Immunity to a Pulmonary Cryptococcus neoformans Infection Requires both CD4+ and CD8+ T Cells
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Summary
The role of CD4+ and CD8+ T cells in mediating pulmonary clearance of a cryptococcal infection was investigated. Intratracheal inoculation of BALB/c and C.B-17 micewith a moderately virulent strain of Cryptococcus neoformans (52D) resulted in a pulmonary infection, which was cleared by a T cell–dependent mechanism. During this clearance, there was a significant influx of both CD4+ and CD8+ T cells into the lungs. Depletion of CD4+ and CD8+ T cells by injections of CD4-specific monoclonal antibody (mAb) prevented pulmonary clearance and also resulted in significant colonization of the brain and spleen of infected mice. CD4 depletion did not prevent the influx of CD8+ T cells into the lungs. Surprisingly, depletion of CD8+ T cells by mAb also ablated pulmonary clearance. CD8-depleted mice also had a small but significant increase in brain and spleen colony-forming unit compared to control mice by the end of the study. CD4+ T cell pulmonary influx was independent of the presence of CD8+ T cells. The lungs of T cell–depleted mice were examined histologically. CD4+ and CD8+ T cells each mediated a degree of inflammatory influx seen in the lungs of infected mice and raised the possibility that CD4+ and CD8+ T cells may synergize to generate the inflammatory response in the lungs. Numerous phagocytized but intact cryptococci were seen in the inflammatory foci of CD8-depleted mice but not in control or CD4-depleted mice. We propose that CD4+ T cells may recruit and activate effector phagocytes while CD8+ T cells predominantly function to lyse cryptococcus-laden unactivated phagocytes similar to the function of CD8+ T cells during listeria and mycobacteria infections.

T cell–mediated immunity (CMI) is a critical component of protective immunity against infection by the yeast Cryptococcus neoformans (1). Both CD4+ (also known as L3T4+ or Lyt-1+2- in the mouse) and CD8+ (murine Lyt-1-2+) T cells are involved in the systemic anticryptococcal immune response (1). CD4+ T cells mediate anticycrococcal delayed type hypersensitivity (DTH) (2–5), the development of Ts (6–8), and clearance of extrapulmonary infection (5). CD8+ T cells, in contrast, may downregulate the systemic anticycrococcal immune response through the development of Ts, which can suppress T cell responses (2, 7, 9, 10) and phagocytosis (11–13). The lungs are the portal of entry for the infectious agent of cryptococcosis and would be the initial site for the development of anticryptococcal CMI. The role of CD4+ and CD8+ T cells in pulmonary clearance is uncertain. Indeed, there is evidence that local and systemic immune responses might differ after pulmonary immunization (14). A previous report involving this laboratory described a murine intratracheal cryptococcal infection model and demonstrated the critical role of CD4+ T cells in mediating systemic protection (5). However, the cryptococcal strain used in those studies grew progressively in the lungs even in immunocompetent mice. We have recently developed and characterized another murine intratracheal cryptococcal infection model using a less virulent strain of C. neoformans in which pulmonary clearance of the infection occurred in immunocompetent mice but not in T cell–deficient mice (14a). Using this murine model, we have addressed the question of whether CD4+ and CD8+ T cells had a positive, a negative, or no effect on T cell–mediated pulmonary clearance.

Materials and Methods

Mice. BALB/c and C.B-17 mice were used throughout these studies. BALB/c mice were obtained from Simonsen Laboratories, Inc. (Gilroy, CA). C.B-17 (BALB/c strain congenic for C57BL6 IgH) were bred at The University of Texas Southwestern Medical
Center (Dallas, TX). Both male and female mice (8-12 wk) were used, and the sex was noted at necropsy. There was no correlation between the sex of the mice and yeast virulence. Mice were housed in sterilized cages within a pathogen-free environment tent. Sterilized food and water was given ad libitum. Mice were observed for any overt signs of secondary infection and randomized mice were tested at the conclusion of the experiments and found to be negative for serum antibodies to mouse hepatitis virus, Sendai virus, and Mycoplasma pulmonis.

C. neoformans. C. neoformans 52D (15, 16) was used for these studies. Strain 52D (24067; American Type Culture Collection, Rockville, MD) is an encapsulated serotype D strain with an in vitro generation time of 2.6 h. Stock cultures were grown on Sabouraud dextrose agar (SDA; 1% neopeptide, 2% dextrose, 1% agar) slants at room temperature and stored at −70°C. Working cultures were prepared monthly by thawing a stock culture and inoculating fresh slants, and the working cultures were stored at 4°C. To prepare an infecting inoculum, yeast were inoculated from a culture slant stored at 4°C into Sabouraud dextrose broth (SDB; 1% neopeptide, 2% dextrose) and grown on a shaker at room temperature until confluent (36 h). An aliquot of culture was collected, washed three times in sterile nonpyrogenic saline (Travenol, Deerfield, IL), counted on a hemocytometer, and diluted to the appropriate concentration.

Route of Infection. Mice were anesthetized with methoxyflurane vapor (Metrane; Pitman-Moore, Washington Crossing, NJ), restrained on a small board, and infected with 10^4 CFU of C. neoformans 52D in nonpyrogenic saline by surgical intratracheal inoculation (IT). A small incision was made through the skin over the trachea. The underlying tissue was separated and, using a bent 30-gauge needle (Becton Dickinson and Co., Rutherford, NJ) attached by polyethylene tubing (Intramedic, Clay Adams, Parsippany, NJ) to a tuberculin syringe (Monoject, St. Louis, MO), the needle was inserted into and parallel with the trachea. A 50-μl inoculum was then dispensed into the lungs. After inoculation, the skin was sutured, and mice were allowed to recover. At periodic intervals during the procedure, mice were over-anesthetized and their lungs were removed to assay the number of CFU deposited in the lungs.

Assay for Organ CFU. At various time points infection, lungs, brain, and spleen were removed, placed in a plastic sample bag (Tekmar Co., Cincinnati, OH) filled with 2 ml sterile water, and homogenized. Duplexes of 50 μl of the homogenate were plated on SDA plates containing 50 μg/ml chloramphenicol (Remel, Lenexa, KS). Dilutions were made in sterile water as necessary. Plates were incubated 3 d, and CFU expressed as log_{10} CFU per organ.

Delayed Type Hypersensitivity (DTH) Assay. Preparation of cryptococcal filtrate antigen (CneF Ag) was adopted from a procedure described previously (17). Briefly, the supernatant, collected from formaldehyde-treated cultures, was filtered through a 0.45-μm filter (Corning Glass Works, Corning, NY) concentrated 50-fold in an ultrafiltration unit (Amicon Corp., Danvers, MA) using a PM-30 membrane (Amicon Corp.), and dialyzed overnight against PBS. The sample was filter sterilized, aliquotted, and stored at −20°C. The sample was plated overnight at 37°C, 7% CO₂ in complete media to remove adherent cells, and the nonadherent cells were passed over a nylon wool column to further remove adherent cells including most B cells.

Histopathology. Day 11-14 post-IT-infected mice were over-anesthetized and their lungs inflated with 1 ml of 10% neutral buffered formalin (NBF; 10% [vol/vol] 37% formaldehyde in PBS) fixed in NBF overnight, and hematoxylin- and eosin-stained sections were prepared.

Tissue samples for immunohistochemistry from separate animals were snap frozen in 2-methyl butane (Kodak, Rochester, NY) in an acetone/dry ice bath. Frozen sections were stained using a Vectorstain ABC peroxidase kit (Vector Laboratories Inc., Burlingame, CA). Sections were blocked 10 min with whole rabbit serum (Vector Laboratories Inc.) PBS, and either hybridoma culture supernatant or stock mAb diluted, as described above, was used as the primary
antibody. The secondary antibody was biotinylated rabbit anti-rat Ig which had been absorbed with mouse Ig. A Nikon photomicroscope was used for photomicroscopy.

Statistics. Statistical significance was analyzed using the t test with the significance level, $p$, modified for multiple comparisons (the Bonferroni method for multivariable, reference 21).

Results

Effect of In Vivo T Cell Depletion on Pulmonary Clearance and Extrapulmonary Dissemination of C. neoformans Strain 52D. Intratracheal inoculation of either BALB/c or C.B-17 mice with C. neoformans strain 52D resulted in a pulmonary infection that was progressively cleared by a T cell-dependent mechanism(s) beginning after anticyptococcal immunity developed (between day 7 and 21 post-IT; reference 14a). Flow cytometric analysis of the lymphocytes found in the lungs of day 28, strain 52D–infected mice indicated that the majority of the lymphocytes were larger, likely blast cells, as expected for lymphocytes found at the site of infection (data not shown). Analysis of the blast cell population revealed that both CD4+ and CD8+ T cells were present in the infected lungs, at a ratio of ~3:1 (data not shown).

To analyze the influence of these two T cell subsets on pulmonary clearance and dissemination, groups of mice treated with control antibody or with mAb to deplete mice of CD4+, CD8+, or both T cell subsets (22) were infected with C. neoformans IT. At different time points during the course of the experiment, splenic lymphocytes from infected mice were analyzed to monitor T cell depletion (Table 1) and revealed that the depletions were nearly complete in the face of infection. An important observation was that CD4-depleted mice had no CD4+ T cells in their lungs and CD8-depleted mice had no CD8+ T cells, but infiltrating CD8+ T cell influx still occurred in the absence of CD4+ T cells and vice versa (Fig. 1). This suggested that CD4+ and CD8+ T cells were recruited to the lungs by a mechanism independent of the other T cell subset.

During the first 7 d of infection, the yeast grew progressively in the lungs of all four groups of mice (Fig. 2). After day 7, control mice began to clear the infection from their lungs while pulmonary clearance failed to occur in CD4/CD8 double-depleted mice, consistent with the results obtained using SCID and nude mice (14a). In addition, neither CD4- nor CD8-depleted mice cleared the infection from their lungs, demonstrating that both T cell subsets were required for effective immune pulmonary clearance.

Prevention of extrapulmonary dissemination and/or control of growth of the organism in the brain and spleen also required both CD4+ and CD8+ T cells (Fig. 3). CD4-depleted mice had significantly greater brain CFU at 21 and 35 d post-IT than control mice (Fig. 3A and Table 2). At day 35, CD8- and CD4/8-depleted mice also had significant dissemination to the brain. In the spleen, dissemination oc-

### Table 1. Flow Cytometric Analysis of Lymphocyte-gated Spleen Cells from in Vivo T Cell-depleted Infected Mice

| Group | Marker | 1-2 wk | 3-4 wk | 5 wk |
|-------|--------|--------|--------|------|
| Control | CD4 | 25.6| 25.5| 23.7 |
| | CD8 | 10.1| 11.2| 11.3 |
| | Thy-1 | 37.7| 51.8| 50.1 |
| | Ig | 38.4| 48.0| 46.4 |
| CD4- | CD4 | <1| 4.2| <1 |
| | CD8 | 17.6| 13.1| 11.4 |
| | Thy-1 | 18.4| 16.1| 15.7 |
| | Ig | 40.2| 41.8| 62.8 |
| CD8- | CD4 | 30.1| 29.4| 29.2 |
| | CD8 | 4.9| 2.6| 4.9 |
| | Thy-1 | 35.5| 31.0| 46.0 |
| | Ig | 40.4| 32.5| 38.5 |
| CD4-/8- | CD4 | 1.2| 4.0| 4.1 |
| | CD8 | <1| <1| 2.4 |
| | Thy-1 | 4.0| 2.7| 7.4 |
| | Ig | 29.1| 57.3| 65.8 |

Pooled results from mice treated in parallel with mice analyzed in Figs. 2-4.

* Same as in Fig. 1.

† Percent positive after subtracting out background staining.

### Figure 1. Quantitation of the CD4+ and CD8+ infiltrating lung lymphocytes in infected, T cell-depleted mice 21 d post-IT. Groups: control, mice treated with sterile dialyse PBS; CD4-, mice treated with mAb GK1.5; CD8-, mice treated with mAb YTS169.4; CD4-/8-, mice treated with both mAbs GK1.5 and YTS169.4. Data shown are from an analysis of a pooled sample from five (control and CD4-/8-) or seven (CD4+ and CD8+) mice for each group.
Days Post-IT Inoculation

Figure 2. Pulmonary clearance of C. neoformans 52D in control and T cell-depleted mice. The groups are the same as in Fig. 1. Error bars have been omitted for clarity. CFUs were detected in all animals studied. Statistics: (*) \( p < 0.008 \) compared with control group. 

Table 2. Incidence of Dissemination of C. neoformans 52D in Control and T Cell-depleted Mice

|     | Brain | Spleen |
|-----|-------|--------|
|     | Control | CD4- | CD8- | CD4-/8- | Control | CD4- | CD8- | CD4-/8- |
| Day 7 | 3/17 | 2/10 | 2/10 | 2/10 | 7/17 | 6/10 | 7/10 | 6/10 |
| Day 21 | 4/22 | 11/18 | 4/17 | 5/15 | 19/22 | 18/18 | 13/17 | 11/15 |
| Day 35 | 1/17 | 6/10 | 6/10 | 6/10 | 7/17 | 7/10 | 7/10 | 6/10 |

Cryptococcal-infected mice has also been described by others (23, 24). Some single, encapsulated yeast were observed within macrophages in the lungs. Overall, relatively few cryptococci were present compared to the number of inflammatory cells.
Immunohistochemistry of the lungs revealed CD4+ and CD8+ T cells scattered throughout the inflammatory exudates (data not shown), with CD4+ T cells outnumbering CD8+, consistent with the 3:1 ratio of CD4+ to CD8+ T cells found by flow cytometry (data not shown).

Inflammatory cells were sparse in the lungs of CD4/8 double-depleted mice (CD4-8-) (Fig. 5 B). An occasional small focus of macrophages was observed, but as expected, a lymphocytic influx was absent and little or no perivascular cuffing occurred. Large masses of cryptococci filled many of the airspaces.

In CD4-depleted mice (CD4-8+), inflammation was intermediate between that seen in the lungs of the control and double-depleted mice (data not shown). Macrophages, neutrophils, and lymphocytes were present in the inflamed tissue, and perivascular cuffs were also observed. There were large masses of encapsulated yeast throughout the lungs with few inflammatory cells inside the cryptococcal masses. Immunohistochemistry confirmed the depletion of CD4+ T cells while CD8+ T cells were scattered throughout the tissue often adjacent to masses of cryptococci (data not shown).

Similar to CD4-depleted mice, the inflammatory response in the lungs of CD8-depleted mice (CD4+8-) was intermediate between control and double-depleted mice and consisted of macrophages, neutrophils, and lymphocytes (data not shown). Perivascular cuffing occurred, but the major difference between the two groups of single T cell-depleted mice was the distribution of cryptococci in the tissue. Numerous organisms were present and many appeared to be encapsulated single cells engulfed by macrophages. As expected, im-

Figure 5. Photomicrograph of the lungs from C. neoformans 52D-infected, T cell-depleted mice 11-14 d post-IT. (A) Control, (B) CD4/8-depleted (x200), hematoxylin and eosin. The alveoli in B appear larger because they have been distended by the cryptococcal polysaccharide.
munohistochemistry demonstrated numerous, CD4+ with only a rare CD8+ T cell in the inflammatory influx (data not shown).

Discussion

There are three major observations in the current study. First, both CD4+ and CD8+ T cells were important in the clearance of the pulmonary infection caused by intratracheally administered C. neoformans 52D. Second, histologic studies demonstrated that at the time clearance was occurring, the maximal influx of nonlymphoid inflammatory cells accumulated only in animals that contained both CD4+ and CD8+ T cells. Third, both CD4+ and CD8+ T cells played a role in the degree of infection detected in extrapulmonary tissues, i.e., spleen and brain. In regard to this last point, prevention of dissemination and dissemination could not be distinguished in these experiments. The assay, brain or spleen CFU, is a measure of the sum of these two processes. However, since splenic dissemination occurred even in control animals but was then cleared, it seems likely that extrapulmonary T cell–mediated defenses function in our model to clear the yeast from the extrapulmonary organ after dissemination has occurred.

An important observation was that both CD4+ and CD8+ T cells were required for effective pulmonary clearance. Mody et al. (5) did not see evidence of CD4+ T cell involvement in pulmonary clearance after intratracheal inoculation. However, the strain of C. neoformans used in those studies (145A) grows progressively even in the lungs of immunocompetent mice and ultimately results in a fatal disease (5, 25, 26). The effect of CD4 depletion was to decrease systemic clearance and survival in an already fatal infection model (5). The major difference between the 145A and the 52D cryptococcal infection models is that a 52D infection is resolved in immunocompetent mice (14a). Thus, the 52D strain is clearly a more relevant strain to use for studying defense mechanisms during opportunistic pulmonary infections.

Another important observation is that CD8+ T cells are necessary for clearance of a C. neoformans 52D pulmonary infection. Although proliferation of human CD8+ T cells has been observed in vitro after culturing PBLs, with heat-killed cryptococci (27), CD8+ T cells have not been documented to be involved in protective anticytotoxic CMI. There are two likely mechanisms for CD8+ T cell–mediated protection.

First, CD8+ T cells might be involved in recruiting effector cells to the site of infection. The nonlymphocytic cellular infiltrate in the lungs of single subset–depleted animals was intermediate between that seen in control and CD4/8 double-depleted animals. In lymphocytic choriomeningitis virus–infected mice, activated CD8+ T cells alone can mediate a DTH reaction by recruiting monocyte/macrophages, other activated or “resting” CD8+ T cells, but not CD4+ T cells into the cerebrospinal fluid of infected mice (28). The secreted cytokine profile of activated CD8+ T cells is very similar to the profile of CD4+ Th1 cells, the classic mediators of DTH. Both of these T cell types can secrete IFN-γ, GM-CSF, IL-3, TNF-α, and TNF-β, although CD4+ T cells produce higher levels of these cytokines after in vitro stimulation (29, 30).

Second, CD8+ T cells may lyse unactivated macrophages containing phagocytized cryptococci allowing activated effector cells to phagocytize and kill the yeast. Macrophages from many tissue sources, including alveolar macrophages, can phagocytize C. neoformans in vitro (31–34). However, once the yeast is engulfed it may not be killed unless the macrophage has previously been activated (35, 36). In one in vivo study, murine liver macrophages initially ingested cryptococci injected intravenously, but did not kill the engulfed yeast (37). A granulomatous reaction developed at the time cryptococci were eliminated. This response did not occur in nude mice, rather, a cystic lesion full of organisms developed. Some reports suggest that C. neoformans can replicate within unactivated macrophages (31, 33); others dispute this notion (32). Nevertheless, encapsulated cryptocoeci can survive inside phagocytes (31–36) potentially protected from other cryptocidal phagocytes, e.g., activated macrophages and neutrophils. Circumstantial evidence for a macrophage lytic role for CD8+ T cells relates to the histologic sections. In infected CD4+8− mice, a large number of apparently engulfed-but-intact encapsulated cryptococci were observed in tissue sections of the lungs (data not shown). In contrast, only a few engulfed encapsulated yeast were seen in lung sections from CD4+8+ mice and almost none were seen in CD4−8+ mice. A scenario that could account for the difference in phagocytosis from the different groups of infected, T cell–manipulated mice is that CD4+ T cells activate phagocytic macrophages for cryptocidal activity while CD8+ T cells lyse unactivated phagocytes that have taken up yeast but cannot kill them, thus releasing organisms to be engulfed and killed by activated phagocytes.

Similar phagocyte lysis models have been proposed for infections by other microorganisms including listeria and mycobacteria (38). The common feature of these latter two infections is that both bacteria are facultative intracellular parasites of macrophages. Ag-specific, MHC-restricted, and non-restricted CD8+ T cells that are cytolytic for mycobacteria-exposed macrophages are generated during a mycobacterial infection (39, 40). Similarly, CD8+ cytolytic T cells specific for listeria-infected macrophages can also be isolated from listeria-infected mice (41–43). In several studies of listeria-infected mice, CD8+ T cells were totally responsible for protective immunity (44–48). In one study, adoptive transfer of listeria-immune splenic T cell subsets demonstrated that in mice given CD4+ T cells, phagocytes accumulated at the sites of infection but the mice could not resolve the infection. In listeria-infected mice who received CD8+ T cells, phagocytes did not accumulate but they resolved the infection. These studies suggest that CD8+ T cells depleted phagocytes that harbored but did not kill the bacteria (44).

During a cryptococcal infection, therefore, CD8+ T cells may have two roles in protective immunity: first, to lyse cryptococcus-laden phagocytes, and second, to recruit resident phagocytes to the site of infection. However, CD4+ T
cells, by virtue of their increased cytokine production relative to CD8\(^+\) T cells, may be more effective at recruiting and activating effector phagocytes from the circulation. As a result of these synergistic interactions, both CD4\(^+\) and CD8\(^+\) T cells would be necessary for pulmonary immunity. Further experiments will determine whether CD8\(^+\) T cells have cytotoxic activity as proposed in this model. In terms of relevance to cryptococcosis in humans, it is interesting to note that pulmonary cryptococcosis is rare in patients with AIDS. CD8 levels are normal or even elevated until relatively late in AIDS. Since CD8\(^+\) T cells can be involved in mediating pulmonary clearance of \textit{C. neoformans} in mice, CD8\(^+\) T cells in the lungs of AIDS patients may continue to provide the signals necessary for activation of effector cells to control a low level pulmonary cryptococcal infection. CD4\(^+\) T cells may be more important in recruiting and activating effector cells to eliminate cryptococci that have disseminated to nonreticuloendothelial organs of the body such as the brain.

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