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Characterization of a Distinct Host Response Profile to *Pneumocystis murina* Asci during Clearance of Pneumocystis Pneumonia

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***Pneumocystis* spp. are yeast-like fungi that cause pneumocystis pneumonia (PcP) in immunocompromised individuals and exacerbate chronic lung diseases in immunocompetent individuals. The *Pneumocystis* life cycle includes trophic forms and asci (cyst forms). The cell walls of *Pneumocystis* asci contain β-1,3-D-glucan, and treatment of PcP with β-1,3-D-glucan synthase inhibitors, such as anidulafungin, results in depletion of asci, but not trophic forms. The pulmonary host response during immune reconstitution (IR)-mediated clearance of PcP in anidulafungin-treated and untreated mice was characterized to identify ascus-specific responses. During IR, similar numbers of trophic forms were present in the anidulafungin-treated and untreated mice; however, asci were only present in the untreated mice. IR resulted in a significant reduction of trophic forms from the lungs in both groups and asci in the untreated group. The presence of asci in untreated mice correlated with increased β-glucan content in the lungs. The untreated mice mounted immune responses associated with a deleterious host inflammatory response, including increased $CD8^+$ T cell influx and expression of macrophage inflammatory response markers. A more robust cellular response was also observed in the untreated mice, with increased numbers of macrophages and neutrophils that were associated with greater lung damage. Markers of a Th17 response were also elevated in the untreated mice. These results suggest that the host mounts unique responses to asci and trophic forms. That these 2 life cycle stages provoked distinct host response profiles has significant implications for clearance and interpretation of the host immune responses to PcP.**

In fungi, the cell wall is a structure external to the plasma membrane that imparts rigidity and participates in morphogenetic and differentiation processes during the fungal life cycle (1, 2). In most fungi, the glucans and chitin form the main structural components of the cell wall. *Pneumocystis* spp. are yeast-like fungi that reside extracellularly in lung alveoli and are hypothesized to replicate via a biphasic life cycle (3). The smaller vegetative trophic forms reproduce asexually through binary fission, and asci are formed by a sexual process. The ascus (cyst form) contains 8 ascospores when mature. Trophic forms have no discernible cell wall or measureable glucan. The cell walls of the asci of *Pneumocystis* have been reported to contain at least 2 types of glucan, β-1,3-D-glucan (β-glucan) and α-1,4 glucan, with little if any chitin component (4, 5).

β-1,3-D-Glucan is a high-molecular-weight polymer consisting of β-(1,3)-linked glucose residues. In *Saccharomyces cerevisiae*, the glucan synthase enzyme is encoded by 2 genes, *FKS1* and *FKS2*, which encode 2 catalytic subunits, while in *Schizosaccharomyces pombe*, these genes are $bgs1^+$ and $bgs2^+$ (2). Consistent with the reported biochemical profiles, homologs of the genes necessary for glucan synthesis, *FKS1*, *FkS2*, and *Rho1* (coding for the small GTP binding regulatory subunit), were identified in the genome of *Pneumocystis carinii* (http://pgp.cchmc.org). One of the glucan synthase subunits, called *Gsc-1* in *P. carinii*, was characterized and found to encode a 214-kDa integral membrane protein with a 12 transmembrane domain, which was preferentially expressed in asci but not in trophic forms (6). It has long been held that the periplasmic space in the ascus was the repository for glucan, as that space increases throughout maturation. However, staining with a monoclonal antibody (MAb) to β-glucan suggests it may be on the surface of the asci as well, which is in keeping with the recognition of these forms via Dectin-1 (7).

β-Glucan is one of the best-characterized host response factors of *Pneumocystis*. Numerous studies have demonstrated that purified *Pneumocystis* β-glucan stimulates expression of inflammatory cytokines and chemokines *in vitro* (8–16). In addition, β-glucan in the cell wall of *Pneumocystis* has been shown to stimulate pulmonary inflammation (17, 18). Recent studies have also demonstrated the potential clinical usefulness of β-glucan as potential marker for pneumocystis pneumonia (PcP) in serum and bronchoalveolar lavage fluid (BALF) (19–25). Inhibition of *Pneumocystis* β-glucan synthase may provide, in part, an alternative treatment option for patients with PcP (2, 7, 27–31).

We have shown that treatment of *Pneumocystis*-infected mice and rats with inhibitors of β-glucan synthesis, the echinocandins, dramatically shifted the mixed ascus and trophic form populations to ones made almost exclusively of trophic forms (7). The mortality was markedly reduced in the mice treated with echinocandins, especially anidulafungin, yet large populations of trophic forms were present in their lungs. Moreover, the ability of the echinocandin-treated mice versus untreated mice to transmit the infection via the airborne route was eliminated, providing evidence that the asci are necessary for transmission of the infection but may also be responsible for the detrimental host responses associated with the pneumonia.

Echinocandins are being broadly used in a clinical setting to treat fungal infections, including PcP (32, 33). Since the observed life cycle stage-specific sensitivity appears to be unique to *Pneu-*

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mocystis in the fungal kingdom, understanding the consequences of echinocandin treatment on the host inflammatory responses will have direct clinical implications. In the present study, the echinocandin anidulafungin was used as a molecular tool to assess host responses to each life cycle phase following immune reconstitution and clearance of infection in a manner that has not been previously available to investigators.

MATERIALS AND METHODS

Animals. Female C3H/HeN (NCI Mouse Repository, Frederick National Laboratory for Cancer Research) mice were handled in strict accordance with good animal practice, as defined by the University of Cincinnati and VAMC IACUC. The animal husbandry and experimental procedures are consistent with the recommendations in the *Guide for the Care and Use of Laboratory Animals* (34), the *Animal Welfare Act Regulations* (35), and the *Public Health Service Policy on Humane Care and Use of Laboratory Animals* (36). To safeguard against environmental exposure of *P. murina* and other microbes, mice were housed under barrier conditions with autoclaved food, acidified water, and bedding in sterilized shoebox cages equipped with sterile microfilter lids (37). Access was limited to animal care and technical personnel, who were required to wear sterile caps, gowns, masks, gloves, and booties while in the animal rooms.

Basic experimental design. The host response during clearance of pneumocystis pneumonia was compared between ascus-depleted (anidulafungin-treated) or ascus-replete (untreated) *P. murina* infections in a corticosteroid withdrawal immune reconstitution (IR) mouse model (38). C3H/HeN mice were infected with *Pneumocystis murina* by exposure to *P. murina*-infected mice on a rotating basis for 2 weeks (a process referred to as "seeding") while receiving immunosuppression with dexamethasone (4 μg/ml) delivered in their drinking water for a total of 5 weeks (39). After 5 weeks of immunosuppression and exposure to infected mice, the mice had moderate PcP (37). Mice were then divided into 2 groups: anidulafungin-treated and untreated mice. Mice in the treated group received anidulafungin (1.0 mg/kg of body weight 3 times per week intraperitoneally [i.p.]) for 3 weeks, while mice in the untreated group did not receive anidulafungin. This anidulafungin regimen was previously shown to reduce asci to below the limit of detection (7). The immunosuppressive regimen was then withdrawn for all groups after 3 weeks. However, mice in the treated group continued to receive the anidulafungin treatment, to inhibit ascus formation, as did the mice in the noninfected anidulafungin group. Mice were sacrificed at 1, 7, and 14 days postwithdrawal of the immunosuppression. *P. murina* infection levels were evaluated at days 1, 7, and 14 by microscopic enumeration as described below. The host response was monitored at days 7 and 14, as described below, to allow for washout of the corticosteroids. Eight mice were included in each untreated group, while 5 mice were placed in each anidulafungin-treated group. This was due to previous studies in which a higher survival rate was observed for anidulafungin-treated mice. A small number of deaths did occur within each group, and the exact numbers of mice used are noted in the figure legends. The number of mice per group had sufficient power to achieve statistical significance. Experiments were performed once in the interest of practicing one of the "3 Rs" of animal experimentation, "reduction," which is defined as "methods that enable researchers to obtain comparable levels of information from fewer animals or to obtain more information from the same number of animals" (40).

To determine whether the anidulafungin had a direct effect on the host responses during IR, a control group of mice was immunosuppressed, as described above, but not infected with *P. murina*. These uninfected control mice were then divided into 2 groups, anidulafungin-treated and untreated mice, as described above. The immunosuppressive regimen was then withdrawn for both groups, while mice in the treated group continued to receive the anidulafungin treatment. The mice were then processed, and the host response was monitored as described below.

Microscopic enumeration of organism burdens. *P. murina* cells were enumerated in the lungs at 1, 7, and 14 days postwithdrawal of the immunosuppression. Lungs were dissociated in 10 ml of phosphate-buffered saline (PBS) by means of a gentleMACS Dissociator instrument (Miltenyi Biotec, Auburn, CA). The lung tissue was then filtered through a 40-μm-pore mesh, and *P. murina* was recovered by centrifugation at 2,000 × *g* for 5 min and reconstituted in 1.0 ml of PBS. Slides were prepared with 3 × 10 μl of the homogenate placed onto pre-etched glass microscope slides (Fisher Scientific Co., Cincinnati, OH) and allowed to air dry, and then the slides were heat fixed and stained with either cresyl echt violet (CEV) to enumerate asci or LeukoStat (Fisher Scientific Co.), a rapid variant of the Wright-Giemsa stain, to enumerate trophic forms. *P. murina* asci and trophic forms were enumerated by counting the numbers of each form in 30 microscopic fields under oil immersion (1,250× power). The limit of *P. murina* detection by microscopic analysis is approximately 1.75×10^4 cysts or trophic forms per lung ($\log_{10}$ 4.2). The data were expressed as $\log_{10}$ asci or trophic forms per lung.

β-1,3-D-Glucan assay. Glucatell assay kits (Associates of Cape Cod, East Falmouth, MA) were used to measure the content of β-1,3-D-glucan in the lung tissue of treated and untreated mice, as previously described (41). Equal weights of tissue were used for these comparative assays.

Flow cytometry. Lungs were dissociated in 10 ml of PBS by means of a gentleMACS Dissociator instrument (Miltenyi Biotec). The homogenate was passed through an 18-gauge needle and filtered through 100-μm-pore nylon mesh. Cells were recovered by centrifugation, and red blood cells were lysed by incubation with lysis buffer on ice for 10 min. Cells were washed two times and reconstituted in PBS with 1% bovine serum albumin (BSA) and 0.1% sodium azide (HNA). Cells were enumerated on a Z2 Coulter Counter (Beckman Coulter, Hialeah, FL).

Approximately 10^7 cells were used per sample, and labeling was performed at 4°C. The samples were incubated with mouse BD Fc block to block nonspecific Fc-mediated binding of antibodies to the cells for 5 to 10 min. Samples were labeled with the following monoclonal antibodies (MAbs) per the manufacturer's instructions: CD3 (145-2C11), CD4 (L3T4), and CD8 (Ly-2). After incubation and washing, cells were reconstituted in 2% paraformaldehyde and analyzed with a FACSCalibur flow cytometry system.

BALF collection. Bronchoalveolar lavage fluid (BALF) was collected at the time of sacrifice. Mice were anesthetized with Telazol (Fort Dodge Animal Health, Fort Dodge, IA), and the mice were bled out by severing the vena cava. The chest was opened to expose the lungs and trachea, and a cannula was placed in the trachea. The lungs were lavaged three times with 1.0 ml of warm PBS. Pulmonary immune cells were collected by centrifugation at 1,000 × *g* for 10 min, and the supernatants were used to analyze soluble pulmonary host response biomarkers (e.g., cytokines, chemokines, β-glucan, and albumin). Lungs were then processed as required for the various analyses described below.

Differential counting of immune cells. Cells in the BALF were enumerated on a Z2 Coulter Counter (Beckman Coulter, Hialeah, FL). Slides for differential counting of the leukocyte subsets were prepared from BALF using a Cytospin II (Thermo Shandon, Inc., Pittsburgh, PA) to concentrate a standard volume, as previously described (42). Cells were stained with modified Wright-Giemsa stain, and the percentages of macrophages, lymphocytes, eosinophils, and neutrophils were determined by counting a total of 200 cells on each slide. The numbers of cells per ml of each type were determined from the cell numbers enumerated on the Coulter Counter.

Luminex multiplex assay. Multianalyte profiling of cytokine and chemokine levels in the BALF was performed with the Luminex-100 system and the XY Platform (Luminex Corporation). Acquired fluorescence data were analyzed by the MasterPlex QT software (version 1.2; MiraiBio, Inc.). A Milliplex MAP mouse cytokine/chemokine panel (Millipore Corporation) was used to measure gamma interferon (IFN-γ), interleukin-6 (IL-6), IL-10, IL-12p40, tumor necrosis factor alpha (TNF-α), IL-17a, IL-1β, IL-4, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, IL-5, IL-12p70, IL-13, and macrophage inflammatory protein 2 (MIP-2) concentrations

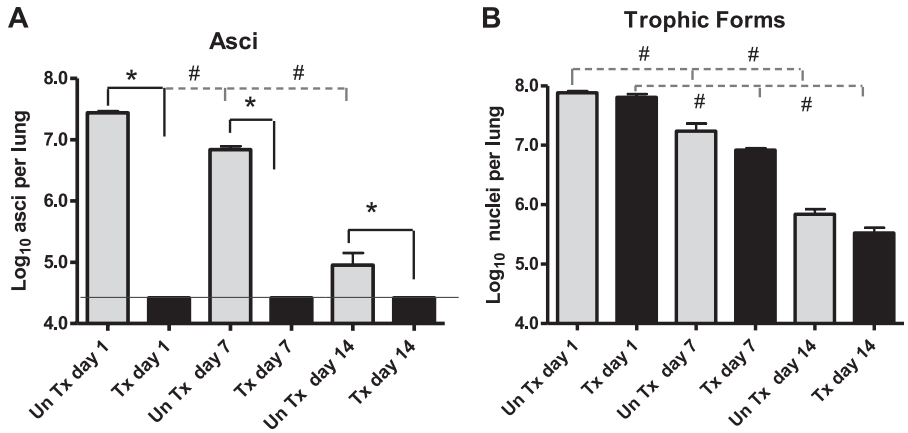

FIG 1 Ascus and trophic burdens in untreated (Un Tx) and anidulafungin-treated (Tx) mice during immune reconstitution. At 1, 7, and 14 days postcessation of dexamethasone treatment, average ascus and trophic form burdens were determined by microscopic enumeration of histologically stained mouse lung homogenates using cresyl echt violet (A) or a rapid Wright-Giemsa stain (B). Gray bars indicate the organism burden in untreated (UnTx) mice that did not receive anidulafungin (ascus replete). Black bars indicate the organism burden in mice treated (Tx) with anidulafungin throughout the experiment (to suppress ascus formation). Black brackets (*) indicate statistically significant differences between the 2 untreated and treated groups at a given time point (1, 7, or 14 days of immune reconstitution [IR]), as determined by the unpaired two-tailed *t* test ($P < 0.05$). Gray dashed brackets (#) indicate significant differences among the 3 time points within the untreated (Un Tx) or treated (Tx) groups, as determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test ($P < 0.05$). Error bars represent the standard errors of the mean within each group. Data are expressed as the $\log_{10}$ asci or trophic forms per lung. There were 8 mice in the untreated groups at days 1 and 7 of IR, 7 mice at day 14, and 5, 5, and 4 mice in the anidulafungin-treated groups at the same time points.

in BALF. The assay was performed at the Research Flow Cytometry Core at Cincinnati Children's Medical Center (Cincinnati, OH).

Albumin quantitation. The mouse albumin enzyme-linked immunosorbent assay (ELISA) quantitation set (Bethyl Laboratories, Inc.) was used to measure albumin concentrations in the BALF of treated and untreated mice. Vendor instructions for reagent, sample preparation, plate washing, coating and blocking, reaction, and detection were followed using 100 μl of lung lavage fluid of a 1:400 dilution of primary BALF (2 ml) per mouse lung in duplicate. Absorbance was measured at 450 nm, and the concentration of mouse albumin was based on a standard curve of known albumin concentrations.

Measurement of MIP-2 expression by alveolar macrophages. Alveolar macrophages were recovered from BALF from nonimmunosuppressed, nontreated C3H/HeN mice by centrifugation. The pelleted cells were reconstituted in RPMI 1640 and plated at a concentration of 2×10^5 cells per well in a 96-well plate. The macrophages were allowed to adhere for 2 h at 37°C, and then the plate was washed to remove nonadherent cells. To determine the effects of blocking the Dectin-1 β-glucan receptor on *P. murina*-mediated activation of alveolar macrophages, cells were preincubated for 30 min at 37°C with 2.0 μg/ml of the monoclonal antibody 2A11, a rat anti-mouse Dectin-1 IgG2b antibody (R & D Systems, Minneapolis, MN) (43). *P. murina* was then added to the wells in the presence or absence of the antibody. The *P. murina* cells used in this experiment were isolated and purified from the lungs of either anidulafungin-treated or untreated mice according to previously published protocols that employed a series of filtrations, an aqueous cell lytic step, and centrifugations to reduce host cell contamination to undetectable levels by microscopic analyses (44). The purified *P. murina* cells were incubated overnight with the cells at 37°C in 5% CO_2 at a concentration of 2×10^5 trophic forms per well. *P. murina* isolated from the untreated mice also contained 1.2×10^5 asci per well. No asci were detected in the *P. murina* isolated from the treated mice. After the overnight incubation, the culture supernatants were harvested and centrifuged to pellet the *P. murina* cells. MIP-2 was measured in the supernatants using the Quantikine mouse CXCL2/MIP-2 immunoassay (R&D Systems, Inc., Minneapolis, MN) per the manufacturer's directions.

Statistical analyses. When 3 time points were compared within either the treated or untreated groups, a one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison test was used to assess significance, set at $P < 0.05$. When considering the untreated versus treated groups at a single time point of 7 or 14 days post-immune reconstitution, the unpaired *t* test (two tailed) was used ($P < 0.05$). All calculations were done with INSTAT (Graph Pad Software for Science, San Diego, CA).

RESULTS

Kinetics of *P. murina* clearance during immune reconstitution in untreated and anidulafungin-treated mice. As expected, at day1 we did not detect any asci in the lungs of mice treated with anidulafungin, while in the untreated mice large numbers of asci were present (Fig. 1A). Importantly, there was no difference in the number of trophic forms between the groups at any time point (Fig. 1B).

Treated mice cleared the *P. murina* trophic forms, while the untreated mice eliminated both the trophic forms and asci from the lungs during immune reconstitution (IR). At day 1, the untreated mice had a mean log ascus per lung burden of 7.4, after which the burden decreased significantly at days 7 and 14. No asci were detected at any time points in the treated mice. (The horizontal line in Fig. 1A represents the level of microscopic detection.) There were no significant differences between the mean log trophic forms per lung in the untreated and treated mice at any of the time points (Fig. 1B). The numbers of trophic forms decreased significantly at days 7 and 14 in the untreated mice and in the treated mice. These decreases were significantly different at each time point within the untreated and treated groups, indicated by the gray dashed brackets.

Since there was no difference in the numbers of trophic forms between the treated and untreated mice at any of the time points and asci were only present in the untreated mice, we were able to identify specific cellular responses to asci and biomarkers of the ascus-specific host response, as described in the following sections.

The presence of *P. murina* asci in the lungs of the untreated mice is associated with increased levels of β-glucan in the lungs. Previous studies demonstrated that the ascus cell wall contains

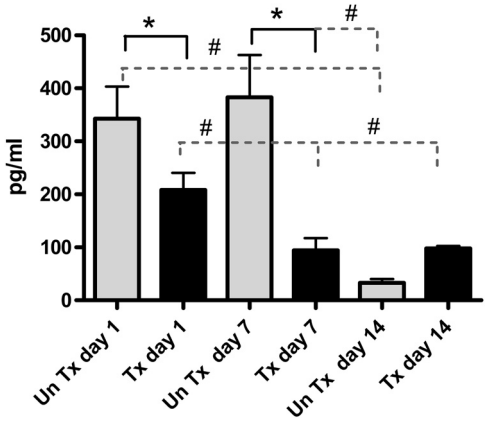

FIG 2 β-1,3-D-Glucan levels in the lungs of untreated (Un Tx) and anidulafungin-treated (Tx) mice during immune reconstitution. Glucatell assay kits (Associates of Cape Cod, East Falmouth, MA) were used to measure the content of β-1,3-D-glucan in the lung tissue 1, 7, and 14 days postcessation of dexamethasone treatment. Gray bars indicate β-1,3-glucan levels in untreated (UnTx) mice that did not receive anidulafungin (ascus replete). Black bars indicate β-1,3-glucan levels in mice treated (Tx) with anidulafungin throughout the experiment (to suppress ascus formation. Black lines (*) indicate statistically significant differences between the 2 untreated and treated groups at a given time point (1, 7, or 14 days of immune reconstitution [IR]), as determined by the unpaired two-tailed *t* test ($P < 0.05$). Gray dashed brackets (#) indicate significant differences among the 3 time points within the untreated (Un Tx) or treated (Tx) groups, as determined by one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison test ($P < 0.05$). Error bars represent the standard errors of the mean within each group. Data are expressed as pg/ml of β-1,3-D-glucan per lung. There were 8 mice in the untreated groups at days 1 and 7 of IR, 7 at day 14, and 4, 5, and 3 mice in the anidulafungin-treated groups at the same time points.

large amounts of β-glucan and that anidulafungin treatment reduces β-glucan levels in the lungs of *P. murina*-infected mice (7). In this experiment, we measured the amount of β-glucan in the lungs during IR in untreated and treated mice to determine whether the presence of asci in the untreated mice was associated with increased levels of β-glucan (Fig. 2). At days 1 and 7, β-glucan was detected in the untreated mice at significantly higher levels than in the treated mice. At day 14, the amount of β-glucan had decreased significantly in the untreated mice to a level comparable to that in the treated mice.

Asci promote an enhanced pulmonary T cell response during IR. Evaluation of T cell populations in the lungs has long been used to study the host response to *Pneumocystis*. In this experiment, the pulmonary T cell response to *P. murina* asci was characterized by quantifying the percentage and number of $CD4^+$ and $CD8^+$ T cells in the lungs during IR in untreated mice (which contained asci) and anidulafungin-treated mice by flow cytometry.

At days 7 and 14, there were more $CD4^+$ T cells in the lungs of untreated mice than in the treated mice still receiving the anidulafungin to prevent ascus formation, although the difference in levels was only significant at day 14 (Fig. 3A). The number of $CD4^+$ T cells decreased significantly in both groups of mice at day 14 (indicated by the gray dashed brackets); however, there were approximately 5 times more cells present in the untreated mice at this time point. There were no differences in the percentages of $CD4^+$ T cells between the 2 groups after 7 or 14 days (Fig. 3C).

More dramatic differences were detected in the numbers of $CD8^+$ T cells between the untreated and treated mice. At both days 7 and 14, the total numbers and percentages of $CD8^+$ T cells (Fig. 3B and D) were significantly higher in the lungs of the untreated mice. In fact, only very low numbers of $CD8^+$ T cells were even detected in the lungs of anidulafungin-treated mice at either time point. During the 2-week period of IR, the total numbers of $CD8^+$ T cells significantly decreased within the untreated and treated mouse groups (indicated by the gray dashed brackets). The finding of significantly lower percentages of $CD3^+$ $CD8^+$ T cells in the treated mice with similar percentages of $CD3^+$ $CD4^+$ T cells in the treated and untreated mice revealed the presence of a large population of $CD3^+$ $CD4^-/CD8^-$ T cells in the treated mice. As this was an unexpected finding, we were not able to further immunophenotype these cells during these studies, but they will be a focus of future investigations.

Interestingly, the untreated and treated mice displayed dramatic differences in the CD4/CD8 ratios present in the lung during IR due to the significantly lower numbers of $CD8^+$ T cells detected in the treated mice. At day 7, the untreated mice had similar numbers of $CD4^+$ and $CD8^+$ cells present in the lungs, with a CD4/CD8 ratio of approximately 1.0. However, significantly fewer $CD8^+$ cells than $CD4^+$ cells were detected at day 7 in the treated mice, with a CD4/CD8 ratio of approximately 9.0. This trend was also observed at day 14, where the CD4/CD8 ratios were 2.0 and 7.8 in the untreated and treated mice, respectively. These findings suggest that *P. murina* asci promote the potentially deleterious $CD8^+$ T cell response associated with the clearance of PcP.

Anidulafungin treatment in the absence of *P. murina* infection had no direct effect on the pulmonary T cell response during IR (Table 1). There was no difference in the total numbers of $CD4^+$ and $CD8^+$ T cells between the anidulafungin-treated and untreated uninfected mice during IR. These results demonstrate the differences in the pulmonary T cell response between the infected treated and untreated mice were related to the presence of asci in the untreated mice and not the effects of anidulafungin. Results from uninfected mice also demonstrate that responses seen during IR were related to *P. murina* and not merely to withdrawal of the corticosteroids. Significantly fewer $CD4^+$ T cells were present in the uninfected control mice compared to the infected mice at days 7 and 14. Fewer $CD8^+$ T cells were also detected in untreated uninfected mice compared to untreated infected mice at both time points; however, similar numbers of $CD8^+$ T cells were present in the uninfected and infected treated mice. This finding further indicates that asci promote an enhanced $CD8^+$ T cell response.

Asci promote cellular pulmonary inflammatory responses in the BALF during IR. Resolution of *Pneumocystis* infection is typically associated with an influx of macrophages, neutrophils, and lymphocytes into the lung. Reports implicate neutrophils as a cause of the lung damage associated with the *Pneumocystis* hyperinflammatory response in humans (45). Here, we enumerated the numbers of leukocytes present in the BALF during IR in untreated and treated mice by using a Coulter Counter and by differential counting (Fig. 4).

Significantly more total mononuclear cells were observed in the BALF from untreated mice than in treated mice at days 7 and 14 (data not shown). The total numbers of cells dropped significantly in both groups of mice at day 14 but remained significantly higher in the untreated mice. Differential counting of leukocytes in the BALF demonstrated that asci promote an enhanced and

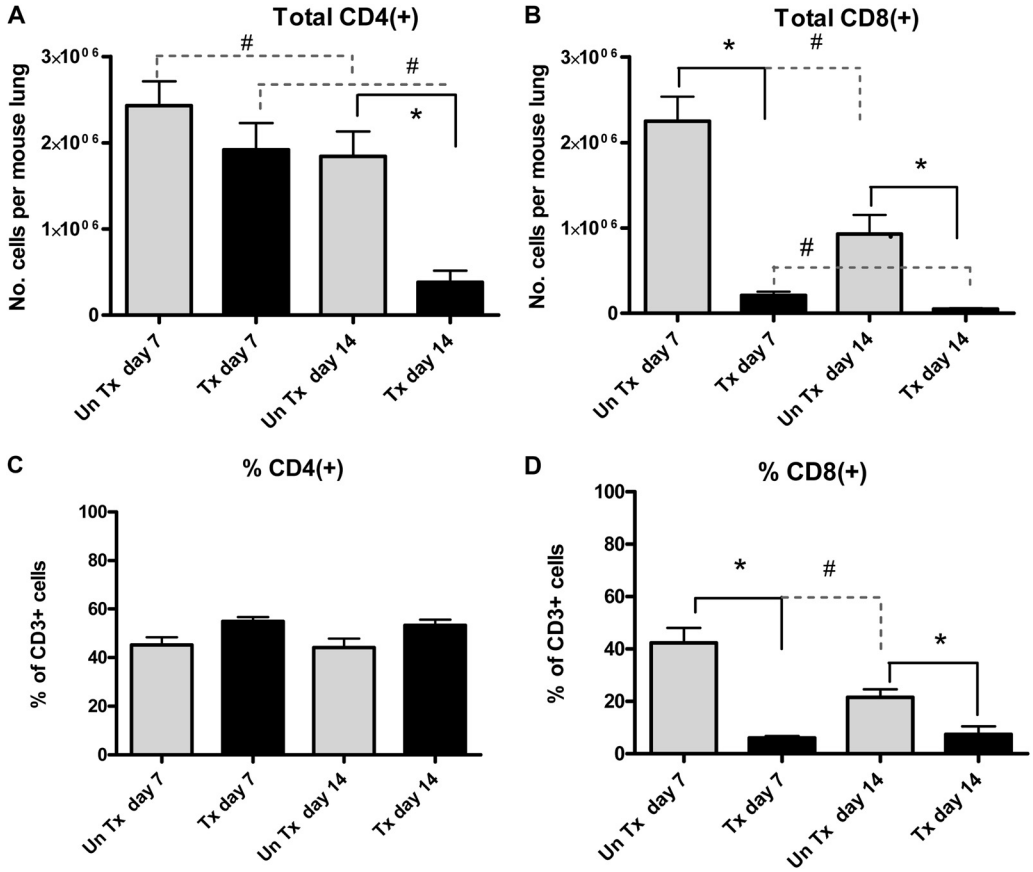

FIG 3 Pulmonary $CD4^+$ and $CD8^+$ T cell responses in untreated (Un Tx) and anidulafungin-treated (Tx) mice during immune reconstitution. Pulmonary T cell populations were characterized by flow cytometry at 7 and 14 days postcessation of dexamethasone treatment. Gray bars indicate organism burden in untreated (Un Tx) mice that did not receive anidulafungin (ascus replete). Black bars indicate organism burden in mice treated (Tx) with anidulafungin throughout the experiment (to suppress ascus formation). (A) Total number of $CD4^+$ T cells; (B) total number of $CD8^+$ T cells; (C) percentage of $CD4^+$ T cells; (D) percentage of $CD8^+$ T cells. Black brackets (*) indicate statistically significant differences between the 2 untreated and treated groups at a given time point (7 or 14 days of immune reconstitution [IR]), as determined by the unpaired two-tailed *t* test ($P < 0.05$). Gray dashed brackets (#) indicate significant differences within either the untreated or treated groups at either 7 or 14 days IR, as determined by the unpaired two-tailed *t* test ($P < 0.05$). Error bars represent the standard errors of the mean within each group. Data are expressed as the total number of $CD4^+$ or $CD8^+$ T cells and as the percentage of $CD3^+$ T cells per lung. There were 8 and 7 mice in the untreated groups at days 7 and 14, respectively, and 5 and 4 mice in the treated groups at the same time points.

prolonged cellular pulmonary inflammatory response during IR-mediated clearance of the infection.

There were significantly higher percentages of neutrophils in the untreated mice, while the percentages of macrophages were significantly higher in the treated mice at 7 and 14 days postwithdrawal of corticosteroids (Fig. 4A and B). The percentage of neutrophils decreased and the percentage of macrophages increased significantly in both groups of mice from day 7 to day 14 (gray dashed brackets).

The percentages determined by differential counting and total

TABLE 1 Pulmonary host response in immunosuppressed anidulafungin-treated and immunosuppressed untreated mice in the absence of *P. murina* infection

Time and group[a]	Mean (SD) total cells/lung					MIP-2 concn (pg/ml)
	T cells					
	$CD4^+$	$CD8^+$	Macrophages	Neutrophils	Lymphocytes	
Day 7						
Un Tx	1.1×10^5 (8.0×10^4)	8.8×10^4 (5.58×10^4)	1.0×10^6 (3.2×10^5)	2.3×10^4 (3.1×10^4)	9.0×10^3 (3.5×10^3)	105 ± 3.8
Tx	5.5×10^4 (4.3×10^4)	4.5×10^4 (3.5×10^4)	1.1×10^6 (1.5×10^5)	3.1×10^4 (2.2×10^4)	1.5×10^4 (1.3×10^4)	99 ± 7.7
Day 14						
Un Tx	4.7×10^4 (4.2×10^4)	3.9×10^4 (3.3×10^4)	1.5×10^6 (8.8×10^5)	2.3×10^4 (1.5×10^4)	8.8×10^3 (5.2×10^3)	107 ± 5.9
Tx	1.1×10^5 (8.8×10^4)	6.7×10^4 (5.5×10^4)	1.3×10^6 (2.4×10^5)	8.0×10^3 (2.5×10^3)	1.0×10^4 (7.68×10^3)	99 ± 12.4

[a] UnTx, immunosuppressed untreated mice that did not receive anidulafungin; Tx, immunosuppressed mice treated with anidulafungin.

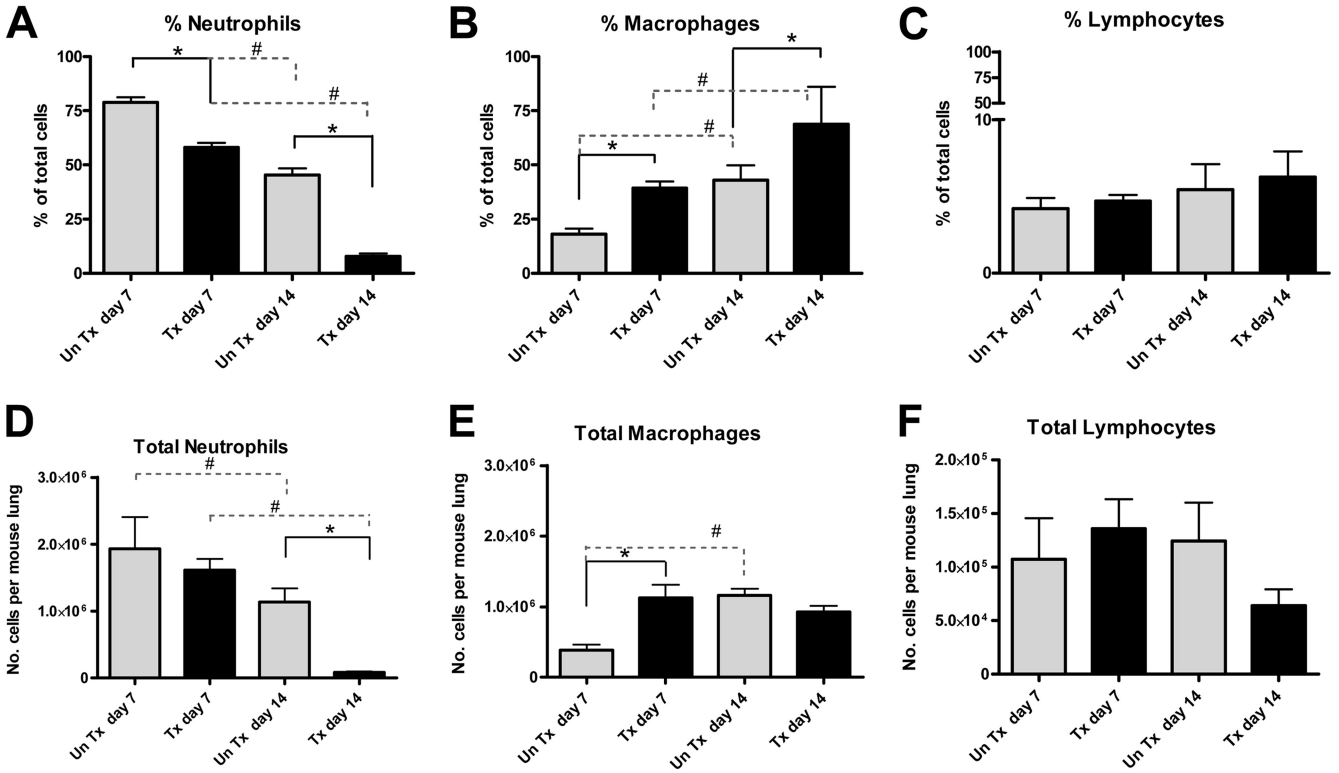

FIG 4 Cellular pulmonary inflammatory responses in untreated (Un Tx) and anidulafungin-treated (Tx) mice during immune reconstitution. The numbers of leukocytes present in the bronchoalveolar lavage fluid (BALF) were enumerated at 7 and 14 days postcessation of dexamethasone treatment by differential counting. Gray bars indicate organism burden in untreated (Un Tx) mice that did not receive anidulafungin (ascus replete). Black bars indicate organism burden in mice treated (Tx) with anidulafungin throughout the experiment (to suppress ascus formation). (A) Percentage of neutrophils; (B) percentages of macrophages; (C) percentage of lymphocytes; (D) total number of neutrophils; (E) total number of macrophages; (F) total number of lymphocytes. Black brackets (*) indicate statistically significant differences between the 2 untreated and treated groups at a given time point (7 or 14 days of immune reconstitution [IR]), as determined by the unpaired two-tailed *t* test ($P < 0.05$). Gray dashed brackets (#) indicate significant differences within either the untreated or treated groups at either 7 or 14 days IR, as determined by the unpaired two-tailed *t* test ($P < 0.05$). Error bars represent the standard errors of the mean within each group. Data are expressed as the total number of neutrophils, macrophages, and lymphocytes or as the percentage of the total number of cells per lung. There were 8 and 7 mice in the untreated groups at days 7 and 14, respectively, and 5 and 4 mice in the treated groups at the same time points.

numbers of cells enumerated with the Coulter Counter were used to determine the total number of leukocytes present in the BALF. At day 7, we detected more neutrophils in the untreated mice than in the treated mice, although the difference did not reach statistical significance (Fig. 4D). However, the day 14 results revealed a dramatic effect of the asci on PMN numbers in the lung. At day 14, the number of neutrophils in the treated mice was 8.2×10^4, compared to 1.1×10^6 in the untreated mice, demonstrating that the asci provoke continued migration of neutrophils into the alveolar space, which could lead to lung damage in the untreated mice (Fig. 4D). Not surprisingly, based on the percentage data, there were significantly more macrophages at day 7 in the treated mice; however, despite the significantly higher percentage of macrophages in the treated mice at day 14, this did not translate to higher numbers of these cells compared to the number in the untreated mice (Fig. 4E). In fact, there was a significant increase in the number of macrophages in the BALF from day 7 to day 14 in the untreated mice that was not detected in the treated mice (gray dashed brackets in Fig. 4E). These results provide more evidence that the host mounts an ascus-specific response.

There were no significant differences in the total numbers or percentages of lymphocytes between the untreated and treated groups at any of the time points (Fig. 4C and F). No eosinophils were detected in any of the samples.

Anidulafungin treatment in the absence of *P. murina* infection had no direct effect on pulmonary inflammatory responses during IR (Table 1). There was no difference in the total numbers of macrophages, neutrophils, or lymphocytes between the anidulafungin-treated and untreated uninfected mice during IR. These results confirm that the differences detected in the inflammatory response between the infected treated and untreated mice were related to the presence of asci in the untreated mice and not the effects of anidulafungin on the inflammatory response. As seen with the T cell responses described above, the results from the uninfected mice demonstrate that the pulmonary cellular response during IR is related to clearance of *P. murina*. Similar numbers of macrophages were present in uninfected and infected mice. This is related to the finding that significantly fewer lymphocytes and neutrophils were detected in the uninfected mice compared to the infected mice, since the total numbers were based on percentages of each cell type determined by differential counting.

Asci promote expression of inflammatory response markers in the lungs during IR. Chemokines and cytokines shape the host response during resolution of PcP (12, 13). When these molecules

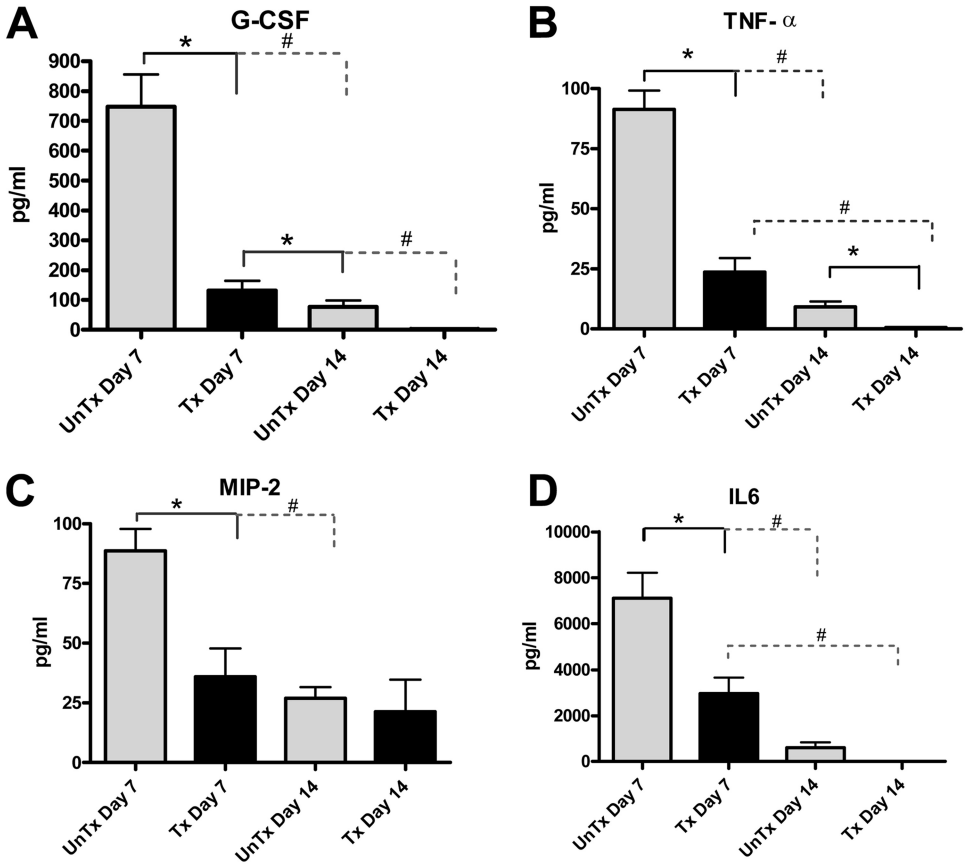

FIG 5 Expression of macrophage inflammatory response markers in the lungs of untreated (Un Tx) and anidulafungin-treated (Tx) mice during immune reconstitution. Multianalyte profiling of cytokine and chemokine levels in the bronchoalveolar lavage fluid (BALF) was performed with the Luminex-100 system. Gray bars indicate untreated (UnTx) mice that did not receive anidulafungin (ascus replete). Black bars indicate mice treated (Tx) with anidulafungin throughout the experiment (to suppress ascus formation). (A) G-CSF; (B) TNF-α; (C) MIP-2; (D) IL-2. Black brackets (*) indicate statistically significant differences between the 2 untreated and treated groups at a given time point (7 or 14 days of immune reconstitution [IR]), as determined by the unpaired two-tailed *t* test ($P < 0.05$). Gray dashed brackets (#) indicate significant differences within either the untreated or treated groups at either 7 or 14 days IR, as determined by the unpaired two-tailed *t* test ($P < 0.05$). Error bars represent the standard errors of the mean within each group. Data are expressed as pg/ml of BALF. There were 8 and 7 mice in the untreated groups at days 7 and 14, respectively, and 4 mice in the treated groups at the same time points.

are produced by cells of the innate immune response, they orchestrate the primary nonspecific host response to PcP and initiate development of the adaptive immune response that leads to protection of immunocompetent hosts from subsequent infection.

We evaluated the cytokine/chemokine expression profile in the BALF during IR-mediated clearance of *P. murina* from the lungs of untreated and anidulafungin-treated mice to identify markers of the host response to asci (Fig. 5). Interestingly, we found that the levels of the inflammatory response markers G-CSF, TNF-α, MIP-2, and IL-6 were all significantly higher in the untreated mice at day 7 (Fig. 5A to D, respectively). These results suggest that *P. murina* asci trigger expression of these markers and initiate the enhanced and prolonged inflammatory cellular response in the untreated mice. MIP-2 levels were measured in the BALF by ELISA from untreated and treated uninfected mice to determine if anidulafungin treatment in the absence of *P. murina* infection affected expression of inflammatory response markers in the lungs. No differences in MIP-2 levels were detected between the untreated and treated noninfected mice (Table 1). The MIP-2 results along with the cellular results described above indicate that the differences seen in the inflammatory response markers between the untreated and treated infected mice were not due to anidulafungin.

IL-17 levels were also higher in the untreated mice at day 7 (Fig. 6). This finding may be related to the host response to the β-glucan associated with the asci as a recent report indicates that *Pneumocystis* β-glucan may drive the activation of the IL-23/IL-17 axis (14). Levels of these cytokines mostly declined within each of the treated or untreated mouse groups over time (significance indicated by gray dashed brackets).

No differences in most markers of Th1 or Th2 responses were detected between the treated and untreated mice, including IFN-γ and IL-4 (Table 2). We detected increased IFN-γ levels at day 7 in both treated and untreated mice, which dropped significantly by day 14. Low IL-2 levels were evident in both groups, and there was no significant change over time. IL-4 and IL-10 levels were increased in both groups of mice at day 7 but decreased by day 14. Levels of IL-5 were significantly higher in the treated mice at day 7.

Albumin concentrations are higher in untreated mouse lungs. To determine whether the host response to asci was linked to lung damage, albumin concentrations within the BALF were measured as a general determinant of capillary leakage related to

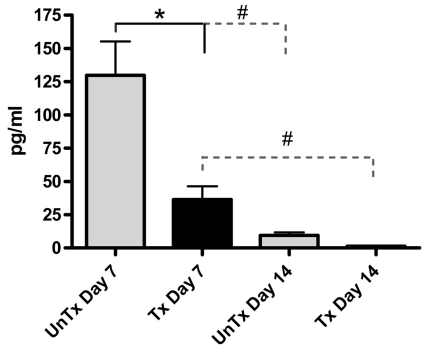

FIG 6 Expression of IL-17 in the lungs of untreated (Un Tx) and anidulafungin-treated (Tx) mice during immune reconstitution. Multianalyte profiling of IL-17 levels in the bronchoalveolar lavage fluid (BALF) was performed with the Luminex-100 system. Gray bars indicate untreated (Un Tx) mice that did not receive anidulafungin (ascus replete). Black bars indicate mice treated (Tx) with anidulafungin throughout the experiment (to suppress ascus formation). Black brackets (*) indicate statistically significant differences between the 2 untreated and treated groups at a given time point (7 or 14 days of immune reconstitution [IR]), as determined by the unpaired two-tailed *t* test ($P < 0.05$). Gray dashed brackets (#) indicate significant differences within either the untreated or treated groups at either 7 or 14 days IR, as determined by the unpaired two-tailed *t* test ($P < 0.05$). Error bars represent the standard errors of the mean within each group. Data are expressed as pg/ml of BALF. There were 8 and 7 mice in the untreated groups at days 7 and 14, respectively, and 4 mice in the treated groups at the same time points.

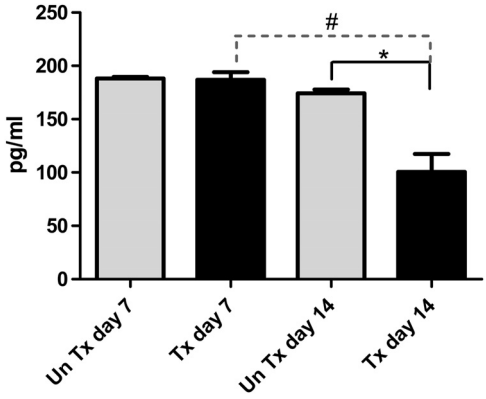

FIG 7 Albumin concentrations in the bronchoalveolar lavage fluid (BALF) of untreated (Un Tx) and anidulafungin-treated (Tx) mice during immune reconstitution. Albumin concentrations were measured in BALF by ELISA. Gray bars, untreated (UnTx) mice that did not receive anidulafungin (ascus replete); black bars, mice treated (Tx) with anidulafungin throughout the experiment (to suppress ascus formation). Black brackets (*) indicate statistically significant differences between the 2 untreated and treated groups at a given time point (7 or 14 days of immune reconstitution [IR]), as determined by the unpaired two-tailed *t* test ($P < 0.05$). Gray dashed brackets (#) indicate significant differences within either the untreated or treated groups at either 7 or 14 days IR, as determined by the unpaired two-tailed *t* test ($P < 0.05$). Error bars represent the standard errors of the mean within each group. Data are expressed as pg/ml of BALF. There were 5 and 4 mice in the untreated groups at days 7 and 14, respectively, and 4 mice in the treated groups at the same time points.

lung damage. Albumin levels were the same in the BALF from mice in both the untreated and treated groups at day 7 of IR (Fig. 7). However, by day 14 of IR, the amount of albumin was significantly higher in the untreated mice than that in the treated mice, suggesting that the host response to asci was associated with lung damage. While albumin levels were the same at days 7 and 14 in the untreated mice, there was a significant difference in mice treated with anidulafungin.

Asci stimulate expression of MIP-2 by alveolar macrophages through Dectin-1. To investigate macrophage activation by asci, alveolar macrophages isolated from BALF were stimulated overnight with *P. murina* isolated from anidulalfungin-treated or untreated mice in the presence or absence of monoclonal antibody to Dectin-1. Following the overnight incubation, MIP-2 was measured in the culture supernatants by ELISA (Fig. 8). Significantly greater levels of MIP-2 expression were detected following stimulation with untreated than with treated *P. murina*. *P. murina* cells isolated from the untreated mice contained 1.2×10^5 asci per well. No asci were detected in the *P. murina* cells isolated from the treated mice. Addition of the anti-Dectin-1 antibody significantly reduced expression of MIP-2 in macrophages stimulated with untreated *P. murina*, but not in macrophages incubated with treated organisms. These results suggest that β-glucan in ascus cell wall is being recognized by Dectin-1 and initiating an inflammatory response by the alveolar macrophages.

DISCUSSION

Our previous studies showed that treatment of *Pneumocystis*-infected corticosteroid-immunosuppressed mice or rats with anidulafungin or caspofungin resulted in a lack of microscopic detection of asci, with little effect on the numbers of trophic forms (7). Anidulafungin treatment does not alter the exposure of β-glucan on the surface of *P. murina* asci, in contrast to the effects of echinocandins on *Aspergillus fumigatus* (46). Caspofungin treatment

TABLE 2 Pulmonary cytokine response in BALF

Time and group[a]	Mean ±SE cytokine level (pg/ml) in BALF				
	IFN-γ	IL-4	IL-5	IL-10	IL-2
Day 7					
Un Tx	623.4 ± 208.5	20.19 ± 4.4	58 ± 10.9[b]	4.873 ± 0.9	6.136 ± 0.3
Tx	714.3 ± 111.5	20.31 ± 8.2	122.9 ± 39.9	5.969 ± 1.2	4.549 ± 0.4
Day 14					
Un Tx	55.9 ± 12.04	9.013 ± 3.5	14.75 ± 4.7	ND[c]	5.065 ± 0.8
Tx	ND	ND	ND	ND	4.409 ± 0.4

[a] Un Tx, untreated mice that did not receive anidulafungin (ascus replete); Tx, mice treated with anidulafungin to suppress ascus formation.
[b] $P < 0.05$ versus Tx on day 7.
[c] ND, not detected.

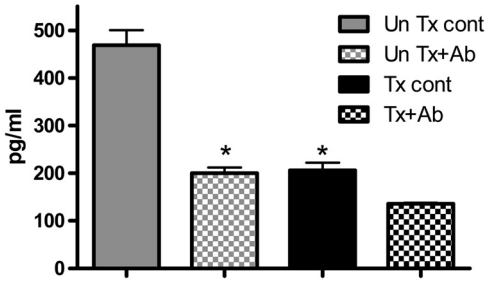

FIG 8 *P. murina* isolated from untreated mice stimulates expression of MIP-2 by alveolar macrophages through Dectin-1. Alveolar macrophages isolated from BALF were stimulated overnight with 2×10^5 trophic forms of *P. murina* isolated from untreated (Un Tx) or anidulafungin-treated (Tx) mice in the absence (cont) or presence (+Ab) of 3 μg per ml of a rat anti-mouse Dectin-1 antibody. *P. murina* isolated from the untreated mice also contained 1.2×10^5. No asci were detected in the *P. murina* isolated from the treated mice. MIP-2 was measured in the culture supernatants by ELISA. Asterisks indicate statistically significant differences between the untreated control group, as determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test ($P < 0.05$). Error bars represent the standard errors of the mean within each group of triplicate wells. Data are expressed as pg/ml of culture supernatant. Results are representative of two separate experiments performed in triplicate.

of *A. fumigatus* alters surface β-glucan exposure in a stage-specific manner and does not completely inhibit growth. Treatment increases β-glucan exposure on the surface of hyphae, enhancing recognition by macrophages through Dectin-1 and subsequent expression of TNF-α and MIP-2 (47). In contrast, echinocandin treatment reduces surface β-glucan exposure on *A. fumigatus* conidia and germlings, repressing their recognition by Dectin-1 and depressing the inflammatory response. In the case of *P. murina*, reduction in *de novo* synthesis of β-glucan by inhibition of β-1,3-glucan synthase with anidulafungin treatment appears to cause lysis of existing asci, in addition to preventing development of new asci (7). The effects of anidulafungin treatment on *P. murina* asci appear to be similar to the fungicidal effects of caspofungin treatment on *Candida albicans* that lead to killing of actively growing cells by inducing changes in the cell wall that cause osmotic instability and eventual cell lysis (48). The lack of activity of anidulafungin treatment against *P. murina* trophic forms supports previous findings that this form of the organism does not contain β-glucan.

The elimination of β-glucan-rich asci and lack of an effect on trophic forms allowed us to use anidulafungin treatment to dissect the host response to intact asci. In the present study, we exploited this effect to dissect the pulmonary host responses to asci during IR in a corticosteroid mouse model of PcP. This is a novel approach and adds to the experimental techniques for investigation of *Pneumocystis*, which are often limited due to the inability to continuously grow these fungi *ex vivo*. By using echinocandin treatment to eliminate asci, we were able to identify the specific pulmonary host responses associated with *P. murina* asci during IR facilitated by withdrawal of corticosteroids. It is important to note that responses to this fulminant, established infection upon immune reconstitution could differ from a model in which fewer organisms were present.

The untreated mice (ascus replete) and anidulafungin-treated mice (ascus depleted) had similar numbers of trophic forms in their lungs at days 1, 7, and 14 during IR, but no asci were detected in the lungs of the treated mice; therefore, any differences detected in the host response at these time points could be directly attributed to the presence or absence of the asci.

Although anidulafungin treatment clearly reduced the numbers of intact asci, the direct measurement of β-glucan in the lungs of the treated mice showed that significant amounts remained in these mice. These results indicate that intact asci are not the only source of β-glucan in the lungs and that β-glucan concentration is not solely a function of ascus burden. Since microscopic evaluation revealed no asci in the treated mice at day 1, the β-glucan that was present would not appear to be associated with asci. This β-glucan may be present in ascus cell wall fragments or soluble molecules remaining after anidulafungin-mediated degradation of the asci. Because Dectin-1 signaling is only activated by particulate forms of β-glucan that are able to cause receptor clustering, this residual β-glucan may not stimulate an inflammatory response through Dectin-1 (49). However, soluble β-glucan may augment other arms of the host immune response, such as the humoral response (50). This finding points to the possibility that soluble β-glucan liberated from ascus cell wall by anidulafungin treatment may act as an adjuvant to promote antibody-mediated clearance of asci.

Initial studies focused on identifying the T cell responses to the asci. The $CD4^+$ T cell responses have long been considered essential for the prevention of PcP and play a major role in its resolution by orchestrating the activities of adaptive immunity through interaction with other cells, production of cytokines, and enhancing antibody production (51). In the absence of $CD4^+$ T cells, $CD8^+$ T cells become the prominent immune cell type detected in the lung and are often associated with a failure to control the infection with a concomitant deleterious inflammatory response leading to lung damage, although they have also been implicated in a beneficial host response (52, 53).

The results presented here suggest that *P. murina* asci promote migration of $CD8^+$ T cells to the lung and prolong the T cell pulmonary response. At day 7 of IR, the untreated mice had significantly higher numbers of $CD8^+$ T cells than the treated mice, and at day 14, the $CD8^+$ T cell levels remained higher in the untreated mice, indicating that the asci stimulate a $CD8^+$ T cell response. Similar levels of $CD4^+$ T cells were present in the lungs of treated and untreated mice at day 7; however, at day 14 the number of $CD4^+$ T cells decreased significantly in the treated mice, but remained elevated in the untreated mice, suggesting that the asci also prolong the $CD4^+$ T cell response. $CD4^+$ and $CD8^+$ T cells trigger the adaptive immune response to *Pneumocystis* and can activate alveolar macrophages to become fully engaged in the host response. We identified an enhanced alveolar macrophage inflammatory response in untreated mice with a mixed infection containing both *P. murina* asci and trophic forms compared to treated mice without asci. The levels of the inflammatory response markers G-CSF, TNF-α, MIP-2, and IL-6 were all significantly higher in the untreated mice at day 7, as were the total numbers of macrophages within the untreated mouse lungs.

These results suggest that the *P. murina* asci induce a proinflammatory response that may be related to Dectin-1 interactions on alveolar macrophages with the β-glucan-rich asci. That the β-glucan levels were higher in the untreated mice was verified by direct measurement of β-glucan, the primary component of *Pneumocystis* ascus walls. The higher levels of β-glucan were also associated with increased lung damage detected by increased lev-

els of albumin in the alveolar environment. The results of these studies also suggest that the presence of β-glucan associated with the asci in the untreated mice also may activate the IL-23/IL-17 axis as IL-17 levels were also higher in the untreated mice at day 7, as has been recently described (14).

P. murina asci do not appear to polarize the immune response to either a Th1 or a Th2 phenotype. No differences were detected in IFN-γ or IL-4 levels between the untreated and treated mice at either time point; however, both cytokines were elevated in both groups of mice at day 7, and the levels dropped significantly at day 14, suggesting that in this model a distinct Th1 or Th2 response is not expressed. The lack of a defined Th1 or Th2 response is not surprising. Most studies indicate that there is not a clear distinction between either a Th1 or Th2 immune response to *Pneumocystis* (54, 55).

Previous studies have interrogated the immune responses to the *Pneumocystis* β-glucans *in vitro* by direct challenge of *Pneumocystis*-derived β-glucans to rat-derived alveolar epithelial cells and human-derived dendritic cells and lymphocytes (14, 56). Here, we eliminated most of the organism-associated β-glucans in an *in vivo* setting and contrasted these responses with an ascus-replete pneumonia containing high levels of β-glucans. Remarkably, these complementary approaches identified similar responses between the β-glucan-exposed *ex vivo* immune cells and the *in vivo* β-glucan-exposed lung cells. Both studies found increased levels of IL-17 and IL-6, which drive the Th17 phenotype. The level of MIP-2 was also increased in both systems in response to β-glucan, which is associated with leukocytic transmigration into lung interstitium and alveolar spaces, leading to lung injury (57). *In vitro* studies in this work showing that ascus-replete *P. murina* preparations stimulate expression of MIP-2 by isolated alveolar macrophages provide additional evidence that asci promote neutrophil accumulation in the lungs. In our model, we observed an increased neutrophil presence in the lungs containing asci, as well as increased lung damage, which is consistent with activation by this chemokine.

After completion of these studies, an intriguing report by Zhang et al. (58) was published that described the increasing levels of myeloid-derived suppressor cells in the lungs of rats and mice immunosuppressed by corticosteroids and infected with *P. carinii* and *P. murina*, respectively. These cells were strikingly similar in morphology to neutrophils but were identified by the Gr-1$^+$ CD11b$^+$ immunophenotype in mice and the CD11bc$^+$ His48$^+$ phenotype in rats. These cells were shown to suppress CD4$^+$ T cell proliferation and to cause lung damage in normal mice upon adoptive transfer. As the morphology of the myeloid-derived suppressor cells closely resembles that of polymorphonuclear leukocytes, we were unable to affirm these findings without a source of freshly derived cells. Moreover, the IR model used here may not result in the same observations as those with the progressive pneumonia model used by Zhang et al (58). In fact, we did not observe a suppression of CD4$^+$ T cells in the untreated mice during IR, which instead had increased numbers of CD4$^+$ T cells.

Our data show a differential pulmonary host response between the ascus life cycle stage and the most numerous forms of the life cycle, the trophic forms. Most previous studies have used the combined life cycle stages to evaluate the host immune responses that were likely driven by the numbers of asci in the preparation. The fact that these 2 life cycle stages are seen differently by the host has significant implications for clearance and interpretation of the host immune responses. Significantly, others have described a Dectin-1-based clearance of the asci that is supported by the presence of β-glucan in the ascus cell wall (43). The lack of β-glucan in the trophic forms implies that host recognition of these forms does not occur via Dectin-1 as for asci, but is likely by different receptors. However, the trophic forms must be cleared in some manner, since their numbers decrease after steroid withdrawal. The trophic forms contain little to no β-glucan, and we predict that the trophic forms will interact with receptors, such as the mannose receptor, on macrophages. Most PcP infections have an overabundance of trophic forms versus asci. The lack of a strong proinflammatory response to the trophic forms may provide a survival advantage to the fungi by dampening the damage to the host while enjoying a less robust immune response. Furthermore, we link the presence of *Pneumocystis* asci and β-glucan with the deleterious immune response that is associated with the pathophysiology of PcP and immune reconstitution syndrome (IRS) (59–61). The results of this study also point to the potential clinical use of echinocandin treatment to limit development of IRS following treatment of PcP in the immunocompromised host (62, 63). If the asci are the major cause of the deleterious host response, as suggested by the data presented here, echinocandin treatment of PcP in conjunction with immune reconstitution may limit development of IRS. However, the present study, as well as our previous study, indicates that echinocandin therapy alone will not sufficiently reduce the number of viable organisms to be considered useful as a monotherapy.

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