In Vivo Role of Dendritic Cells in a Murine Model of Pulmonary Cryptococcosis

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Dendritic cells (DC) have been shown to phagocytose and kill Cryptococcus neoformans in vitro and are believed to be important for inducing protective immunity against this organism. Exposure to C. neoformans occurs mainly by inhalation, and in this study we examined the in vivo interactions of C. neoformans with DC in the lung. Fluorescently labeled live C. neoformans and heat-killed C. neoformans were administered intranasally to C57BL/6 mice. At specific times postinoculation, mice were sacrificed, and lungs were removed. Single-cell suspensions of lung cells were prepared, stained, and analyzed by microscopy and flow cytometry. Within 2 h postinoculation, fluorescently labeled C. neoformans had been internalized by DC, macrophages, and neutrophils in the mouse lung. Additionally, lung DC from mice infected for 7 days showed increased expression of the maturation markers CD80, CD86, and major histocompatibility complex class II. Finally, ex vivo incubation of lung DC from infected mice with Cryptococcus-specific T cells resulted in increased interleukin-2 production compared to the production by DC from naive mice, suggesting that there was antigen-specific T-cell activation. This study demonstrated that DC in the lung are capable of phagocytosing Cryptoccus in vivo and presenting antigen to C. neoformans-specific T cells ex vivo, suggesting that these cells have roles in innate and adaptive pulmonary defenses against cryptococcosis.

Cryptococcus neoformans is an opportunistic fungal pathogen that predominantly causes disease in immunocompromised patients, including individuals with AIDS, transplant recipients, and individuals with lymphoid and hematological malignancies (43, 57, 70, 72). Initial exposure to C. neoformans normally occurs via inhalation, and it is thought that most infections are asymptomatic (1, 15, 26). This observation implies that in a normal host, a component(s) of the immune system in the lung plays a role in protection from infection. Therefore, examining interactions of C. neoformans with immune cells in the lung is essential for understanding the initial immune response to this organism.

Recent research on the host immune response to C. neoformans has focused on T-cell-mediated immunity (32, 34–36, 58, 60) and antibody-mediated immunity (68, 78, 82). Additional studies have examined cryptococcal uptake by macrophages (16, 17, 40, 46, 47) and neutrophils (14, 23, 40, 51), but little has been learned about the in vivo role of dendritic cells (DC) in the initial interactions with the organism in the lungs. DC are antigen-presenting cells (APC) that act as sentinels in the peripheral tissues, constantly sampling the antigens in their environment. They ultimately present foreign antigens to T cells in the lymphoid tissues, initiating an adaptive immune response against these antigens (3, 71, 77). An adaptive Th1-type immune response is required for protection against cryptococcal infection (32, 34–36, 58). Because DC are the most effective APC for inducing cell-mediated immune responses (18), it is important to investigate lung DC and their potential to initiate an adaptive anticryptococcal immune response. The respiratory tract, in particular, contains dense networks of DC specialized for antigen uptake (12, 33, 69).

Dendritic cells have previously been shown to phagocytose C. neoformans in vitro (38, 76) by a process which requires opsonization with either complement or antibody. Following phagocytosis, DC are capable of activity against C. neoformans (38). Additionally, studies using cryptococcal antigens have demonstrated the importance of DC for inducing an anticytoccal immune response in vivo and in vitro (4, 5, 52). However, it is not known whether DC in the lung phagocytose C. neoformans and are capable of inducing protective immunity in an in vivo infection model. For other pulmonary pathogens, including Mycobacterium tuberculosis (28, 62, 64) and Aspergillus fumigatus (11), lung DC are important for phagocytosis and for initiating an adaptive immune response.

Alveolar macrophages, another type of APC in the lung, have long been regarded as the phagocytes that initially encounter inhaled C. neoformans. In vitro studies have demonstrated that alveolar macrophages efficiently phagocytose C. neoformans and have fungicidal activity (8–10, 44). Additionally, alveolar macrophages that have internalized C. neoformans have antigen-presenting activity and produce proinflammatory cytokines (30, 48, 79). In vivo, depletion of alveolar macrophages resulted in decreased resistance to cryptococcal infection (59). However, in these studies, no distinction was made between alveolar macrophages and pulmonary DC, and it is possible that some of the activity attributed to macrophages actually was mediated by DC. Neutrophils also phagocytose and kill C. neoformans in vitro (14, 40, 51, 56) and can modulate the immune response by the production of proinflammatory cytokines (67). In animal studies, neutrophil influx...
into tissues following cryptococcal challenge is associated with rapid partial clearance of organisms (24, 63) but is not necessarily associated with protective responses against a pulmonary cryptococcal infection (37, 55).

In the present study, we tried to determine the contribution of lung DC to innate and adaptive host defenses against cryptococcosis during the initial stages of an in vivo infection. Accordingly, we examined uptake of C. neoformans by pulmonary DC in vivo using a murine model of intranasal infection.

The relative contributions of DC, alveolar macrophages, and neutrophils to C. neoformans uptake were then determined during the first 24 h following fungal challenge. Finally, we investigated the effect of in vivo exposure to C. neoformans on the expression of DC maturation markers, as well as the capacity of DC to present antigen to Cryptococcus-specific T cells following infection.

MATERIALS AND METHODS

Reagents. Unless indicated otherwise, chemical reagents that were the highest quality available were obtained from Sigma Chemical Co. (St. Louis, MO), tissue culture media were purchased from Gibco Life Technologies (Rockville, MD), and plastic ware was purchased from Fisher Scientific (Pittsburgh, PA). The medium used in bone marrow dendritic cell (BMDC) experiments was RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U penicillin/ml, 100 mg of streptomycin/ml, and 50 mM 2-mercaptoethanol (complete medium). All cell cultures were incubated at 37°C in a humidified environment supplemented with 5% CO2.

Staining of Cryptococcus. C. neoformans serotype A encapsulated strain 145 (≈ ATCC 62070; American Type Culture Collection, Manassas, VA) was cultured overnight in YPD containing 2% glucose. Yeast cells were heat killed by incubation at 56°C for 1 h. Cultures of both live and heat-killed C. neoformans were washed with sterile 0.1 M sodium bicarbonate buffer (pH 8.0) (staining buffer), and cells were counted and resuspended to obtain a concentration of 5 × 106 cells/ml. C. neoformans was incubated with 2 μg/ml Oregon green 488 (Molecular Probes, Eugene, OR) at room temperature in the dark for 1 h. The organisms were then washed three times with staining buffer, counted, and resuspended in staining buffer to obtain the concentration needed for each experiment.

Mice. C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and were housed in microisolator cages at the Boston University Medical Center Laboratory Animal Sciences Center. Mice were housed and handled according to institutionally recommended guidelines.

BMDC cultures. BMDC cultures were grown as described previously (38, 50). Briefly, bone marrow was flushed from femurs and tibias of C57BL/6 mice. Cells were collected, counted, and plated at a confluence of 2 × 105 cells/ml in complete medium supplemented with 10% filter-sterilized supernatant from the J558L cell line (which constitutively produces granulocyte-macrophage colony-stimulating factor) (65). Cells were used on day 9 or 10 following plating. This method of culture yielded an average of 77% CD11c+ cells (DC), as assessed by flow cytometry (data not shown).

BMDC phagocytosis of Cryptococcus. BMDC were harvested, counted, and incubated with Oregon green-labeled Cryptococcus. BMDC and Cryptococcus were added at a ratio of 1:1 and were incubated in the presence of 1 μg/ml of 3C2, an opsonizing antiserum monoclonal antibody (74) (a gift from Thomas Kozel, University of Nevada, Reno) for 1 h at 37°C in 1.7-ml microcentrifuge tubes (Costar, Corning, NY). Oposning antibody was used so that the BMDC could phagocytose the encapsulated C. neoformans cells (38). Following incubation, mouse cells were examined for Cryptococcus uptake by flow cytometry and fluorescent microscopy. Phagocytosis was defined as the association of fluorescent C. neoformans cells with BMDC or lung cells as determined by flow cytometry or the presence of intracellular C. neoformans by microscopy.

Mouse infection. For intranasal infection, mice were anesthetized with halothane (Samuel Perkins, Quincy, MA) and subsequently inoculated intranasally with 1 × 107 Oregon green-stained C. neoformans cells in 50 μl of sterile staining buffer. At specific times following inoculation, mice were euthanized by CO2 inhalation, and the pulmonary artery of each mouse was perfused with 1 ml heparin in phosphate-buffered saline (143.4 USP units/ml) to clear the lungs of peripheral blood. The lungs were then removed, minced, and incubated in digestion buffer consisting of 1 mg/ml collagenase type IV (Sigma, St. Louis, MO), 75 μg/ml bovine pancreatic DNase (Sigma), and 5% fetal bovine serum in RPMI 1640 (Gibco Life Technologies Rockville, MD) for 1.5 h at 37°C. Following digestion, lung tissue was passed through a sterile 70-μm filter in order to obtain single-cell suspensions, and red blood cells were lysed with red blood cell lysis buffer (eBioscience, San Diego, CA). Cells were then examined by fluorescent microscopy, confocal microscopy, or flow cytometry.

Isolation of lung DC. Lung DC were isolated from digested whole lungs by positive selection with CD11c-MACS magnetic beads (Miltenyi Biotec, Auburn, CA). Lung DC were defined by expression of intermediate levels of CD11c on the surface, as previously described (64, 75).

Cell surface marker analysis. Flow cytometry was performed with BMDC and single-cell suspensions of lung tissue. The antibodies used included CD11c (DC), F4/80 (macrophages), mouse neutrophil-specific antigen clone 7/4 (neutrophils), and CD80, CD86, and major histocompatibility complex class II (MHC-II) (DC maturation). Antibodies and isotype controls were obtained from eBioScience; the exception was the 7/4 antibody, which was obtained from Serotec, Oxford, United Kingdom. Antibodies were diluted in FACS buffer (2% fetal bovine serum in phosphate-buffered saline) to obtain the concentration recommended by the manufacturer, and 100 μl of diluted antibody was added to 1 × 106 cells and incubated for 1 h on ice, protected from light. After three washes, cells were analyzed with a flow cytometer (BD FACScan, San Jose, CA). Data were analyzed using the WIN MDI software (Joseph Trotter, The Scripps Research Institute, La Jolla, CA).

Microscopy. BMDC or lung DC were incubated with Oregon green-labeled C. neoformans as described above and then visualized with an epifluorescence microscope (Nikon Diaphot 300; Nikon Instruments, Inc.) or a confocal microscope (Leica TCS-SP8; Leica, Wetzlar, Germany). For the confocal studies, DC were stained with CD11c phycocerythrin antibody (eBioScience). The microscope used in this system was a Nikon TE2000-U inverted microscope with a Nikon 100× 1.4NA DIC lens and type A immersion oil (Nikon, Melville, NY). The camera used in these experiments was a Hamamatsu Orca ER camera (model C4742-95-12ERG). The Perkin-Elmer Ultraview software was used for acquisition. Individual images were saved as tiff files and were processed using the Perkin-Elmer Ultraview software. Optimal contrast was obtained by adjusting the gray levels for the entire image.

Cell activation. Lung DC isolated from naive or infected mice were incubated overnight with Cryptococcus-specific hybridoma P1D6 T cells (45). P1D6 cells secrete interleukin-2 (IL-2) when they are stimulated with APC presenting the C. neoformans mannoprotein MP98. Supernatants were harvested and then incubated with the IL-2-dependent cell line CTL-LL-2 (25) for 24 h. AlamarBlue (TREK Diagnostic Systems, Cleveland, OH) was added during the final 8 h. Absorbance was determined with an automated plate reader, and data were compared to an IL-2 standard curve. The results were expressed as units of IL-2/ml.

Statistical analysis. For statistical comparisons we used the Student t test. P values of <0.05 were considered significant. Data were analyzed using the GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

RESULTS

BMDC phagocytosis of fluorescently labeled Cryptococcus. In order to establish that fluorescently labeled C. neoformans could be detected by flow cytometry and fluorescent microscopy, live and heat-killed organisms were labeled with Oregon green 488 and subsequently incubated with BMDC and opsonizing antibody for 1 h to examine the initial phagocytosis. Opsonization is required for BMDC to phagocytose encapsulated C. neoformans (38). The results showed that after 1 h of incubation at 37°C, CD11c+BMDM were able to bind and/or phagocytose both live and heat-killed C. neoformans, as determined by flow cytometry (Fig. 1A) and fluorescence microscopy (Fig. 1B).

In vivo uptake of Cryptococcus by lung phagocytic cells. To determine the population of cells in the lungs that phagocytose C. neoformans in vivo, live and heat-killed organisms were fluorescently labeled with Oregon green and then used to intranasally inoculate mice. The lung DC from naive mice did not express CD8α, as previously reported (75). Additionally,
lung CD11c<sup>+</sup> cells rarely expressed the macrophage marker F4/80 or the T-cell markers CD8 and CD3 (data not shown). In order to examine the early stages of *C. neoformans* uptake, the lung cells were examined soon after inoculation. At 2, 8, and 24 h postinoculation, mice were sacrificed, lungs were removed, and single-cell suspensions of lung tissue were prepared and stained for phagocyte cell surface markers. The percentages of DC, macrophages, and neutrophils present in the lungs during the initial stages of infection are shown in Table 1. Flow cytometric analysis demonstrated that both live and heat-killed organisms were phagocytosed in vivo by pulmonary DC (Fig. 2A). In order to verify that *C. neoformans* cells were internalized by the DC, confocal microscopy was performed. Lung DC containing phagocytosed *C. neoformans* cells were observed by confocal microscopy (Fig. 2B). Analysis of Z-stack images confirmed the intracellular location of the fungus. We next examined the capacity of the two other major phagocytic cell populations in the lung, macrophages and neutrophils, to interact with *C. neoformans* (Fig. 3). Both live and

![Images and graphs](http://iai.asm.org)
heat-killed organisms were phagocytosed in vivo by macrophages and neutrophils at 2, 8, and 24 h postinoculation.

**DC expansion and expression of maturation markers during pulmonary infection.** In order to further study the role of lung DC with *C. neoformans*, we examined lung DC at later times postinoculation. First, we monitored the percentage of lung cells expressing the dendritic cell marker CD11c to determine if infection caused DC expansion or infiltration. Lung cells examined at 3 and 7 days postinfection contained significantly higher percentages of CD11c+ DC than lung cells from uninfected mice contained (Fig. 4). Upon maturation, DC upregulate MHC-II and the costimulatory molecules CD80 and CD86. Thus, we examined the levels of these surface molecules on CD11c+ DC during the course of a cryptococcal infection (Fig. 5). The percentage of DC positive for CD80 (Fig. 5A) increased throughout infection and on day 7 postinoculation was significantly greater than the percentage of DC positive for CD80 in control animals. Additionally, the percentages of DC positive for CD86 (Fig. 5B) and MHC-II (Fig. 5C) also exhibited a trend, albeit not significant, toward increased expression on day 7.

**Cryptococcus-specific T-cell activation by pulmonary DC.** In the final set of experiments we tried to determine whether lung DC from infected animals acquired the capacity to present cryptococcal antigen to T cells (Fig. 6). Lung DC were isolated on days 1, 3, and 7 postinfection and incubated with the *C. neoformans*-specific T-cell hybridoma P1D6. DC obtained from infected mice at all times were able to induce T-cell activation, as measured by IL-2 production, indicating that there was cryptococcal antigen presentation by lung DC.

**DISCUSSION**

Macrophages and neutrophils have long been thought to be the first phagocytes to encounter *Cryptococcus* following inhalation; however, the role of lung DC in phagocytosis and killing of *C. neoformans* during infection has not been studied. An immunization model using cryptococcal antigens showed that DC are important for induction of protective immunity against *C. neoformans* (4, 5), and work in our laboratory and by other researchers has shown that DC can phagocytose *C. neoformans* in vitro (38, 76). Therefore, we postulated that lung DC are important for phagocytosis of *C. neoformans*, presentation of cryptococcal antigens to T cells, and ultimately directing the immune response to the organism. Accordingly, in this study, we examined in vivo phagocytosis of *C. neoformans* by lung cells, lung DC maturation, and ex vivo T-cell activation following intranasal inoculation of mice.

This study demonstrated that lung DC can indeed phagocytose *C. neoformans* in vivo. Additionally, our studies confirmed previous findings which demonstrated that macrophages (8–10, 44) and neutrophils (14, 40, 51, 56) can also phagocytose *C. neoformans*. The relative importance of the three phagocytic cell types to host defenses cannot be gauged from our studies. Since DC are thought to be more important in the initial presentation of antigens to naïve T cells and can thus initiate
an adaptive immune response, we focused on the role of DC in the uptake of *C. neoformans*. In order to verify that the DC were capable of internalizing *C. neoformans* rather than just binding extracellularly to the surface of the organism, we examined lung cells from infected mice by confocal microscopy. By examining confocal images, we determined that the *C. neoformans* cells were indeed inside the DC. In studies of other pathogens that infect the lung, such as *M. tuberculosis* (28, 62, 64, 66) and *A. fumigatus* (11), lung DC have been shown to phagocytose the organisms in vivo and are responsible for initiating an adaptive immune response. Moreover, we have shown previously that DC phagocytose *C. neoformans* in vitro when it is opsonized by complement or antibody (38). In our in vivo study, the mice were not previously exposed to *C. neoformans*, and thus it is unlikely that they had anticytotoxic antibodies. Therefore, we speculate that opsonization and phagocytosis of *C. neoformans* in vivo are probably the result of binding of complement components, such as iC3b, to the surface of the organism (40–42, 81).

Initially, we examined the uptake of both live and heat-killed *C. neoformans* following intranasal inoculation. We found that at several early times postinoculation, organisms were taken up by DC, macrophages, and neutrophils. We hypothesized that live and killed organisms would be phagocytosed at different

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**FIG. 5.** Expression of costimulatory molecules on pulmonary DC following infection with *C. neoformans*. Mice were not challenged (naive) or were inoculated with live *C. neoformans*. At 1, 3, and 7 days postinoculation, mice were sacrificed, and the percentages of CD11c+ lung cells coexpressing CD80 (A), CD86 (B), and MHC-II (C) were determined by flow cytometry. The bar graphs show the means ± standard errors of the means from three independent experiments. Lung cells from four mice per group were pooled for each experiment. The asterisk indicates that there was a significant difference as determined by the Student t test for a comparison with naive mice (P = 0.006). The histograms show the results of one representative experiment. Gray area, naive mice; thin line, day 3 infected mice; thick line, day 7 infected mice.
DC were purified and separated by cell sorting or by CD11c bead positive selection. DC were then incubated with a T-cell hybridoma specific for C. neoformans mannoprotein (P1D6), and IL-2 production in the supernatant, a measure of T-cell activation, was determined. P1D6 cells alone (without DC) served as a negative control. Lung cells from four mice per group were pooled for each experiment. The data are representative of three experiments. An asterisk indicates that there was a significant difference as determined by the Student t test for a comparison with naive mice (P = 0.004 for day 1; P = 0.002 for day 3; P = 0.002 for day 7).

In order to determine if cryptococcal infection could affect DC infiltration and maturation, we examined the percentage and maturation of lung DC after several days of infection. Our data showed that the percentage of CD11c+ DC in the lungs increased throughout infection, suggesting that there was DC infiltration into the lungs during infection. DC infiltration into the lungs has also been reported with other proinflammatory stimuli in the lung, including gamma interferon (27), cigarette smoking (73), and allergen inhalation (53, 54, 62). Also, the percentage of these DC that expressed the maturation marker CD80 significantly increased, and the levels of CD86 and MHC-II increased moderately throughout the course of the infection, suggesting that pulmonary cryptococcal infection leads to DC maturation. However, the increases were modest, and it is possible that many of the mature DC began to traffic to the regional lymph nodes, where they encounter naive antigen-specific CD4+ T cells (29). If the mature DC indeed traffic to the regional lymph nodes, they could present cryptococcal antigens to T cells and thus initiate the adaptive immune response.

In agreement with other studies of pulmonary DC, we defined immature pulmonary DC by surface expression of intermediate levels of CD11c (64, 75), low to intermediate levels of MHC-II (31, 75), and undetectable levels of CD8α (75). Recent studies have suggested that under some conditions, CD11c can be expressed on macrophages (64) and activated CD8+ T cells (6). However, the CD11c+ cells that we isolated from naive and infected mice only rarely expressed the macrophage marker F4/80 and did not coexpress the T-cell markers CD3 and CD8. Thus, the vast majority of the CD11c+ cells isolated from the lung in this study appeared to be DC. Additionally, two major subtypes of CD11c+ DC have been described, plasmacytoid and myeloid DC. Myeloid DC are CD11c+, CD11b+, CD8α−, and B220− (2, 3, 19, 80), whereas plasmacytoid DC are CD8α− and B220+ and lack expression of costimulatory molecules (2, 3, 7, 13, 61, 80). In preliminary studies with both naive and infected mice, less than 3% of lung DC expressed B220 (Wozniak and Levitz, unpublished observations), suggesting that the vast majority of these DC are myeloid DC.

Finally, we examined the potential for pulmonary DC from infected mice to present antigen ex vivo to Cryptococcus-specific T cells. We showed that the Cryptococcus-specific T cells were activated when they were incubated with CD11c+ lung DC from infected mice but not when they were incubated with DC from uninfected mice. This suggests that lung DC from infected mice are capable of cryptococcal antigen presentation to T cells, which probably occurs once the DC traffic to the regional lymph nodes. Interestingly, the T cells stimulated by DC from mice infected for only 24 h produced more IL-2 than the T cells stimulated by DC from mice infected for 3 or 7 days. This is especially intriguing, as the highest percentage of DC was present in the lungs at 7 days postinfection. However, it is possible that by days 3 and 7 postinoculation, the mature DC that present cryptococcal antigen may already be trafficking out of the lung and into the regional lymph nodes, and the increase in DC in the lung may be due to immature DC trafficking into the lung.

In these experiments, we showed that lung DC are involved in cryptococcal phagocytosis in vivo and antigen presentation to Cryptococcus-specific T cells. This suggests not only that DC are important for initiating an adaptive immune response to the organism but also that DC may play a previously unappreciated role in innate defenses. We are performing studies to determine the interaction between C. neoformans and lung DC in development of protective immunity in the lung.

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REFERENCES


are recruited into the airway epithelium during the inflammatory response to a broad spectrum of stimuli. J. Exp. Med. 184:2429–2432.