Role of Dendritic Cells and Alveolar Macrophages in Regulating Early Host Defense against Pulmonary Infection with Cryptococcus neoformans

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Successful pulmonary clearance of the encapsulated yeast Cryptococcus neoformans requires a T1 adaptive immune response. This response takes up to 3 weeks to fully develop. The role of the initial, innate immune response against the organism is uncertain. In this study, an established model of diphtheria toxin-mediated depletion of resident pulmonary dendritic cells (DC) and alveolar macrophages (AM) was used to assess the contribution of these cells to the initial host response against cryptococcal infection. The results demonstrate that depletion of DC and AM one day prior to infection results in rapid clinical deterioration and death of mice within 6 days postinfection; this effect was not observed in infected groups of control mice not depleted of DC and AM. Depletion did not alter the microbial burden or total leukocyte recruitment in the lung. Mortality (in mice depleted of DC and AM) was associated with increased neutrophil and B-cell accumulation accompanied by histopathologic evidence of suppurative neutrophilic bronchopneumonia, cyst formation, and alveolar damage. Collectively, these data define an important role for DC and AM in regulating the initial innate immune response following pulmonary infection with C. neoformans. These findings provide important insight into the cellular mechanisms which coordinate early host defense against an invasive fungal pathogen in the lung.

Cryptococcus neoformans, an opportunistic fungal pathogen acquired through inhalation, causes significant morbidity and mortality primarily in patients with impairments in host defense, including those with AIDS, those with lymphoid or hematological malignancies, or those receiving immunosuppressive therapy secondary to autoimmune disease or organ transplantation (31, 33, 60). The development of a T1 antigen-specific immune response characterized by gamma interferon production and classical activation of macrophages is required to eradicate the organism (4, 8, 21, 23, 24, 28). This adaptive immune response takes 2 to 3 weeks to develop and coincides with the CCR2-mediated recruitment of additional pulmonary dendritic cells (DC) and T cells to the lung (51, 66, 67). The role of initial, innate immune responses against the organism (prior to the development of adaptive immunity) is not well understood.

Resident lung phagocytic cells, primarily DC and alveolar macrophages (AM), are likely the first immune cells exposed to C. neoformans upon inhalation of the organism into the lung. Both DC and AM express lectin receptors, including macrophage mannose receptor and DC-specific non-ICAM3 grabbing nonintegrin (DC-SIGN) (14, 15), which bind C. neoformans glycoantigens, including mannoproteins (42, 55). DC and AM phagocytose the organism in vitro and in vivo (29, 34, 63, 77, 78), and phagocytosis (and/or exposure to soluble glycoantigens or cryptococcal DNA) is associated with cytokine and chemokine production (5, 29, 40, 48, 49, 55, 61) and yeast lysis (77). It is unclear whether phagocytosis by resident DC and AM contributes to early clearance and/or the later development of adaptive immunity.

DC represent an important interface between innate and adaptive immunity (reviewed in references 25, 53, 59, and 62). DC-cryptococcal interactions alter DC antigen-presenting functions and modulate resultant T-cell responses in vitro (10, 19, 55, 71). Following cryptococcal infection in vivo, DC migrate to thoracic lymph nodes (4, 52, 67). Thereafter, newly recruited DC colocalize with T cells within bronchovascular infiltrates in the lung (51). This is associated with interleukin-12 and gamma interferon production, yet direct evidence that DC–T-cell interactions modulate anticytococcal responses in vivo remains sparse.

A well-described murine model of in vivo DC depletion has been used to evaluate the contribution of DC to the development of dynamic, antigen-specific immune responses against a variety of antigens and microbial pathogens (16, 26, 27, 37, 56, 57, 69). Depletion results from the administration of diphtheria toxin (DT) to transgenic (Tg) mice in which the DT receptor (DTR) has been linked to the CD11c promoter. DT administration transiently depletes tissue DC (which express CD11c) for up to 96 h. This model has helped in assessment of the role of pulmonary DC in mediating adaptive immune responses against inhaled antigen (ovalbumin) and influenza virus (16, 69). In these studies, DT administration was associated with transient depletion of AM (which also express CD11c). The effect of CD11c cell depletion on innate immune responses has not been assessed (or reported) in these studies.

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In the current study, this established model of DT-mediated depletion of resident DC and AM was used to assess the in vivo contribution of these cells to the initial host response against cryptococcal infection. This objective is clinically relevant, as studies modulating DC numbers are in development for the treatment of patients with asthma (30, 32), autoimmune (65, 68, 74), organ transplantation (12, 39, 43), and cancer (3, 11, 73). It is unknown whether manipulating the number of tissue DC will alter the innate or adaptive antifungal host defense in these patients. Our results demonstrate that DC and AM are critical regulators of the initial immune response against C. neoformans within the lung. Early mortality in DC- and AM-depleted mice precluded our ability to assess the role of these cells in the development of adaptive immunity.

MATERIALS AND METHODS

Mice. Speciﬁc-pathogen-free inbred female C57BL/6 (designated wild type [WT]) mice were purchased from Charles River Laboratory Inc. (Wilmington, MA). B6.FVB-Tg(Ifgαx-DRG/EGFP)S7j mice (26), designated Tg(CD11c-ΔTUR) in this study, were bred on site. Mice were housed at the University of Michigan Unit for Laboratory Animal Medicine facilities (Ann Arbor, MI), which is fully accredited by the American Association for Accreditation of Laboratory Animal Care. Mice were kept under speciﬁc-pathogen-free conditions in enclosed ﬁlter top cages and provided standard animal chow and chlorinated tap water ad libitum. Mice were 8 to 12 weeks of age at the time of infection, and there were no age-related differences in the responses of these mice to C. neoformans infection. Experiments were approved by the Animal Care and Use Committee at the University of Michigan.

C. neoformans. C. neoformans strain 52D was obtained from the American Type Culture Collection (ATCC 24067); this strain displayed a smooth colony morphology when grown on Sabouraud dextrose agar. For the infection, yeast cells that were recovered from 10% glycerol stocks were grown to stationary phase (at least 72 h) at 36°C in Sabouraud dextrose broth (1% peptone, 2% dextrose; Difco, Detroit, MI) on a shaker. The cultures were then washed in nonpyrogenic saline (Travenol, Deerﬁeld, IL), counted on a hemocytometer, and diluted to 3.3 × 10^7 yeast cells/ml in sterile nonpyrogenic saline.

Experimental design. Primary cryptococcal infection was induced in the lungs by intratracheal (i.t.) inoculation of mice with strain 52D of C. neoformans. The number and phenotype of CD11c-expressing cells (DC and AM) in the lungs of WT mice were assessed at baseline (uninfected, day 0) and at days 4 and 7 postinfection using ﬂuorescence-activated cell sorting (FACS). Next, a well-described model utilizing Tg mice was used to deplete lung CD11c-expressing cells prior to infection with C. neoformans. Briefly, Tg(CD11c-ΔTUR) mice contain a Tg construct linking the DTR to the CD11c promoter (26). Systemic (intraperitoneal) administration of DT induces the irreversible death of CD11c-expressing cells (up to 96 h) (16, 69). Conﬁrmation of CD11c cell depletion (in Tg mice) was performed by FACS. To assess the effect of CD11c cell depletion on host defense, primary cryptococcal infection was induced in the lungs by i.t. inoculation of WT or Tg(CD11c-ΔTUR) mice with C. neoformans 24 h after DT treatment. Tg(CD11c-ΔTUR) mice receiving saline (sham) in place of DT served as an additional control in preliminary experiments. WT mice receiving saline (sham) in place of DT served as an additional control in all experiments. Thereafter, the following experiments were performed: (i) survival postinfection, (ii) analysis of pulmonary cryptococcal burden (by lung CFU assay) and serum antigen level (by agglutination assay) at day 4 postinfection, (iii) enumeration and phenotype of lung leukocytes (by visual identiﬁcation and FACS) at day 4 postinfection, and (iv) histological assessment of the lungs (by hematoxylin and cosin staining and light microscopy) at day 5 postinfection.

In preliminary experiments comparing the effects of cryptococcal infection in infected sham-treated (i.e., no DT pretreatment) WT or Tg(CD11c-ΔTUR) mice, no signiﬁcant differences were observed in (i) mortality (n = 3 mice per group observed at >21 days postinfection) or (ii) lung CFU or total lung leukocytes (including total polymorphonuclear PMNs, lymphocytes, or macrophage/monoocytes) (n = 4 mice per group harvested at day 4 postinfection). Therefore, the control group of infected, sham-treated Tg(CD11c-ΔTUR) mice was omitted from further studies.

Surgical i.t. inoculation. Mice were anesthetized via i.p. injection of a ketamine-xylazine mix (100-6.8 mg/kg of body weight [BW]) and were restrained on a foam plate. A small incision was made through the skin over the trachea, and the underlying tissue was separated. A bent 30-gauge needle (Becton Dickinson, Rutherford, NJ) was attached to a tuberculin syringe (BD & Co, Franklin Lakes, NJ) ﬁlled with the diluted C. neoformans culture. The needle was inserted into the trachea and 30 μl of inoculum dispensed into the lungs (10^7 yeast cells). The skin was closed with cyanoacrylate adhesive. The mice recovered with minimal visible trauma.

Lung leukocyte isolation. The lungs from each mouse were excised, washed in phosphate-buffered saline (PBS), minced with scissors, and enzyme digested at 37°C for 30 to 35 min in 15 ml/digestion buffer (RPMI, 5% fetal calf serum, antibiotics, 1 mg/ml collagenase [Boehringer Mannheim Biochemical, Chicago, IL], and 30 μg/ml DNase [Sigma]). The cell suspension and tissue fragments were further dispersed by repeated aspiration through the bore of a 10-ml syringe and were centrifuged. Erythrocytes in the cell pellets were lysed by addition of 3 ml of NH4Cl buffer (0.329% NH4Cl, 0.1% KHC03, 0.0372% Na2EDTA, pH 7.4) for 3 min, followed by a 10-fold excess of RPMI. Cells were resuspended, and a second cycle of syringe dispersion and ﬁltration through a sterile 100-μm nylon screen (Nitek, Kansas City, MO) was performed. The ﬁltrate was centrifuged for 25 min at 1,500 × g in the presence of 20% Percoll (Sigma) to separate leukocytes from cell debris and epithelial cells. Leukocyte pellets were resuspended in 5 ml of medium and enumerated on a hemocytometer following dilution in Trypan Blue.

Antibody staining and ﬂow cytometric analysis. Staining, including blockade of Fc receptors (with antimurine CD16/CD32, rat immunoglobulin G2b [IgG2b]), and analysis by ﬂow cytometry were performed as described previously (50). All antibodies were purchased from BD Pharmingen. Data were collected on a FACS Vantage ﬂow cytometer using Cell Quest software (both from Becton Dickinson Immunocytometry Systems, Mountain View, CA) and analyzed using FlowJo software (Tree Star Inc., San Carlos, CA). A total of 10,000 to 100,000 cells were analyzed per sample.

Gating strategy using FACS to identify lung CD11c-expressing cells. Single-cell populations were obtained from enzyme-digested lungs, Fc blocked, and stained with anti-CD11c (Armenian hamster IgG1a2-fluorescein isothiocyanate; FL-1) and anti-CD11b (Rat IgG2b-phycocerythrin; FL-2). Initial gates were set based on light scatter characteristics to eliminate debris, red cells, granulocytes, and lymphocytes, and DCs. The FACS-versus-FL-2 (CD11c) scatter plot was used to identify large CD11c-expressing cells (Fig. 1A, gate R1). Using this R1 population, a second plot of FL-1 (CD11c) versus FL-2 (CD11b) was used to differentiate AM (CD11c-positive/CD11b-negative/low) from lung DC (CD11c+/CD11b+). As has been previously reported (50, 51, 75, 76), to maintain complete consistency, the cytometer parameters and gate position were held constant during analysis of all samples. The percentage of AM and DC obtained from flow cytometry was used to calculate the total number of these cells from each lung by multiplying the frequency of each population by the total number of leukocytes identified within that sample.

DT administration. To deplete CD11c-expressing cells, Tg (CD11c-ΔTUR) mice were injected (i.p.) with 5 mg/g BW of DT (Sigma D-0564) in 0.25 ml PBS as reported previously (26). WT mice that received either DT or PBS served as a sham treatment. Cell effectiveness in the lungs was assessed using FACS. The population recovered to normal by day 5 after DT treatment as reported previously (16, 26, 69).

Survival studies. Parallel survival studies were performed using matched WT versus Tg mice. Mice received an injection (i.p.) of DT (5 mg/g BW) or PBS (sham, WT only) followed 24 h later by i.p. challenge with C. neoformans (as described above). General health evaluation was performed daily, and mice were followed for 10 days or until death.

Lung CFU assay. Aliquots of the lung digest solutions were collected for lung CFU assays. Lung suspensions were serially diluted in sterile water. Dilution samples (10 μl each) were plated on Sabouraud dextrose agar and incubated at room temperature for 48 h. Recovered colonies retained their smooth morphol-

 Colony counts were performed and adjusted to reﬂect the total lung CFU. Serum antigen assay. Serum from infected mice was obtained at 4 days postinfection and assayed for the presence of cryptococcal antigen using the Latex-Cryptococcus Antigen Detection System (Immuno-Mycologies Inc., Nor- man OK) per the manufacturer’s instructions. Antigen agglutination reactions were visually scored on a scale from 0 (no clumping reaction) to 4 (large clumping reaction).

Analysis of lung leukocyte subsets. Isolated lung cells (10^4) in complete medium were cytospun onto glass slides (Shandon Cytospin, Pittsburgh, PA). The slides were ﬁxed for 2 min in the one-step, methanol-based Wright-Giemsa staining (Harleco; EM Diagnostics, Gibbstown, NJ), followed by steps 2 and 3 of the Diff-Quik whole-blood stain kit (Baxter Scientiﬁc, Miami, FL). Large mono
cell nuclei (macrophages, monocytes, and DC), neutrophils, and eosinophils were visually counted under a microscope from randomly chosen high-power
fields, for a total of 200 cells. Lymphocytes were identified within single-cell suspensions using FACS as either CD4+ T cells, CD8+ T cells, or B220+ B cells. The percentage of a specific leukocyte subset was multiplied by the total number of leukocytes to calculate the absolute number of the subset in the sample.

**Histology.** The trachea was cannulated with PE50 tubing (Clay-Adams, Parsippany, NJ) and inflated with 1 ml of 10% neutral buffered formalin. The fixed lung specimens were stored in 10% neutral-buffered formalin until being dehydrated in 70% ethanol and then paraffin embedded. Sections (5 μm) were cut, deparaffinized, stained with hematoxylin and eosin, and viewed by light microscopy.

**Statistical analysis.** All data were expressed as mean ± standard error of the mean (SEM). Continuous ratio scale data were evaluated by an unpaired Student t test (for comparison between two groups) or by analysis of variance (for multiple comparisons) with post hoc analysis by a two-tailed Dunnett test, which compares treatment groups to a specific control group (79). Survival data were analyzed by the Kaplan-Maier method (percentage of surviving animals) with log rank analysis (to compare survival curves). Statistical calculations were performed on a Dell 270 computer using GraphPad Prism version 3.00 for Windows (GraphPad software, San Diego, CA). Statistical significance was accepted at a P value of <0.05.

**RESULTS**

**CD11c-expressing DC and AM populations in the lung at baseline and following pulmonary infection with C. neoformans.** The first objective was to identify the number and phenotype of CD11c-expressing cells in the lungs of uninfected mice. Our previous work (50-52) and the work of others (9, 69, 70, 75, 76) have demonstrated that lung DC and AM are the primary cells which express high to moderate levels of CD11c in the lung. CD11b expression has been previously shown to distinguish conventional DC (which express both CD11c and CD11b) from AM (which express CD11c and minimal CD11b). DC identified in this manner express major histocompatibility complex class II and costimulatory molecules, including CD40, CD80, and CD86 (51, 76). Based on these previous studies, a specific gating strategy was applied to cells obtained from enzymatically digested lungs harvested from uninfected mice (see Materials and Methods). Briefly, we first identified a population of large cells exhibiting moderate to high expression of CD11c (Fig. 1A, left scatter plots, R1 gate). Thereafter, these CD11c-expressing cells (Fig. 1A, R1 gate) were assessed for CD11b expression. AM were defined as cells expressing minimal amounts of CD11b (Fig. 1A, right scatter plots, AM gate), whereas DC were defined by their strong CD11b expression (Fig. 1A, right scatter plots, DC gate). In uninfected mice, CD11c-expressing cells accounted for 5.1% ± 1.8% (mean ± SEM) of the total population of lung cells (1.1 × 10^6 ± 0.2 × 10^6 total CD11c cells/lung) (Fig. 1B). The majority (87%) of these CD11c-expressing cells were AM (4.5% ± 1.7% of total lung cells; 9.7 × 10^5 ± 2.2 × 10^5 total AM/lung). A population of resident lung DC were also present at baseline (0.4% ± 0.2% of total lung cells; 9.6 × 10^4 ± 3.0 × 10^4 total DC/lung), consistent with other reports (50, 72).

We next determined whether the numbers and/or composition of the CD11c-expressing cell population was altered following pulmonary infection with C. neoformans. The same gating strategy was applied to total cell populations obtained from the lungs of mice at days 4 and 7 postinfection (Fig. 1B). At day 4, there was no significant difference in the total numbers of CD11c-expressing cells (AM or DC) in the lung compared with uninfected mice. However, by day 7, the percentages and total numbers of CD11c-expressing cells were significantly increased (7.8% ± 1.1% of total lung cells; 2.7 × 10^6 ± 1.2 × 10^6 total CD11c cells/lung). This was primarily attributable to a significant increase (15-fold) in DC (4.3% ± 1.1% of total lung cells; 1.5 × 10^6 ± 0.8 × 10^6 total DC/lung), whereas the numbers of AM remained unchanged. Collectively, these data suggest that AM are the dominant CD11c-expressing population in uninfected lungs and through the first 4 days following infection with C. neoformans. Thereafter, the CD11c-expressing cell population expands rapidly due to an accumulation of additional pulmonary DC.

**Effect of depleting resident lung DC and AM on survival after cryptococcal infection.** The second objective of this study was to examine the role of DC and AM in vivo in mediating...
host defense against pulmonary cryptococcal infection. We utilized a well-described murine model of in vivo CD11c−/− cell depletion (DC and AM) (16, 26, 27, 37, 56, 57, 69) to determine whether ablating resident DC and AM would fundamentally alter the host defense against C. neoformans. Our results confirm that administration of DT to Tg(CD11c-DTR) mice eliminates approximately two-thirds of CD11c-expressing cells within the lung (at 24 h following DT administration to WT (left panel) or Tg(CD11c-DTR) (right panel) mice. (B) In separate experiments, WT or Tg(CD11c-DTR) mice were infected (i.t.) with 10⁴ C. neoformans (Cneo) organisms at 24 h following saline (sham) or DT (i.p.) administration. Data represent survival at the indicated days postinfection for three treatment groups (n = 6 to 9 mice per group from three separate experiments; P < 0.05 versus WT + sham + Cneo [*] and WT + DT + Cneo [**] by log rank analysis).

FIG. 2. Depletion of resident DC and AM markedly impairs survival following pulmonary infection with C. neoformans. WT or Tg(CD11c-DTR) mice were injected (i.p.) with DT (5 ng/g BW). (A) Representative histograms displaying the CD11c− population present in the lungs at 24 h following DT administration to WT (left panel) or Tg(CD11c-DTR) (right panel) mice. (B) In separate experiments, WT or Tg(CD11c-DTR) mice were infected (i.t.) with 10⁴ C. neoformans (Cneo) organisms at 24 h following saline (sham) or DT (i.p.) administration. Data represent survival at the indicated days postinfection for three treatment groups (n = 6 to 9 mice per group from three separate experiments; P < 0.05 versus WT + sham + Cneo [*] and WT + DT + Cneo [**] by log rank analysis).

Effect of depleting resident lung DC and AM on microbial burden after cryptococcal infection. To ascertain whether the rapid deterioration and death of mice depleted of DC and AM were due to overwhelming cryptococcal infection, we evaluated the burden of cryptococcus within the lung (by CFU assay) at day 4 postinfection (24 h prior to the time at which mice began to expire) (Fig. 3A). There was no significant difference in the microbial load within the lungs of DC- and AM-depleted mice (Tg(CD11c-DTR) + DT) and two control groups of mice (WT + sham and WT + DT). Mortality (in the DC- and AM-depleted mice) was not attributable to phenotypic switch-
Depletion of DC and AM does not alter the absolute number of lung leukocytes at 4 days following pulmonary infection with *C. neoformans*. WT or Tg(CD11c-DTR) mice were injected (i.p.) with either saline (sham) or DT (5 ng/g BW) and infected (i.t.) with 10⁶ *C. neoformans* organisms 24 h later. At 4 days postinfection, lungs were harvested and leukocytes were enumerated by visual inspection (n = 6 to 9 mice per group from three separate experiments; error bars, SEM; *P* < 0.05 versus WT + sham + Cneo [*] and WT + DT + Cneo [**] by unpaired Student t test).

In the absence of significant changes in pulmonary microbial burden in DC- and AM-depleted mice, we hypothesized that an overabundance of cryptococcal antigen within the bloodstream could account for the rapid death of infected Tg mice. Fungal glycosylated and mannosylated antigens (including glucuronoxylomannan, galactoxylomannan, and mannoproteins 1 and 2) are efficiently bound by DC and AM, resulting in immunostimulatory and immunomodulatory effects (10, 34–36, 42, 55, 63, 64, 71). To evaluate this hypothesis, serum cryptococcal antigen levels in infected DC- and AM-depleted mice [Tg(CD11c-DTR) + DT] and control mice (WT + sham and WT + DT) were compared. No significant differences (in serum antigen levels) were observed (Fig. 3B). Collectively, these results indicate that the rapid death of infected mice depleted of resident DC and AM was not a result of increased expansion of *C. neoformans* in the lungs or changes in circulating cryptococcal antigen level.

**Effect of depleting resident lung DC and AM on lung leukocyte populations after cryptococcal infection.** Since the depletion of resident DC and AM did not alter the microbial load in *C. neoformans*-infected lungs, we next investigated whether differences in the magnitude and/or leukocyte composition of the resultant inflammatory response might account for the mortality observed in mice depleted of CD11c-expressing cells. Total pulmonary leukocyte numbers were evaluated in all treatment groups at day 4 postinfection [24 h prior to onset of mortality in DT-treated Tg(CD11c-DTR) mice]. No differences in the total number of pulmonary leukocytes were observed between mice depleted of resident DC and AM [Tg(CD11c-DTR) + DT] and control mice (WT + sham and WT + DT) (Fig. 4).

Next, a subset analysis was performed on lung leukocytes obtained from enzyme-digested lungs of infected mice from the three treatment groups [WT + sham, WT + DT, and Tg(CD11c-DTR) + DT]. The results demonstrate that the absolute numbers of PMNs were significantly increased in the lungs of DC- and AM-depleted mice [Tg(CD11c-DTR) + DT] relative to control mice (WT + sham and WT + DT) (Fig. 5A). Few eosinophils were identified at this early time point, and there were no differences (in eosinophil numbers) between treatment groups. A significant increase in B cells but not CD4 or CD8 T cells was observed in infected mice depleted of resident DC and AM (Fig. 5B). There were no differences between treatment groups in the number of large mononuclear cells (AM, DC, and recruited inflammatory monocytes) (data not shown); note that these populations were evaluated 5 days after DT treatment, when the effect of DC and AM depletion had resolved. Collectively, these data suggested that differences in total lung leukocyte recruitment did not account for the increased mortality observed in cryptococcal mice depleted of resident DC and AM. The relative increase in PMNs and B cells (in the lungs of DC- and AM-depleted mice) implicated these cells as potential mediators of lung injury in response to cryptococcal infection.

**Effect of depleting resident lung DC and AM on histopathology after cryptococcal infection.** We next sought to examine whether depletion of resident DC and AM altered morphological patterns of lung inflammation in response to *C. neoformans*. First, to determine whether DT treatment alone caused lung damage, we performed a histopathologic assess-
ment of lung sections obtained from WT or Tg(CD11c-DTR) mice treated with DT alone (in the absence of cryptococcal infection) (Fig. 6). The results demonstrate that DT treatment of Tg(CD11c-DTR) mice resulted in little discernible inflammation when assessed 1 day (Fig. 6A and B) or 6 days (Fig. 6C and D) later. As expected, there was no appreciable inflammation in WT mice treated with DT when assessed at the same time points (data not shown).

Second, we performed a comparative histopathologic assessment of lung sections from DC- and AM-depleted mice [Tg(CD11c-DTR) /H11001 DT] and control mice (WT /H11001 DT) obtained at 5 days postinfection (Fig. 7). The overall numbers of pulmonary leukocytes within lung sections from both groups appeared to be similar (consistent with our findings comparing total leukocyte recruitment in enzyme-digested lungs [Fig. 4]). However, morphological patterns of inflammation differed between treatment groups. Inflammatory infiltrates in the lungs of WT mice were tightly organized (Fig. 7A) and contained a mixture of leukocytes, including larger mononuclear cells (many containing intracellular cryptococci), neutrophils, and lymphocytes. The lumens of small airways were widely patent (Fig. 7C), and the borders between inflamed lung and normal alveoli were distinct (Fig. 7E). In contrast, less organized inflammatory infiltrates were spread diffusely through larger regions of the lung in mice depleted of DC and AM (Fig. 7B). Neutrophils were more abundant, especially within aggregates of PMNs, cryptococcus, and cell debris that almost entirely filled small airways (Fig. 7D). Margins between inflamed and uninvolved alveoli were less distinct (Fig. 7F), and extracellular cryptococci were more numerous.

Three specific severe pathological lesions were identified in mice depleted of DC and AM (Fig. 8). First, we noted suppurative neutrophilic bronchopneumonia as evidenced by plugs of inflammatory debris almost entirely filling numerous small airways (Fig. 8A). As noted above, these infiltrates contained numerous PMNs (some with intracellular yeast), extracellular cryptococci, and inflammatory debris. Second, we noted conglomerates of extracellular cryptococci suspended in transparent capsular material (or biofilm) suggestive of cyst formation (Fig. 8B). Protein-rich exudates surrounded by numerous neutrophils and macrophages were adjacent to these structures. Cysts are typically late manifestations of uncontrolled infections and are associated with failure of the host response to C. neoformans. Third, we observed proteinaceous exudates filling alveoli adjacent to areas of intense inflammation, often in association with free red blood cells suggestive of inflammatory lung injury and hemorrhage (Fig. 8C). Collectively, these histopathologic findings demonstrate that cryptococcal infection of mice depleted of resident DC and AM results in diffuse pulmonary inflammation involving both airways and alveolar
structures. These findings were sufficiently severe to account for the early mortality observed in these mice.

**DISCUSSION**

This study defines, for the first time, the effect of DT-mediated CD11c\(^+\) cell depletion on the host response against a subsequent pulmonary challenge with a pathogenic fungus. We report the following novel findings. (i) Depletion of the CD11c-expressing population, including both DC and AM, immediately prior to pulmonary cryptococcal infection results in rapid clinical deterioration and death of mice (within 6 days postinfection). (ii) Death was not attributable to differences in pulmonary microbial burden, serum antigen, or the total number of leukocytes recruited to the lung. (iii) Depletion of DC and AM was associated with increased neutrophil and B-cell accumulation, suppurative neutrophilic bronchopneumonia, and evidence of severe lung damage. Collectively, these findings demonstrate that DC and AM play a previously unrecognized and critical role in regulating the developing immune response immediately following cryptococcal infection within the lung.

The results of our flow cytometric analysis confirm that DC and AM are the dominant resident cells in the lung expressing CD11c. Additional DC are recruited to the lung during the first 7 days following infection with *C. neoformans*. This agrees with our previous study (51) and the findings of Wozniak et al. (78). Wozniak et al. demonstrated that lung DC phagocytose *C. neoformans* in vivo and that recovered lung DC could stimulate cryptococcus-specific T-cell responses in vitro. They inferred that DC might contribute to both innate and adaptive immune responses against the organism. Collectively, these studies suggested that DC and AM might participate in the early host response against *C. neoformans* in the lung. Our study utilizing DT-mediated CD11c\(^+\) cell depletion allowed us to directly test this hypothesis using an in vivo model of cryptococcal infection.

Strikingly, our results demonstrate that mice depleted of DC and AM rapidly become moribund and die within the first week after infection with *C. neoformans*. These findings shed new insight into the importance of the initial immune response against the organism. The observed kinetics (mice died prior to the onset of adaptive immunity) suggests that DC and/or AM critically influence innate immune responses to the infection. Our study investigated the mechanism(s) whereby DC and AM exerted this previously unrecognized protective effect.

Our findings demonstrate that early mortality in cryptococcus-infected mice depleted of DC and AM is not attributable to an increase in the microbial burden within the lung. This

![FIG. 8. Depletion of DC and AM results in distinct patterns of lung damage following pulmonary infection with *C. neoformans*. Tg(CD11c-DTR) mice were injected (i.p.) with DT (5 ng/g BW) and infected (i.t.) with 10\(^4\) *C. neoformans* organisms 24 h later. At 5 days postinfection, lungs were harvested and the morphological pattern of inflammation was assessed by light microscopy. Representative high-power photomicrographs (hematoxylin and cosin stained; magnification, ×400) demonstrating three distinct patterns of lung pathology are shown.](http://iai.asm.org/)

(A) Neutrophilic bronchopneumonia; note the foci of inflammation within the airway (AW) containing numerous neutrophils, individual cryptococci, and exudative debris. (B) Cyst formation; note the numerous extracellular cryptococci within the cyst (Cy) and the protein-rich (pink) exudate surrounded by neutrophils and macrophages immediately superior to the cyst. (C) Alveolar exudates containing protein and hemorrhage; note the protein-rich (pink) exudates and evidence of parenchymal hemorrhage (arrows identify collections of red blood cells).
finding was surprising since both DC and AM phagocytose the organism in vitro and in vivo (29, 34, 63, 71, 77, 78). Phagocytosis is associated with the production of numerous inflammatory cytokines, including tumor necrosis factor alpha, and expression of inducible nitric oxide synthase, a potent mediator of fungal cytotoxicity (2, 17, 20, 41, 46). Thus, one might have predicted that depletion of AM and DC might impair clearance of *C. neoformans* from the lung. This was not observed. In addition, DC and AM depletion did not increase the amount of circulating cryptococcal antigen.

Our observation that DC and AM depletion did not result in increased pulmonary microbial burden is similar to the findings reported by Shao et al. (58). In their study, three strains of mice were depleted of AM by i.t. instillation of clodronate liposomes prior to infection with *C. neoformans*. They observed either (i) no change (in A/J mice) or (ii) a decrease in lung CFU (in BALB/c and C57 mice) at 3 days postinfection in AM-depleted mice. In marked contrast to our results, they did not observe early mortality (in the clodronate-treated mice) following infection. The use of clodronate liposomes to deplete AM in this manner has since been shown to also deplete the alveolar but not the interstitial subset of pulmonary DC (44). This might imply that the early mortality observed in our study is specifically attributable to depletion of the interstitial subset of DC. However, differences in the mechanism, efficacy, and duration of depletion limit the direct comparison of our data with those of Shao et al. Both our study and theirs utilized cell depletion strategies that were not specific for either DC or AM; thus, we cannot conclude with certainty whether the observed effects are specifically attributable to the reduction in DC, AM, or both. The development of techniques to more specifically target DC or AM are needed, yet that is beyond the scope of this study. Nonetheless, both studies reveal that resident phagocytic cells are not essential for limiting the initial microbial burden in the lung. However, the markedly increased mortality (in DC- and AM-depleted mice) observed in our study suggests that these cells are nonetheless essential to the survival of the host in the first week following cryptococcal infection.

Our data reveal that PMNs are increased in the lungs of cryptococcus-infected mice depleted of CD11c-expressing cells. This finding provides important insight into the potential mechanism(s) whereby equivalent fungal clearance is achieved despite the depletion of resident DC and AM. PMNs can phagocytose and kill *C. neoformans* (38, 45, 47, 78). PMNs are rapidly recruited in response to *C. neoformans* (22), and their influx into tissue has been associated with transient reductions in microbial burden (13, 54). In our study, we observed PMNs containing intracellular cryptococcus, most notably in the lungs of mice depleted of DC and AM. B cells were also increased; we believe this to be of questionable significance, as their appearance precedes the onset of adaptive immunity.

Our comparative histopathologic analysis identified differing patterns of inflammation (in response to cryptococcal infection) between WT mice and mice depleted of DC and AM. Specifically, we observed evidence of suppurative neutrophilic bronchopneumonia filling small airways with inflammatory debris. In addition, we observed evidence of alveolar damage, including hemorrhagic and proteinaceous exudates lining terminal alveoli. These changes are observed in PMN-mediated lung injury associated with respiratory failure and death (1, 80). In the study by Shao et al. (discussed above), they also noted increased inflammation in clodronate-treated mice (although a quantitative subset analysis was not reported). The collective results of our subset and histopathologic analysis suggest that PMN accumulation (in the lungs of mice depleted of resident AM and DC) is detrimental to the infected host. This hypothesis is further supported by a study performed by Mednik et al. (45) in which PMN were depleted immediately prior to cryptococcal infection. They demonstrated that PMN-depleted mice had a significant survival advantage (compared to nondepleted controls) in response to pulmonary infection with *C. neoformans*. Collectively, our results and those of Mednik et al. reveal that early PMN-mediated immune responses against *C. neoformans* are harmful and potentially lethal. The evidence we report of PMN-mediated lung injury in the lungs of infected mice depleted of resident DC and AM does not exclude the possibility that additional cell types might contribute to the histopathology and mortality observed. B cells were increased in the lungs of (infected) DC- and AM-depleted mice. They could mediate lung injury through the production of inflammatory cytokines, chemokines, or naturally occurring autoantibodies. Although our subset analysis of leukocyte recruitment did not identify differences in total macrophage/monocyte recruitment (at day 4 postinfection), it remains possible that accumulation of a subset of monocytes (specifically Ly-6C^high^ inflammatory monocytes) could also contribute to lung injury. We believe that this model system is well suited to further dissect the complicated interrelationships between DC and/or AM and the recruitment of additional leukocyte subsets to the lung.

The results of our study, in concert with the important contributions of Shao et al. (58) and Mednik et al. (45), support a new paradigm regarding the role of the initial immune response against pulmonary infection with *C. neoformans*. They provide compelling evidence that this response is critically important and tightly regulated by resident DC and AM. The data suggest that these cells are not essential for early clearance of the organism from the respiratory tract. Rather, we propose that they function to limit deleterious innate immune responses until more efficient (and less damaging) adaptive responses are generated. The consequences of DC and AM depletion can be better understood in the context of the “damage response” framework of microbial pathogenesis whereby the intensity of the inflammatory response contributes to the pathophysiology of infection (6, 7). Traditionally, this implies that inherent or acquired characteristics of the microbe define the severity of the resultant immune response. As an example, we recently demonstrated that a microbial factor (cryptococcal urease) contributes to enhanced inflammation and resultant pathophysiology in a manner that allows *C. neoformans* to be considered a type III (versus a type II) pathogen (52). In our current model, we propose that an alteration in the infected host (CD11c cell depletion) can similarly allow *C. neoformans* to function as a type III pathogen as a result of the increased host response-mediated lung injury that results in the absence of DC and AM.

In summary, these findings reveal that DC and/or AM are critical determinants of the initial host response against *C. neoformans* in the lung. Our data support a model in which
these resident phagocytic cells regulate early innate immune responses. In their absence, PMN and B-cell accumulation was associated with evidence of increased histopathologic damage to the pulmonary microenvironment. The early mortality observed in mice depleted of CD11c-expressing cells limits our use of this model system to investigate the role of DC and AM in mediating adaptive immune responses against Cryptococcus neoformans. However, our results reveal that the initial host response (prior to the onset of adaptive immunity) against this pathogenic fungus is active, tightly regulated, and vitally important. This information enhances our understanding of the host's early response against the organism and has important implications for disease states or applied therapeutics whereby pulmonary DC and AM populations may be depleted.

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