

Mucus production by airway epithelium was evaluated using PAS stain. Goblet cells were absent in the bronchial epithelium of uninfected mice (not shown). PAS-positive goblet cells were abundant within the bronchial epithelial layer in GM^{+/+} mice at weeks 4 and 5 after infection (Figure 6A). Furthermore, bronchial epithelium in the infected areas of the lungs appeared cylindrical, and PAS-positive goblet cells showed PAS-positive granules separating into the lumen of the airways. These changes are consistent with epithelial cell metaplasia and increased mucus production observed in allergic airway responses. In contrast, these alterations were not observed in the airways of GM^{-/-} mice (Figure 6B). PAS-positive cells were absent from the airways in infected areas of the lung. The shape of the bronchial epithelium was cuboidal. Thus, GM-CSF is required for effective recruitment of leukocytes into the alveolar compartment and containment of fungus within inflammatory foci. GM-CSF is also required for the development of ABPM pathology in the lungs and in the airways after *C. neoformans* infection in C57BL/6 mice.

Production of Serum IgE by GM^{+/+} and GM^{-/-} Mice during ABPM

The presence of pulmonary eosinophilia and ABPM pathology in GM^{+/+} but not GM^{-/-} mice suggested that GM^{+/+} mice developed a Th2 response and GM^{-/-} mice did not. Accumulation of serum IgE is a systemic indicator of an adaptive immune response driven by Th2 cytokines. We analyzed levels of serum IgE in GM^{+/+} and GM^{-/-} mice to determine whether this element of systemic Th2 response was regulated by GM-CSF. Blood from GM^{+/+} and GM^{-/-} mice was collected, and total IgE levels in serum were assayed by ELISA. At week 3, serum IgE levels in GM^{+/+} mice were significantly higher than those in GM^{-/-} mice (Figure 7). Thus, in contrast, GM^{-/-} mice did not demonstrate systemic increase in IgE level, suggesting that the Th2 response to *C. neoformans* did not develop in the absence of GM-CSF.

GM-CSF Deficiency Results in Defective Th1 and Th2 Cytokine Production in the Lungs of GM^{-/-} Mice

The absence of Th2 hallmarks could be a result of increased Th1 cytokine production, decreased production of Th2 cytokines, or both. Production of cytokines by pulmonary leukocytes isolated from infected GM^{+/+} and GM^{-/-} mice was measured. Culture supernatants (24 hours) were tested for IL-4, IL-5, and IFN- γ production by ELISA. GM^{-/-} mice produced very limited amounts of cytokines (Figure 8). Leukocytes from GM^{-/-} mice produced significantly lower amounts of IL-4 and IL-5 at weeks 1 and 3. Leukocytes from GM^{-/-} mice also produced very little IFN- γ at weeks 1 and 2; IFN- γ was

present at week 3. GM^{+/+} mice produced significant amounts of IFN- γ over the period of infection. Taken together, these results demonstrated that the production of both Th1 and Th2 cytokines in the lungs of *C. neoformans*-infected mice was impaired in the absence of GM-CSF.

Production of MCP-1 and TARC Chemokines by Lung Leukocytes from C. neoformans-Infected GM^{+/+} and GM^{-/-} Mice

As described above, pulmonary macrophages and CD4⁺ T cells were markedly increased at week 3 in infected GM^{-/-} mice. We thought that MCP-1, a C-C chemokine for monocytes and T lymphocytes, was probably responsible for this phenomenon. To verify this, we determined the level of MCP-1 in the lung cultures from infected GM^{+/+} and GM^{-/-} mice. The lung cells from GM^{-/-} mice produced 3.5-fold greater amounts of MCP-1 than did cells from GM^{+/+} mice (1149.1 $\pm$ 120.6 versus 330 $\pm$ 132.7 pg per 5 million lung leukocytes, respectively). Thus, the production of MCP-1 correlated with the numbers of macrophages and T cells infiltrating into the lungs of GM^{-/-} mice. TARC/CCL17, a Th2 chemokine, is a product of alternatively activated macrophages³¹ implicated in the development of Th2 driven pathologies.³² We determined the level of TARC in the lung cultures and bronchial lavage fluid from infected GM^{+/+} and GM^{-/-} mice at weeks 3 and 4. Marked increase in TARC levels in both bronchial lavage fluid and pulmonary leukocyte cultures were observed in GM^{+/+} mice compared with GM^{-/-} mice at weeks 3 and 4 after infection (week 3, Figure 8A; week 4 not shown). Thus, the robust induction of TARC protein in the infected lungs of GM^{+/+} mice correlated well with the development of ABPM pathology in the *C. neoformans*-infected lungs of GM^{+/+} mice.

Delayed Inflammatory Response in GM^{-/-} Mice Is Associated with Decreased Production of TNF- α by Pulmonary Leukocytes

TNF- α is one of the proinflammatory cytokines produced by the cells of the innate immune system after pathogen recognition, and TNF- α is critical for driving the afferent phase of immune response to *C. neoformans*.³³ We evaluated TNF- α production in the absence and in the presence of GM-CSF during the early stage of infection. TNF- α levels in culture supernatants (24 hours) of pulmonary leukocytes isolated from GM^{+/+} and GM^{-/-} mice at weeks 0 and 1 after infection was measured by ELISA. Leukocytes from GM^{-/-} mice produced significantly lower amounts of TNF- α compared with those of GM^{+/+} mice at weeks 0 and 1 (Figure 9B). Thus, TNF- α deficiency in GM^{-/-} mice correlated with the delayed pulmonary inflammation during the early stage of infection.

Figure 6. Airway pathology in $GM^{+/+}$ (A) and $GM^{-/-}$ (B) mice infected with *C. neoformans* (week 5). Lungs were processed for histology and stained with PAS method with H&E counter stain. Digital images were taken at $\times 40$ objective magnification. Note the extended cylindrical shape of bronchial epithelium, the presence of PAS-positive goblet cells producing mucus (pink) in $GM^{+/+}$ mice, and absence of these alterations in the airway from the infected $GM^{-/-}$ mice.

Exogenous GM-CSF Specifically Enhances Chemokine Production by Cultured AM in Response to *C. neoformans*

We next determined whether the addition of GM-CSF to cultures of AMs from $GM^{-/-}$ mice could specifically increase the production of TARC and MDC [also a product

Figure 7. Total serum IgE levels in *C. neoformans*-infected $GM^{+/+}$ and $GM^{-/-}$ mice. At week 3 after inoculation, total serum IgE levels were determined by ELISA, as described in Materials and Methods. Values are means $\pm$ SEM. * $P < 0.01$, compared with $GM^{+/+}$ mice at the same time point. $n = 6\sim 14$ per data point, pooled from three separate experiments.

of alternatively activated macrophages (AAMs)] in these cells. We added GM-CSF to $GM^{+/+}$ and $GM^{-/-}$ AMs cultured *in vitro* and assayed chemokine levels in the culture supernatants. AMs from $GM^{+/+}$ mice produced significantly higher levels of TARC than did AM from $GM^{-/-}$ mice, in all conditions, whereas AM from $GM^{-/-}$ mice produced significant amounts of TARC only in the presence of exogenous GM-CSF (Figure 10). AM from $GM^{+/+}$ mice produced significant levels of MDC in the absence or presence of heat-killed *C. neoformans*. The addition of exogenous GM-CSF increased MDC production by AM from $GM^{-/-}$ mice reached to the level generated by AM of $GM^{+/+}$ mice. Thus, our data are consistent with *in vivo* results indicating that GM-CSF specifically enhanced TARC and MDC production by AM.

Discussion

Our study provides novel information concerning the role of endogenous GM-CSF in host response to *C. neoformans* during the development of ABPM. GM-CSF plays a complex role in the development of anticryptococcal immunity in the lungs. GM-CSF is required for early recruitment of leukocytes into the lung, movement of recruited leukocytes into the alveolar space, and the formation of inflammatory foci in the lungs. However, GM-CSF also contributed to the development of a Th2 phenotype and

Figure 5. Lung pathology in $GM^{+/+}$ and $GM^{-/-}$ mice infected with *C. neoformans*. Lungs were collected from infected mice at 2, 4, and 5 weeks after infection, fixed, processed for histology, and stained with H&E. Digital images were taken at the specified objective magnification. **A:** Lung of a *C. neoformans*-infected $GM^{+/+}$ mouse (week 2, $\times 20$). Note the tight leukocyte infiltrate forming granuloma around the yeast cells and a clear demarcation between normal and infected lung tissue. **B:** Lung of a *C. neoformans*-infected $GM^{-/-}$ mouse (week 2, $\times 20$). Note leukocyte concentration in perivascular tissue and diffuse leukocyte infiltrate with the minimal presence of leukocytes within the alveoli. **C:** Lung of a *C. neoformans*-infected $GM^{+/+}$ mouse (week 4, $\times 20$). Note the tight leukocyte infiltrate within the alveolar compartment and clear demarcation between normal and infected lung tissue. **D:** Lung of a *C. neoformans*-infected $GM^{-/-}$ mouse (week 4, $\times 20$). Note the uncontrolled *C. neoformans* growth in the absence of inflammation within the alveolar compartment and the presence of inflammatory cells in the interstitium around airways and blood vessels. **E:** Inflammatory focus in $GM^{+/+}$ (week 5, $\times 40$); note the absence of airspace/alveolar architecture, giant cells, and macrophages containing ingested yeast cells and concentrated foci of eosinophilic granulocytes. **F:** High-power examination of an inflammatory focus in $GM^{+/+}$ mouse lung (week 5, $\times 100$). Note accumulation of YM1 crystals within enlarged macrophages extracellular YM1 deposits, eosinophils. **G:** Inflammatory focus in $GM^{-/-}$ (week 5, $\times 40$); note the preserved alveolar architecture, majority of free cryptococci within the airspace, dense mononuclear infiltrate in the vessel wall, and many fewer macrophages migrating into the airspace. **H:** High-power examination of an inflammatory focus in $GM^{-/-}$ mouse lung (week 5, $\times 100$). Note the lack of YM1 crystals and very few ingested cryptococci.

subsequent development of ABPM pathology in the lungs and in the airways.

Pulmonary *C. neoformans* infection of C57BL/6 mice is an established model of an allergic bronchopulmonary mycosis, ie, a chronic pulmonary fungal infection that is accompanied by an "allergic" response (Th2) to the active infection. This model has been used to address the role of immunomodulatory agents such as OX40, *Mycobacterium* bacillus Calmette-Guérin, α -galactosylceramide (a CD1 ligand), IL-5 antagonists, and anticapsular antibodies and to address the role of antifungal drugs in modulating immunity and promoting protective host responses.^{19,20,34–39} C57BL/6 mice develop a chronic pulmonary *C. neoformans* infection that is accompanied by a pulmonary eosinophil infiltrate.^{2,4} Between weeks 5 and 7 after infection, significant amounts of the eosinophilic crystalline protein YM1 begin to accumulate in the lungs, and the macrophages harbor large numbers of cryptococci.^{4,40} C57BL/6 mice can survive >12 weeks while harboring a stable *C. neoformans* burden in the lungs of 10^6 to 10^7 CFU.^{2,4}

C. neoformans-infected C57BL/6 mice produce both Th1 and Th2 cytokines in their lungs. C57BL/6 lung leukocytes secrete significant levels of IFN- γ , IL-12, and TNF- α .³⁰ However, despite the secretion of IFN- γ , C57BL/6 mice develop Th2 responses in their lungs. Lung leukocytes from C57BL/6 mice produce high levels of IL-4 and IL-5, and high serum IgE levels are noted.³⁰ Thus, lung leukocytes from C57BL/6 mice secrete IFN- γ , IL-12, and TNF- α as well as IL-4, IL-5, and IL-13, creating a mixed Th1/Th2 environment in the lungs. The inflammatory response that develops contains a high percentage of eosinophils, similar to that observed in asthma models.⁴¹ The end result is a chronic pulmonary fungal infection with features of ABPM pathology.

Our study shows that the absence of GM-CSF results in several alterations in the pulmonary response to *C. neoformans* in this model. Severe defects exist in migration of leukocytes into infected pulmonary airspaces. The development of ABPM/Th2 lung pathology and mucus overproduction by airway epithelium are absent in GM^{-/-} mice. Unlike GM^{+/+} mice, GM^{-/-} mice do not form inflammatory foci, and *C. neoformans* continues its unopposed growth in the absence of inflammatory cells in the alveolar compartment. The absence of leukocytes in the infected alveolar space, despite their recruitment into the lung tissue, suggests a novel role for GM-CSF in alveolar host defenses against *C. neoformans*. Our studies demonstrate that GM-CSF plays a crucial role in leukocyte migration across the alveolar epithelium into sites infected with *C. neoformans*.

Despite rapidly progressing infection, Th2-dependent ABPM pathology does not develop in GM^{-/-} mice. GM^{-/-} mice do not develop the robust Th2 response observed in GM^{+/+} mice. Th2 cytokines IL-4 and IL-5 are produced to a much lesser extent and the hallmarks of a Th2 response (pulmonary eosinophilia and increased levels of serum IgE) are much less prominent. Mucus-producing goblet cells were abundantly present in the bronchial epithelium of GM^{+/+} mice but not in the airways of GM^{-/-} mice. In the mouse, IL-4, IL-9, and IL-13 are

Figure 8. Production of Th1- and Th2-type cytokines by lung leukocytes from *C. neoformans*-infected GM^{+/+} and GM^{-/-} mice. At weeks 1, 2, 3, and 5 after inoculation, total lung leukocytes were isolated and cultured at 5×10^6 cells/ml for 24 hours *in vitro* without additional stimulus. Culture supernatants were analyzed by ELISA for cytokines, as described in Materials and Methods. * $P < 0.05$, # $P < 0.01$, compared with GM^{+/+} mice at the same time point; $n = 12$ per data point, pooled from four separate experiments; values are means $\pm$ SEM.

known to induce mucin gene expression *in vivo*.^{42,43} It is not clear whether the higher level of IL-4 production in GM^{+/+} mice is directly or indirectly responsible for inducing goblet cell metaplasia. Induction of AAM markers, YM1 crystal deposition, and production of TARC/CCL17 are markedly decreased in GM^{-/-} mice compared with GM^{+/+} mice. The absence of AAM markers is consistent with the absence of a Th2 response in the lungs of GM^{-/-} mice,^{29,30} whereas the hallmarks of AAMs are clearly observed in the lungs of GM^{+/+} mice. These hallmarks include macrophages harboring numerous microbes

Figure 9. TARC/CCL17 and TNF- α production by pulmonary leukocytes of GM^{+/+} and GM^{-/-} mice. TNF- α and TARC levels were measured by ELISA. **A:** Pulmonary TARC production at week 3 after infection (levels in *C. neoformans*-pulsed 24-hour lung leukocyte culture and in bronchial lavage fluid collected from infected mice). **B:** TNF- α production by *C. neoformans*-pulsed lung leukocytes isolated from uninfected animals (week 0) and at week 1 after infection with *C. neoformans*. Values are means $\pm$ SEM. * P < 0.01, compared with GM^{+/+} mice. n = 6–10 per data point, pooled from two separate experiments.

(Figure 5E), intracellular and extracellular YM1 crystal deposits (Figure 5F), and an increased induction of TARC/CCL17 (Figure 9A). In the infected areas of GM^{+/+} mice, alveoli are consolidated, and the alveolar septa are destroyed (Figure 5, C and E), whereas the alveolar architecture remains intact in GM^{-/-} mice despite the heavy cryptococcal load (Figure 5, D and G).

The development of a Th2 response and alternative activation of macrophages is an important element of *C. neoformans* persistence in the infected lungs. AAMs harbor *C. neoformans* and support intracellular growth of *C. neoformans*.²⁹ In GM^{-/-} mice, however, growth of *C. neoformans* occurs in the absence of AAMs. In GM^{-/-} mice, the majority of, if not all, growth of *C. neoformans* occurs in the extracellular compartment. This unopposed growth of *C. neoformans* probably occurs because of severe impairment in leukocyte migration into the alveolar compartment and diminished phagocytosis as reported previously for *C. neoformans* and *Pneumocystis carinii*.^{24,44}

The stable *C. neoformans* burden in the lungs of GM^{+/+} mice contrasts with the progressive growth in GM^{-/-} mice. GM^{+/+} mice control but do not eradicate the infec-

Figure 10. GM-CSF addition specifically enhanced TARC and MDC production by AM *in vitro*. AMs were purified from both GM^{+/+} and GM^{-/-} mice and cultured at 3×10^5 cells/well in 48-well plates in the presence or absence of heat-killed *C. neoformans* supplemented with or without 20 ng/ml GM-CSF. After 48 hours of culture, supernatants were collected, and chemokine levels were analyzed by ELISA. Values are means $\pm$ SEM. * P < 0.05, * P < 0.01, compared with GM^{+/+} mice or in the absence of GM-CSF. n = 6–10 per data point, pooled from three separate experiments.

tion. GM-CSF is an important component of this protection and is associated with induction of IFN- γ , which starts at week 1 after infection. IFN- γ induction is absent (weeks 1, 2, and 5) or significantly restricted (week 3) in GM^{-/-} mice. Unlike GM^{-/-} mice infected with *Mycobacterium tuberculosis*,⁴⁵ *C. neoformans*-infected GM^{-/-} mice recruit similar or significantly greater numbers of CD4⁺ and CD8⁺ into their lung than do GM^{+/+} mice. CD4 and CD8 T cells are predominant cellular source of IFN- γ during the effector phase of immune response required for control of *C. neoformans*.^{7,46} Our previous studies suggest that a non-T-cell source of IFN- γ is responsible for the low-level protection of C57BL/6 during pulmonary cryptococcosis.²⁹ NK and NKT cells have been identified as sources of IFN- γ during *C. neoformans* infection.^{47–49} GM-CSF could directly or indirectly affect the ability of IFN- γ production by these cellular sources during infection. GM^{-/-} mice recruit large numbers of CD4 and CD8 T cells into the lung, but they do not develop a Th2 response. Interestingly, a deficiency in production of both Th1 and Th2 cytokines is observed in the absence of GM-CSF. The fact that both CD4 and CD8 T cells are recruited into the lungs in large numbers (albeit somewhat delayed) suggests that GM^{-/-} mice develop a T-cell-mediated immune response and that GM-CSF deficiency does not result in tolerance. The lack of Th1 and Th2 cytokine responses suggests that the effector function of these T cells is largely absent. The mechanism of this defect is unknown, but we believe that the defect is most likely related to the GM-CSF effects on dendritic cells. GM-CSF is crucial for proliferation, maturation, and migration of dendritic cells, and antigen presentation by dendritic cells is also required during the

effector phase to induce cytokine production by T cells.^{45,50–54}

A significant reduction of both elements of Th1 and Th2 responses in the absence of GM-CSF suggests that GM-CSF promotes early events in the development of the immune response that occur upstream of T-cell polarization. Reduced TNF- α induction, delayed recruitment of inflammatory cells into the lungs, and defect in granuloma formation are all consistent with the early defect in the development of the immune response.

TNF- α is one of the first cytokines produced by the cells of the innate immune system after pathogen recognition, and TNF- α is a critical “danger signal” driving the afferent phase of immune response to *C. neoformans*. Recognition of *C. neoformans* and subsequent signaling that leads to the early induction of TNF- α and other cytokines is thought to be mediated by TLR2 and MyD88 pathway.⁵⁵ Because GM-CSF regulates the induction of pathogen pattern recognition receptors expression, including TLR2,⁵⁶ our data corroborate these findings. Severe defects in TNF- α production in response to *C. neoformans* infection in GM-^{-/-} mice support the view that *C. neoformans* recognition is impaired in these mice. Paradoxically, in our model of ABPM, the failure to recognize pathogen results in the absence of pathological Th2 immunity responsible for the profound ABPM lung pathology. GM-CSF has been demonstrated to play a critical role in pulmonary homeostasis⁵⁷ and in stimulating AM antimicrobial activity against a variety of pathogens.^{24–26,58} In the absence of GM-CSF, mice are more susceptible to infection with Group B *Streptococcus*,²⁵ *P. carinii*,²⁶ *Plasmodium chabaudi* AS,⁵⁹ *Pseudomonas aeruginosa*,⁵⁸ and *C. neoformans* (present study), even though GM-^{-/-} mice have increased numbers of leukocytes in the lungs. The unique role of GM-CSF in the restoration of functional activity of AM from GM-^{-/-} mice through GM-CSF administration has been extensively studied.^{26,58} Our *in vitro* data demonstrate that GM-CSF specifically stimulates TARC/CCL17 production by AM, a likely cellular source of this chemokine.

In conclusion, GM-CSF plays a dual role in the immune responses to *C. neoformans*-induced model of ABPM. GM-CSF is required for the recognition of pathogen, timely development and proper compartmentalization of the immune response, and the control of pulmonary growth of *C. neoformans*. On the other hand, GM-CSF is also required for the development of ABPM-associated pathology. Our studies document a complex role for GM-CSF in a strain of mouse that develops a chronic *C. neoformans* infection. These findings suggest that the use of GM-CSF replacement therapy should be approached with caution. GM-CSF replacement therapy may lead to the development and/or enhancement of Th2 pathology in the lungs in individuals genetically predisposed to Th2 responses.

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