CD4+ T Cells Cause Multinucleated Giant Cells to Form around Cryptococcus neoformans and Confine the Yeast within the Primary Site of Infection in the Respiratory Tract

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Summary
The possible mechanisms by which CD4+ T cells prevent the dissemination of Cryptococcus neoformans from the primary site of infection in the respiratory tract were examined. It was found that even before fungicidal mechanisms are fully induced in the lungs, the host generates a CD4+ T cell–dependent inflammatory response that sequesters yeast within the pulmonary alveoli. This confinement is evident histopathologically and demonstrable objectively as a rapid decline in the ability to dislodge yeast from the lungs by bronchopulmonary lavage. One striking component of this response is the enclosure of cryptococci within multinucleated giant cells in granulomas. Studies in severe combined immunodeficient mice that were engrafted with selected lymphocyte subpopulations show that B cells, and hence anti-Cryptococcus antibodies, are not necessary for the CD4+ T cell–dependent responses that isolate and subsequently destroy this opportunistic pathogen in the lung parenchyma.

Meningoencephalitis caused by Cryptococcus neoformans is a common opportunistic infection of AIDS patients (1). Presumably because of their CD4+ T cell deficiency, infected individuals fail to prevent the yeast from disseminating from the primary site of infection in the lung and establishing metastatic foci in the brain. In support of clinical findings are the results of experimental studies in T cell–depleted mice, which show that while both CD4+ and CD8+ T cells help to eliminate the yeast from the respiratory tract, CD4+ T cells appear to be very important in preventing this pathogen from disseminating to the brain (2-4).

How CD4+ T cell–mediated immunity protects the host against systemic disease is not known. On the one hand, such immunity may function by killing yeast cells that enter the circulation. On the other hand, it has been speculated (2) that CD4+ T cells could function locally within the lung to prevent viable organisms from gaining access to the vasculature in the first place. The results presented in this paper support the latter hypothesis by showing that even before fungicidal mechanisms are expressed, the T cell–competent host generates a CD4+ T cell–dependent response that leads to the sequestration of the still viable cryptococci within the pulmonary alveoli. This is demonstrable as a rapid decline in the ability to dislodge viable yeast cells from the alveoli by bronchoalveolar lavage. One component of the CD4+ T cell–dependent response is the formation of multinucleated giant cells (MGC). MGC probably develop from “histiocyte rings” (5), in which encapsulated organisms too large to be ingested by alveolar macrophages become surrounded by host cells in the inflammatory exudate (6).

Materials and Methods
Mice. BALB/cJ and C.B-17/Smn mice were obtained from the Trudeau Institute Animal Breeding Facility and were maintained in standard wired-topped cages, with food and water provided ad libitum. C.B-17-scid/scid (SCID) mice were produced at the Trudeau Institute from foundation stock obtained from Dr. Leonard Schultz (The Jackson Laboratory, Bar Harbor, ME). When established as a breeding colony, the SCID mice were endogenously infected with Pneumocystis carinii (PC), making them unsuitable for use in reconstitution studies. It had been shown (7) that when given an infusion of purified T cells, PC-infected SCID mice develop a severe acute pneumonia resulting from an immune response against their large PC lung burden. A PC-free subcolony of SCID mice was therefore established using SCID mice reconstituted intravenously with 5 × 107 C.B.-17 spleen cells. Breeders were rested for 3 wk to allow them to clear PC from their lungs (8) before being mated in enclosed micro-isolator cages (Lab Products, Maywood, NJ).

1 Abbreviations used in this paper: MGC, multinucleated giant cells; PC, Pneumocystis carinii; TLB, total lung burden.
Depletion of Lymphocyte Subsets. The hybridoma GK1.5 (Dr. Frank Fitch, University of Chicago) secreting rat IgG2b L3T4 (CD4-) mAb, and the hybridomas 30-H12 and TIB-210 (American Type Culture Collection, Rockville, MD) producing IgG2b Thy-1.2 and Ly-2.2 (CD8-) mAbs, respectively, were grown as ascites in pristane-primed, irradiated BALB/c mice (3). The rat IgG2b content of ascites was quantitated by ELISA, and the mAbs were stored at -70°C until needed. To deplete CD4+ or CD8+ T cells, mice were injected intravenously with 1 mg of the appropriate mAb 1 wk before inoculating them with C. neoformans, as previously described (3). Mice were treated again every 10 d thereafter with an additional 500 μg of mAb. Control mice received an equivalent amount of normal, affinity-purified rat IgG (ICN Immunobiolog-}

als, Lisle, IL).

To purify populations of T cells for infusion into SCID mice, C.B-17 spleen cells were depleted of Ia+ cells (macrophages, B cells) by in vitro treatment with 23-16-82 mAb (9). The 23-16-82 hybridoma, which secretes I-Aβ mAb crossreacting against I-Aκ, was obtained from the American Type Culture Collection (HB35) and used in conjunction with rabbit C (10). In some experiments, purified T cells were infused into mice that had been injected intravenously 1 h earlier with CD8 or CD4 mAbs, to prevent the engraftment of the corresponding T cell subpopulation. Mice so treated are referred to as “4” or “8” mice, respectively, in the tables.

The efficiency of both T cell depletion and reconstitution was determined by flow cytofluorometric analysis of cells obtained from the blood and alveolar exudate, as described (3). Data are expressed either as the percentage or as the total number of Thy-1.2+, CD4+, CD8+, and Ig+ cells.

Cryptococcus neoformans. Serotype A, strain 184 (11), was main-
tained as already described (3). All mice were infected intratracheally with 10⁶ C. neoformans. At various times after inoculation, four or five mice from each experimental group were killed with halothane vapor and the lungs lavaged via the trachea with five 1.0-ml volumes of Ca²⁺- and Mg²⁺-free HBSS-EDTA (3). To pre-
serve the integrity of the host cell/yeast aggregates thus obtained from infected lungs, the lavage samples were not triturated or washed before loading them into the cytocentrifuge. Cytocentrifuge slides were stained with Diff-Quik (3).

To enumerate viable yeast (CFU) in the lungs and brains, homogenized tissues and samples of lavage fluid were diluted and plated on Sabouraud’s agar (5). The total lung burden (TLB) of yeast was calculated by adding the CFU in the lung lavage fluid to the number in the tissue homogenate. The numbers (log₁₀) of viable C. neoformans are expressed as the mean (± SD) of four or five mice. Samples were compared with the Student’s t test.

Lung Histopathology. Mice were killed by halothane gas and the lungs were fixed in situ by infusing 10% neutral-buffered formalin into the trachea under a constant 15 cm of fluid pressure. After 15 min, the trachea was tied off and the inflated lungs and the heart were removed en bloc and immersed in fixative. The following day, tissues were rinsed in tap water and stored in 70% ethanol. Tissues were embedded in paraffin, and sections were stained as described in Results and photographed with a Nikon MicroPhot-Fx microscope.

Results

Normal T Cell-competent Mice Rapidly Sequester C. neoformans within the Lung. Fig. 1 shows changes, with time, in the total lung burden (A) and the number of viable yeast that can be lavaged from the lungs (B) of infected mice. It can be seen that during the first 3 wk after the instillation of 10⁶ yeast cells into the trachea, there was no change in the number of viable cryptococci in the lungs. Only thereafter did mice acquire the capacity to eliminate the organisms from the site of infection. However, the number of cryptococci that could be washed out of these lungs began to decline almost immediately after depositing the organisms in the respiratory tract. Thus, while it takes 3 wk for fungicidal mechanisms to be induced, the host rapidly confines the yeast within the lung parenchyma. This is illustrated in Fig. 1 C, where the fraction of the TLB that can be washed from the lungs is plotted against time of infection.

Fig. 1 also shows the fate of C. neoformans in the lungs of mice depleted of CD4+ T cells by anti-CD4 mAb treatment. There was a 1-wk delay in the onset of elimination of the yeast and the rate of elimination is retarded compared with normal controls. Moreover, the fraction of the TLB that could be lavaged from the lungs did not decline as it did in controls, but actually increased with time. This indicates that the early host response that confines C. neoformans within the lungs of normal mice is dependent on CD4+ T cells. This conclusion is supported by the data presented in Table 1, which show the calculated fraction of the TLB that can

| Group      | TLB*          | Lavage fraction |
|------------|---------------|-----------------|
| Normal     | 5.66 ± 0.51   | 0.018 ± 0.011   |
| CD4 depleted | 5.63 ± 0.08   | 0.258 ± 0.012   |
| CD8 depleted | 5.40 ± 0.41   | 0.073 ± 0.051   |
| SCID       | 5.39 ± 0.24   | 0.281 ± 0.120   |

* TLB expressed as Log₁₀ viable C. neoformans.
† Mean(± SD) of five mice.

Table 1. Fraction of the TLB of C. neoformans Washed from the Lungs of Normal and T Cell-deficient Mice at 3 wk of Infection
Table 2. Distribution of C. neoformans among Host Cells Washed from the Lungs of Normal and T Cell-deficient Mice at 3 wk of Infection

| Group      | Macrophages | MGC     | Extracellular |
|------------|-------------|---------|---------------|
| Normal     | 43.7 ± 29.9 | 56.3 ± 30.0 | <0.1*         |
| CD4 depleted | 12.7 ± 4.8  | <0.1    | 79.3 ± 11.3   |
| CD8 depleted | 27.7 ± 22.0 | 33.0 ± 8.1 | 39.3 ± 10.0   |
| SCID       | 31.0 ± 6.6  | <0.1    | 68.5 ± 6.6    |

* None seen in cytocentrifuge smears.

Table 3. An Infusion of Thymocytes or Spleen Cells Engrafts CD4+ and CD8+ T Cell Subpopulations in C.B-17-scid/scid (SCID) Mice and Protects the Recipients against a Pulmonary C. neoformans Infection

| Mice        | Cells infused | Percentage of PBMC at 6 d | No. in alveolar exudate at 6 wk | Log10 viable C. neoformans at 6 wk |
|-------------|---------------|---------------------------|---------------------------------|-----------------------------------|
|             |               | Thy+  CD4+  CD8+  Ig+     | Thy+  CD4+  CD8+  Ig+           | Lung  Brain                        |
| SCID        | None          | 13.5  0.6  <0.1  <0.1     | 3.1  <0.1  <0.1  <0.1           | 6.16  0.15  3.52  0.34             |
| SCID        | Spleen cells  | 34.0  28.6  2.3  33.3     | 23.0  21.0  1.6  0.6            | <2.48  <1.48                       |
| SCID        | Thymocytes    | 13.7  5.4  0.3  0.0       | 14.0  11.0  2.2  <0.1           | <2.48  <1.48                       |
| C.B-17      | None          | 54.3  47.3  6.4  24.0     | 39.0  22.0  15.6  1.8           | <2.48  <1.48                       |

8-10-wk-old SCID mice were infused intravenously with 5 × 10⁷ thymocytes (98% Thy+ 0.1% Ig+) or 5 × 10⁷ C.B-17 spleen cells (24.4% Thy+ 22.6% CD4+ 4.1% CD8+ 59.1% Ig+). 6 d later, blood samples were taken from the tail artery and pooled. PBMC were isolated on density gradients and analyzed by flow cytofluorometry. The following day, the recipients along with a group of normal C.B-17 mice were infected intratracheally with 10⁶ C. neoformans. At 6 wk of infection, all mice were killed. Cytocentrifuged analyses were performed on alveolar exudate cells (3).

* Viable yeast (CFU) in the lungs and brains of individual mice were enumerated by plating aliquots of homogenized tissue on agar. Yeast burdens are expressed as the mean (± SD) of four mice. In this experiment, the detection limit was 300 (2.48 logs) and 30 (1.48 logs) in the lungs and brains, respectively.
Figure 2. Photomicrographs of foci in the lungs at 3 wk of infection. (A) Section through normal lung showing alveolar exudate cells organized into granulomas (hematoxylin-eosin) (×200). (B) Twofold enlargement of A showing yeast cell in an alveoli surrounded by a histiocyte ring (×400). (C) Section through normal lung stained with PAS to show yeast cells within intraalveolar granulomas (×200). (D) Focus of infection in lung of CD4⁺ T cell-depleted mouse showing lesion consisting of diffuse accumulations of alveolar macrophages (hematoxylin-eosin) (×200).
Figure 3. Changes with time of infection in the composition of the alveolar exudates obtained from the lungs of normal mice by bronchopulmonary lavage. Each point represents the mean of five mice. Mac, alveolar macrophages; PMN, polymorphonuclear leukocytes.

Cells or spleen cells obtained from normal C.B-17 donors endows SCID mice with the capacity to eliminate the yeast from the lung. In Table 3 it can be seen that T and B lymphocytes were present in the blood of SCID mice within 6 d of infusing spleen cells. At 6 wk of the infection, when the mice were killed to enumerate viable cryptococci, the numbers of CD4⁺, CD8⁺, and Ig⁺ cells obtained from the alveoli were comparable with those found in normal C.B-17 mice. Table 3 also shows that an infusion of thymocytes, on the other hand, reconstituted the CD4⁺ and the CD8⁺ T cell populations only.

Despite differences in their B cells status, both reconstituted groups were protected against pulmonary Cryptococcus infection. While >10⁶ yeast were present in the lungs of uninfused SCID mice and the infection had disseminated to the brain, no yeast were detected at either site in the reconstituted mice. It is therefore concluded that B cells are not necessary for the elimination of C. neoformans from the respiratory tract of SCID mice.

To examine the capacity of CD4⁺ vs. CD8⁺ T cells to protect SCID mice, the two T cell subpopulations were independently engrafted by infusing normal splenic T cells into recipients that had been injected 1 h earlier with either CD4 or CD8 mAb. Table 4 shows that both T cell populations were capable of partially restoring anti-Cryptococcus immunity. However, as judged by the amount of protection conferred, CD4⁺ T cells were significantly better than CD8⁺ T cells in reducing the yeast TLB. It was also found that only mice engrafted with CD4⁺ T cells sequestered yeast in the alveoli. It deserves mention, in addition, that MGC were found only in the alveolar exudates of SCID mice whose CD4⁺ T cell subpopulation had been restored. The results

| Mice     | mAb treatment | Cells infused | Cells engrafted | TLB*   | Fraction of TLB in Lavage | Log₁₀ Protection | p  ≤ |
|----------|---------------|---------------|----------------|--------|---------------------------|-----------------|------|
|          |               |               |                |        |                           |                 |      |
|          |               |               | Thy⁺ CD4⁺ CD8⁺ Ig⁺ |        |                           |                 |      |
|          |               |               | × 10⁴           |        |                           |                 |      |
| C.B-17   | None          | None          | 45.0 40.0 5.2 2.6 4 + 8 + B | 1.91 ± 0.75 | <0.001¹ | 4.36 | 0.001 |
| SCID     | None          | None          | 8.1 <0.4 <0.2 <0.2 | –     | 6.27 ± 0.21 | 0.190 ± 0.050 | –    |
| SCID     | None          | Whole spleen  | 22.0 19.0 2.3 2.5 4 + 8 + B | 2.42 ± 0.26 | <0.001 | 3.85 | 0.001 |
| SCID     | None          | Enriched T cells | 26.0 20.0 5.5 <0.9 | 4 + 8 | 2.48 ± 0.71 | 0.011 ± 0.004 | 3.79 | 0.001 |
| SCID     | Anti-CD8      | Enriched T cells | 27.0 26.0 <0.7 <0.2 | 4     | 4.62 ± 0.49 | 0.001 ± 0.001 | 1.65 | 0.02 |
| SCID     | Anti-CD4      | Enriched T cells | 58.0 <0.8 50.0 <0.1 | 8     | 5.21 ± 0.18 | 0.310 ± 0.120 | 1.06 | 0.001 |

8-10-wk-old SCID mice were treated with CD4 or CD8 mAbs and then given an infusion of whole C.B-17 spleen cell populations or a splenic equivalent of cells that had been enriched for T cells by anti-Ia (+ C) treatment in vitro. 1 wk later, the recipients along with a group of normal C.B-17 controls were infected intratracheally with 10⁶ C. neoformans. All mice were killed 6 wk later, at which time flow cytometric analyses were performed on alveolar exudates and tissue burdens of viable yeast determined (3).

* TLB at 6 wk. Mean ± SD of five mice.

¹ Log₁₀ protection = Log₁₀ viable yeast in lung of uninfused SCID mice – Log₁₀ viable yeast in reconstituted SCID mice, as per reference (10).

² No viable yeast were present in the lavage fluid.

³ 5 × 10⁷ whole spleen cells containing 1.4 × 10⁷ Thy-1⁺, 1.1 × 10⁷ CD4⁺, 2.6 × 10⁶ CD8⁺, and 2.6 × 10⁷ Ig⁺ lymphocytes.

⁴ 2.9 × 10⁷ whole spleen cells containing 1.6 × 10⁷ Thy-1⁺, 1.5 × 10⁷ CD4⁺, 3.5 × 10⁶ CD8⁺, and 1.0 × 10⁷ Ig⁺ lymphocytes.
of these reconstitution studies are thus in agreement with the results of our studies of normal mice selectively depleted of T cell subpopulations. They show that the early host response that focuses MGC at the site of infection and thereby helps to confine C. neoformans to the lung is dependent on CD4+ T cells.

Discussion

As the numbers of CD4+ T cells decline in AIDS patients, opportunistic C. neoformans infections cause significant morbidity and mortality (12). Given that C. neoformans enters the host via the respiratory tract, it needs to be explained how the loss of CD4+ T cells leads to the dissemination of the yeast from the lung to extrapulmonary sites via the blood. Perhaps in the immunocompetent host, CD4+ T cell–mediated immunity causes the destruction of C. neoformans after yeast cells have gained access to the systemic circulation. However, experimental evidence argues against this possibility, in that studies from several laboratories (2, 13–15) have shown that normal, CD4+ T cell–competent mice succumb to meningoencephalitis when small numbers of yeast cells are introduced directly into the vasculature.

Another possibility is that CD4+ T cells may prevent the development of systemic disease by blocking the yeast’s access to the systemic circulation (2). The results presented in this paper support this proposition by showing that a CD4+ T cell–mediated granulomatous response serves to sequester C. neoformans within the lung alveoli. The confinement of the yeast cells within granulomas precedes the decline in the number of viable cryptococci and thus must serve to immobilize the organisms in foci of infection until local fungicidal mechanisms fully develop. The response begins as macrophages and lymphocytes accumulate in the alveoli. While apparently some yeast cells are phagocytosed by macrophages, larger, thickly encapsulated organisms are surrounded by host cells, giving rise to “histiocyte rings” (6). Under the influence of CD4+ T lymphocytes, alveolar macrophages and associated yeast become organized into discrete granulomas containing MGC.

Regarding the subsequent destruction of the yeast, there is published evidence that some damage occurs while cryptococci are extracellular (16, 17). However, given the observation that yeast accumulate within multinucleated giant cells during the first 3 wk, the potential role of MGC in protective immunity needs to be examined. Commonly found at sites of Cryptococcus infection (6, 18, 19), MGC probably emerge from the fusion of macrophages (16). While it has always been assumed that lymphocytes and their soluble products play a role in MGC formation (20), this paper provides strong evidence that the process is mediated by T cells of the CD4+ subset.

Because of an autosomal recessive mutation, SCID mice lack immunocompetent T and B cells in their peripheral lymphoid organs (21). The results presented here confirm that susceptible SCID mice can be rendered resistant to pulmonary C. neoformans infection by an infusion of T cells obtained from immunocompetent donors (22). We furthermore show that an infusion of thymocytes, which reconstitutes only T cell populations in SCID mice, is as effective as an infusion of spleen cells, which reconstitutes both T and B cells, in protecting SCID mice from infection. It is almost certain, therefore, that B cells, and thus Cryptococcus antibodies, are not necessary in the protective host responses that confine and subsequently destroy this opportunistic pathogen at the site of primary infection.

Reconstituted SCID mice can provide a model with which the CD4+ and the CD8+ T cell components of the host response can be separately studied in vivo. Although others (23) have been unable to engraft small numbers (<5 × 10⁴) of postthymic CD8+ T cells in SCID mice, results presented here, where infected SCID mice were reconstituted with a larger splenic equivalent number of cells, demonstrate that the CD8+ T cell as well as the CD4+ T cell compartments can be established independent of each other. Similar findings have been reported by Harmsen and Stankiewicz (8).

Over a 6-wk test period, SCID mice reconstituted with CD8+ T cells expressed less resistance than mice reconstituted with CD4+ T cells. Although this could be related to the number of cells infused, a simple explanation is that Cryptococcus-induced CD8+ T cells lack some protective functions. It is pointed out that while rings of host cells surrounded yeast in the lungs of CD4+ T cell–depleted mice, MGC did not form in these animals. This could be indicative of a critical deficiency among CD8+ T cells to produce IL-3 or IL-4 (24), or IFN-γ (25), which have been shown in vitro to mediate macrophage fusion.

The significance of the findings presented will lie in the identification of the cytokines produced at the site of infection and the ultimate development of cytokine immunotherapy for disseminated disease (26). It should also be mentioned that our results have implications with regard to the reliability of bronchoalveolar lavage in the diagnosis of pulmonary Cryptococcus infections in AIDS (27). Inasmuch as our results show that CD4+ T cells cause this pathogen to be confined within the lung parenchyma, it may be difficult to wash cryptococci from infected lungs until CD4+ T cell–mediated immunity decays. A patient whose lavage culture is negative may be infected nonetheless and may be a candidate for antifungal chemotherapy.
Figure 4. Photomicrographs of C. neoformans obtained from the alveoli of mice by bronchopulmonary lavage (×1,000). (A) C. neoformans within alveolar macrophages obtained from normal mice; (B) encapsulated, extracellular yeast surrounded by host cells obtained from the lungs of normal mice. Note the heterogeneity of the host cells that comprise the ring; (C) yeast within MGC obtained from the lungs of normal mice; (D) encapsulated, extracellular yeast surrounded by host cells obtained from the lungs of SCID mice. Note that the ring is composed exclusively of alveolar macrophages.
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