Immunologic Homeostasis during Infection: Coexistence of Strong Pulmonary Cell-Mediated Immunity to Secondary Cryptococcus neoformans Infection While the Primary Infection Still Persists at Low Levels in the Lungs

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Immunologic Homeostasis during Infection: Coexistence of Strong Pulmonary Cell-Mediated Immunity to Secondary Cryptococcus neoformans Infection While the Primary Infection Still Persists at Low Levels in the Lungs

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Maintenance of immunity to persistent pathogens is poorly understood. In this study, we used a murine model of persistent pulmonary fungal infection to study the ongoing cell-mediated immune response. CBA/J mice with low-level persistent Cryptococcus neoformans infection had CD4+ T cells of effector memory phenotype present in their lungs. Although unable to eliminate the primary infection to sterility, these mice displayed hallmarks of immunologic memory in response to rechallenge with C. neoformans: 1) the secondary cryptococcal challenge was controlled much more rapidly, 2) the inflammatory response developed and resolved more rapidly, 3) CD4+ T and CD8+ T cell responses were higher in magnitude, and 4) effector cytokine production by T cells was greatly enhanced. Depletion of CD4+ T cells at the time of secondary challenge adversely affected clearance of C. neoformans from the lungs. These results demonstrate that persistent low-level infection with C. neoformans does not impair the cell-mediated response to the fungus. Although they are relatively free of overt disease, these mice can respond with a rapid secondary immune response if the burden of C. neoformans increases. These data support the concept that immunologically healthy individuals can maintain low numbers of cryptococci that can become a nidus for re-activation disease during immunodeficient states such as AIDS. The Journal of Immunology, 2006, 177: 4652–4661.

Cryptococcus neoformans is a pathogenic fungus that causes overt disease primarily in immunocompromised patients. Evidence suggests, however, that C. neoformans exposure and infection in humans is common, if not universal. Serologic data indicate that all adults tested in an urban area, regardless of known exposure, have Abs reactive to C. neoformans (11). Additionally, the majority of children over the age of two have reactivity against a number of C. neoformans proteins (12). Although exposure to C. neoformans is common, disease is relatively rare. Data from epidemiologic studies suggest that cryptococcosis in humans is likely a disease of reactivation of latent infection (13, 14). Thus, immune mechanisms serve to protect the overwhelming majority of individuals from cryptococcosis, but immune suppression facilitates outgrowth of the pathogen.

An emergent view of microbial pathogenesis is the damage-response framework (15–17). This model views host damage as the relevant outcome of the host-pathogen interaction, rather than the traditional determinants of microbial growth versus clearance. Whether protective immunity to C. neoformans is maintained in the presence of low-level persistent infection is largely unknown. Our objective was to determine whether mice that had controlled primary C. neoformans infection, and now had a low-level persistent infection, could generate an effective cell-mediated response upon secondary exposure, and to determine the nature of the cellular response. These findings are discussed in the context of the damage-response framework.

Materials and Methods

Mice

Female CBA/J mice were obtained from The Jackson Laboratory. Mice were housed under pathogen-free conditions in enclosed filter-topped cages. Clean food and water were given ad libitum. The mice were handled and maintained using microisolator techniques, with daily veterinarian

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monitoring. Bedding from the mice was transferred weekly to cages of uninfected sentinel mice that were subsequently bled at weekly intervals and found to be negative for Abs to mouse hepatitis virus, Sendai virus, and Mycoplasma pulmonis. All studies involving mice were approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan.

C. neoformans

C. neoformans strain 52D was obtained from the American Type Culture Collection (ATCC 24067). For infection, yeasts were grown to stationary phase (48–72 h) at 35°C in Sabouraud dextrose broth (1% peptone and 2% dextrose; Difco) on a shaker. The cultures were then washed in non-pyrogenic saline (3), counted on a hemocytometer, and diluted to 3.34 × 10⁶ (or 10⁵) CFU/ml in sterile nonpyrogenic saline. The precise number of organisms delivered was determined by a CFU count of inoculum plated on Sabarua dextrose agar (Difco).

Intratracheal inoculation of C. neoformans

Mice were anesthetized by i.p. injection of ketamine (100 mg/kg; Fort Dodge Laboratories) and xylazine (6.8 mg/kg; Lloyd Laboratories), and restrained on a small surgical board. A small incision was made through the skin over the trachea and the underlying tissue was separated. A 30-gauge needle was attached to a 1-ml tuberculin syringe filled with diluted C. neoformans culture. The needle was inserted into the trachea, and 30 μl of inoculum was dispensed into the lungs. The needle was removed, and the skin was closed with cyanoacrylate adhesive. The mice recovered with minimal visible trauma.

Generation of secondary mice

CBA/J mice were infected intratracheally with 10⁶ CFU of C. neoformans strain 52D. Mice were housed in specific pathogen-free conditions with food and water given ad libitum for 12 wk to allow clearance of the primary infection. A portion of the immunized mice was characterized to determine the level of residual infection, lung inflammatory cellularity, and the presence of T cells of memory phenotype in the lungs. For secondary infections, mice and age-matched controls were then given a second intratracheal inoculation of 10⁵ CFU C. neoformans (10-fold higher than the primary inoculation).

Lung and lymph node leukocyte isolation

Lung leukocytes were isolated as previously described. In brief, lungs from each mouse were excised, washed in PBS, minced and digested enzymatically for 30 min in 15 ml of digestion buffer (RPMI 1640, 5% FCS, 1 mg/ml collagenase (Boehringer Mannheim Biochemical), and 100 units/ml DNase (Sigma-Aldrich)). Following erythrocyte lysis using NH₄Cl buffer, cells were washed, resuspended in complete medium, and centrifuged for 30 min at 2000 × g in the presence of 20% Percoll (Sigma-Aldrich) to separate leukocytes from cell debris and epithelial cells. Total lung leukocyte numbers were assessed in the presence of trypsin blue using a hemocytometer; viability was >85%. Subsets of isolated leukocytes (neutrophils, eosinophils, macrophages, and total lymphocytes) were determined by Wright-Giemsa staining of samples cytopun onto slides. Lung-associated lymph nodes (hilar and/or mediastinal nodes) were excised, and cells were dispersed with the plunger of a 3-ml syringe. Erythrocytes were lysed using NH₄Cl buffer, and cells were resuspended in complete medium.

Flow cytometry

For surface staining alone, leukocytes were washed and resuspended at a concentration of 10⁶ cells/ml in FA buffer (Difco) + 0.1% NaN₃ (Sigma-Aldrich). Fc receptors were blocked by the addition of anti-CD16/32 (Fc receptor antibody; BD Pharmingen) for 20 min at 4°C. Leukocytes were stained with the following Abs, per manufacture’s instructions: CD4 (RM-4-4 and H129.19), CD8α (5H10-1), CD8β(53-58), anti-βTCR (H57-597), CD25 (7D4), CD44 (IM7), CD45RB (16A), CD46L (MEL-14), CD69 (H1.2F3), and CD154 (MR1) (BD Pharmingen). Cells were washed twice with FA buffer, resuspended in 100 μl, and 200 μl of 4% formalin was added to fix the cells. A minimum of 20,000 events was acquired on a FACScan calibrated flow cytometer (BD Pharmingen) using CellQuest software (BD Pharmingen). For activation markers (i.e., CD25, CD44, CD46, CD62L, CD45RB), gates were set based on positive (splenocytes cultured with high-dose PMA (50 ng/ml) and ionomycin (500 ng/ml)) and negative (isotype controls).

Intracellular flow cytometry

Leukocytes were cultured for 12 h at 2 × 10⁶ cells/ml in 12-well plates in the presence of 0.1 μg/ml soluble anti-CD3 (145-2C11; BD Pharmingen) with or without 0.1 μg/ml anti-CD28 (37.51; BD Pharmingen). Brefeldin A or monensin (in the form of Golgi-stop or Golgi-block) were added for the last 4 h of culture per manufacturer’s instructions (BD Pharmingen). Nonadherent cells were harvested, washed twice with FA buffer, and staining for cell surface molecules was done as described above. For intracellular staining, cells were washed of excess surface stains, fixed, and permeabilized using Cytofix/Cytoperm (BD Pharmingen), and stained using anti-IFN-γ (XMG1.2) and anti-TNF-α (MP6-XT22) (BD Pharmingen) in permeabilization buffer (FA buffer + 0.1% saponin (Sigma-Aldrich)) at 4°C for 30 min. Flow cytometry was performed as for surface staining above, except that >50,000 events per sample were collected. The specificity of anti-cytokine Abs was tested by comparing staining of experimental samples to a minimum of two of three negative controls: 1) isotype control, 2) excess unlabeled Ab, and/or 3) preincubation of Ab with recombinant cytokine.

Lung cytokine ELISA

Lung samples were snap-frozen in liquid nitrogen and stored at −70°C until use. Frozen lungs were homogenized in buffered saline containing protease inhibitors. The concentrations of IFN-γ and TNF-α protein were determined by sandwich ELISA using commercially available kits (OptEIA; BD Biosciences).

T cell depletion using monoclonal antibodies

Depletion of CD4⁺ and CD8⁺ T cell subsets was accomplished via i.p. administration of mAbs. Anti-CD4 (GLK.1, rat IgG2b) and anti-CD8 (YTS 169.04, rat IgG2b). Abs were prepared from ascites by dilution in sterile saline and filtering through 0.45-μm syringe filter. Mice received 200 μg of each GLK.1, both YTS 169.04, or saline alone in a volume of 200 μl. Abs were administered at days −1, 0, and 4 of secondary infection. The efficiency of T cell depletion was assessed by flow cytometric analysis using Abs anti-CD4 (RM-4-4) and anti-CD8 (53-5-8), which bind regions of CD4 and CD8 distinct from GK1.5 and YTS 169.04. Efficiency of T cell depletion in the lungs (CD4⁺ >95%, CD8⁺ >92%) and spleen (CD4⁺ >99%, CD8⁺ >98%) of mice was calculated by comparison of T cell numbers in treated mice with those in controls.

C. neoformans-specific Ab assays

C. neoformans-specific Ab titers were determined by flow cytometry. Serum from uninfected and C. neoformans-infected mice was obtained from tail veins, separated via centrifugation, and frozen at −20°C until use. Heat-killed C. neoformans (HKC) ³ 52D was inactivated by incubation at 55°C for 2 h, washed three times, and resuspended in PBS at a concentration of 2 × 10⁶/ml. HKC were plated on Sabarua dextrose agar to confirm nonviability. To measure C. neoformans-specific IgG, serum was serially diluted 3-fold from 1/50 to 1/1,350. A total of 50 μl of diluted serum + 10⁶ C. neoformans cells (in 50 μl of PBS) was incubated at 37°C for 50 min. Serum Ab-coated HKC were washed once with 4 ml of PBS, and incubated with FITC rabbit anti-mouse IgG or control FITC rabbit IgG (final concentration of 1/200; Jackson ImmunoResearch Laboratories) for 20 min at 4°C. Abs-bound HKC were washed again and resuspended in 4% formalin, and analyzed by flow cytometry as described above. The mean fluorescence intensity (MFI) and median fluorescence intensity (MedFI) were calculated for anti-IgG and control Ab-stained HKC. Ab titers were scored positive when the MFI (anti-mouse IgG)-MFI (control Ig) > 1.00 and MedFI (anti-mouse IgG)-MedFI (control Ig) > 1.00.

Statistical analysis

All values are means ± SEM, unless otherwise indicated. Differences between two means were evaluated using the Student t test, (assuming unequal variance where dictated by F test) with p < 0.05 considered to be statistically significant.

³ Abbreviations used in this paper: HKC, heat-killed C. neoformans; MFI, mean fluorescence intensity; MedFI, median fluorescence intensity.
Results

Persistent pulmonary infection in mice 12 wk post-C. neoformans infection

Our first objective was to determine the level of persistent infection in CBA/J mice 12 wk after pulmonary C. neoformans infection. Mice were infected intratracheally with $10^3$ CFU of C. neoformans and housed in specific pathogen-free conditions for 12 wk to follow the clearance of C. neoformans. The fungus grew rapidly in the lungs during the first week of infection, increasing by 1000-fold (Fig. 1). Between weeks 1 and 12, the pulmonary infection was progressively cleared, and by week 12, pulmonary CFU levels were ~5000-fold lower than the levels at the peak of infection (week 1). However, at week 12, the mice had not cleared the initial pulmonary infection to the point of sterility.

Resolution of lung inflammation in mice 12 wk post-C. neoformans infection

The next objective was to determine whether mice resolve the pulmonary inflammation that accompanies the pulmonary immune response to C. neoformans infection. At the peak of inflammation during primary infection with C. neoformans (week 2), large areas of consolidation were present, and numerous Airways were occluded with inflammatory foci (Fig. 2, C and D). In comparison, the lungs of 12 wk mice were relatively clear of inflammation, although a few inflammatory foci of predominantly mononuclear cells persisted (Fig. 2, E and F). Thus, the pulmonary inflammatory response resolved as the infection in the lungs was controlled.

To identify the cells present in the small residual inflammatory foci and to provide a more quantitative measure of overall residual inflammation, we compared the numbers of total leukocytes, CD4$^+$ T cells, and CD8$^+$ T cells from the lungs of mice 12 wk postinfection with cell numbers in age-matched, unaffected mice. The numbers of total leukocytes, CD4$^+$ T cells, and CD8$^+$ T cells in the lungs at week 2 postinfection were significantly higher than cell numbers in either uninfected controls, or at week 12 (data not shown). The number of CD8$^+$ T cells in the lungs of mice at week 12 postinfection was not significantly different from in uninfected controls (Fig. 3). However, the number of CD4$^+$ T cells was significantly higher in the lungs of mice at week 12 postinfection (Fig. 3). The number of total leukocytes (CD45$^+$) was different between the two groups, but when CD4$^+$ T cell numbers were subtracted out, differences in total leukocyte cell numbers were not significant (Fig. 3). These results demonstrate that the majority of the leukocytic inflammation present in mice at week 2 postinfection had been resolved by week 12, but a small number of CD4$^+$ T cells still remained.

Fungal clearance in the primary versus secondary immune response to pulmonary C. neoformans infection

Enhanced clearance of a pathogen upon subsequent encounter is one of the hallmarks of immunity. Therefore, our next objective was to determine whether mice that had controlled a primary C. neoformans infection (secondary mice) display enhanced clearance upon challenge relative to naive mice challenged with C. neoformans (primary mice). To this end, secondary mice and age-matched naive controls were given an intratracheal inoculation of $10^4$ CFU of C. neoformans, and pulmonary fungal burden was assessed at days 4, 7, and 10 postinfection. This second intratracheal inoculation was 10-fold higher than the primary inoculation ($10^3$). We chose this inoculum because the hallmarks of an anamnestic secondary response (compared with a primary response) are that it is 1) faster, 2) more vigorous, and 3) more effective. Following primary infection, the fungus grew rapidly during the first 4 days (~100-fold), whereas growth of the fungus in secondary mice was more limited (3-fold; Fig. 4A)). However, between days 4 and 10, the pulmonary burden continued to increase in primary mice, whereas >90% of the organisms were cleared in secondary mice (Fig. 4A). Thus, mice that have controlled a primary infection clear C. neoformans far more rapidly upon a second pulmonary challenge.
FIGURE 3. Total lung leukocyte (CD45<sup>+</sup>) numbers, and CD4<sup>+</sup> and CD8<sup>+</sup> T cell numbers in mice at weeks 0, 2, and 12 post-C. neoformans infection. Pulmonary cell counts were determined from enzymatic digests of whole lungs as described in Materials and Methods. The absolute number of each subset was determined by flow cytometry. Data points represent the mean ± SEM of 6 mice. Data are from two independent experiments. †, p < 0.05 vs week 0; ††, p < 0.001 vs week 0 and week 12.

Lung leukocyte recruitment in primary versus secondary immune responses to pulmonary C. neoformans infection

Clearance of C. neoformans from the lungs during primary infection is dependent upon the T cell-dependent recruitment and activation of monocytes and macrophages (18–21). To evaluate leukocyte recruitment in primary and secondary mice, we determined pulmonary leukocyte recruitment during primary and secondary immune responses to C. neoformans. Total lung leukocyte numbers and cell differential analysis were done as described in Materials and Methods. In primary mice, little recruitment of pulmonary leukocytes occurred during the first 4 days of infection (Fig. 4B). Leukocyte recruitment was apparent in primary mice by day 7, and further increased by day 10. At day 10 postinfection, the leukocyte cellularity in the lungs of primary mice was ~4-fold that in uninfected controls (Fig. 4B). The leukocyte infiltrate in the lungs of primary mice contained significant numbers of neutrophils and eosinophils (Fig. 5C). In the secondary response, mice had a significant influx of lung leukocytes as early as day 4 postinfection, and peaking at day 7 (Fig. 4B). The increased recruitment to the lungs of immune mice at this early time point was composed of significantly larger numbers of monocytes and macrophages (Fig. 5, A and B). By day 10, total leukocyte, monocyte, and macrophage numbers in the lungs of secondary mice were similar to those in primary mice, but had significantly fewer granulocytes (Fig. 5C). Neutrophils are associated with both protective and non-protective responses to C. neoformans, and adversely affect fungal clearance (22, 23). The accelerated recruitment of monocytes and macrophages to the lungs in secondary mice was concomitant with clearance of C. neoformans (Fig. 4, A and B). Therefore, the secondary response to pulmonary C. neoformans in mice displays enhanced kinetics in the recruitment of leukocytes to the lungs, which correlates temporally with clearance of the fungus.

We also compared histological sections of lungs from the two groups at days 4, 7, and 21 postinfection. At day 4 postinfection very little leukocyte infiltrate was present in the lungs of primary mice (Fig. 6). In contrast, the lungs of secondary mice had numerous inflammatory foci (Fig. 6). These foci were concentrated between perivascular areas and airways, and consisted predominantly of mononuclear cells. At day 10 postinfection, similar levels of inflammatory foci were evident in the lungs of both primary and secondary mice (Fig. 6). However, at day 21 postinfection, there was a striking difference in the inflammatory response between primary and secondary mice. Robust inflammation was evident throughout the lungs of primary mice (Fig. 6), but pulmonary inflammation had largely resolved in secondary mice (Fig. 6). Thus, the development of the inflammatory response during the secondary response was more rapid, clearance of the infection was enhanced, and the resolution of the inflammatory response was more brisk.

CD4<sup>+</sup> and CD8<sup>+</sup> T cell recruitment kinetics in primary versus secondary responses to C. neoformans

Our next objective was to determine whether CD4<sup>+</sup> and CD8<sup>+</sup> T cells participate in the secondary response to C. neoformans. To this end, the kinetics of the pulmonary CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses in primary versus secondary responses were compared. First, we evaluated CD8<sup>+</sup> T cell responses in primary versus secondary infection. During primary infection, little recruitment of CD8<sup>+</sup> T cells occurred to the lungs during the first week of infection (Fig. 7A). By day 10 postinfection, a significant increase in the numbers of CD8<sup>+</sup> T cells were present, relative to uninfected controls (Fig. 7A). In the secondary response, similar numbers of CD8<sup>+</sup> T cells were present at the time of infection, but CD8<sup>+</sup> T cells were recruited to the lungs in higher numbers at days 4 and 7 postinfection, relative to the primary response (Fig. 7A). Conversely, at day 10 postinfection, more CD8<sup>+</sup> T cells were present in the lungs of mice undergoing a primary response (Fig. 7A). Thus, during the secondary response, CD8<sup>+</sup> T cells were recruited.

FIGURE 4. Pulmonary fungal burden (A) and total lung leukocyte numbers (B) in primary vs secondary C. neoformans infection. Pulmonary fungal burden and total lung leukocyte (CD45<sup>+</sup>) numbers were assessed as described in Materials and Methods. Data points represent the mean ± SEM of 6 mice. Data are from two independent experiments. Note that the y-axis in A is in log scale. †, p < 0.05; *, p < 0.01
to the lungs more rapidly than in the primary response, but only at early time points.

Next, we determined the kinetics of the CD4+ T cell response during primary and secondary responses. In primary mice, very little lung CD4+ T cell recruitment occurred during the first 4 days of infection (Fig. 7B). By day 7, significantly higher CD4+ T cells numbers were present in the lungs, compared with the time of infection, and continued to increase by day 10. In secondary mice, CD4+ T cells numbers in the lungs were higher at the time of infection (Fig. 7B). A significant increase in CD4+ T cells was present in the lungs of secondary mice by day 7, relative to CD4+ T cell numbers at the time of infection, and continued to increase through day 10 (Fig. 7B). Compared with primary mice, significantly larger numbers of CD4+ T cells were present in the lungs of secondary mouse at all time points (Fig. 7B). Greater than 10-fold more CD4+ T cells present in the lungs of mice during the secondary response at day 7 postinfection (Fig. 7B). Thus, during the secondary response to C. neoformans, more CD4+ T cells were present at the time of infection, and CD4+ T cells were recruited to the lungs in higher numbers than in the primary response.

Expression of activation and memory-associated markers in the lungs of C. neoformans-infected mice

Our next objective was to determine the phenotype of CD4+ T cells that were maintained in the lungs of mice that had resolved primary infection (Fig. 7A). To this end, lung T cells from mice 12 wk postinfection were stained for a variety of cell surface markers (CD44, CD69, CD62L, CD25, CD154, CD45RB) that are associated with an activated or memory phenotype. CD4+ T cells in the lungs of mice 12 wk postinfection were CD44highCD69highCD62LlowCD25-CD154-CD45RBlow (Fig. 8), consistent with an “effector memory” phenotype (despite not completely sterilizing the primary infection). It should be noted that, although high expression of CD69 is classically associated with an activated phenotype (24), a number of studies have demonstrated that T cells in non-lymphoid sites, or effector memory cells, display a more activated phenotype (25, 26). Low expression of CD25 distinguishes these cells from lung CD4+ T cells found earlier in primary C. neoformans infection (data not shown). In contrast, CD8+ T cells in the lungs of mice 12 wk postinfection were virtually indistinguishable from CD8+ T cells from uninfected control lungs, based on the expression of any of the phenotypic markers assayed (Fig. 8). Thus, CD4+ T cells, but not CD8+ T cells, that express cell surface markers consistent with an effector memory phenotype are maintained in the lungs of mice 12 wk post-C. neoformans infection.
FIGURE 7. Absolute numbers of CD4+ (A) and CD8+ (B) T cells in the lungs of mice during primary and secondary C. neoformans infection. Total lung CD4+ and CD8+ T cell numbers were assessed as described in Materials and Methods. Each time represents the mean ± SEM of 6 mice. Data are from two independent experiments. *p < 0.05 vs time of infection; †, p < 0.05 vs primary response.

Lung T cells from immunized mice express enhanced effector function in the secondary immune response to pulmonary C. neoformans infection

The next objective was to determine whether CD4+ and/or CD8+ T cell population in the lungs had enhanced effector function in the secondary response. To this end, effector cytokine production by T cells from the lungs of mice at day 4 postinfection was determined. TNF-α and IFN-γ production by T cells was assessed by intracellular flow cytometry as described in Materials and Methods. We first examined cytokine responses without in vitro restimulation. CD4+ or CD8+ T cells in uninfected lungs (1st day 0) did not produce either cytokine (Fig. 9A). CD4+ and CD8+ T cells from primary mice (1st day 4) also did not produce IFN-γ or TNF-α (Fig. 9A). Few CD4+ or CD8+ T cells from the lungs of mice 12 wk postinfection (2nd day 0) produced effector cytokines (Fig. 9A). In contrast, CD4+ T cells from mice with secondary infection (2nd day 4) produced IFN-γ or TNF-α, without in vitro restimulation (Fig. 9A). A smaller increase in CD8+ T cells producing IFN-γ was observed in mice with secondary infection (2nd day 4), relative to 12 wk mice (2nd day 0, Fig. 9A). Increased TNF-α and IFN-γ production by T cells in secondary mice translated into significantly higher levels of TNF-α and IFN-γ in the lungs of secondary mice, compared with primary mice (Fig. 9C). Thus, without in vitro restimulation, CD4+ and CD8+ T cells from the lungs of secondary, but not primary, mice produced effector cytokines.

To determine the potential for effector cytokine production in each of these groups, we also determined the cytokine responses by T cells restimulated with low dose αCD3/αCD28 Abs (0.1 μg/ml soluble, as described in Materials and Methods). Following in vitro restimulation, IFN-γ and/or TNF-α were produced by CD4+ and CD8+ T cells from uninfected lungs (1st day 0). In the lungs of primary mice (1st day 4), similar frequencies of IFN-γ- and TNF-α-positive T cells were found, relative to uninfected lungs (Fig. 9B). Although few IFN-γ+ T cells were detected in the lungs of 12 wk mice before restimulation (2nd day 0, Fig. 9A), a substantial proportion of CD8+ T cells in the lungs of 12 wk mice were IFN-γ+ following in vitro restimulation (2nd day 0, Fig. 9B). CD4+ T cells from the lungs of 12 wk mice (2nd day 0) produced IFN-γ and also produced TNF-α (Fig. 9B). In secondary mice (2nd day 4), similar frequencies of effector cytokine production by CD4+ and CD8+ T cells were found, compared with week 12 mice (Fig. 9B). These results suggest that a portion of the CD4+ and CD8+ T cells in the lungs of 12 wk mice were “primed” for effector cytokine production, but were not actively producing cytokines. Either secondary infection (2nd day 4, Fig. 9A) or TCR/CD28 restimulation (2nd day 0, Fig. 9B) resulted in effector cytokine production by T cells. Thus, effector cytokine production by T cells in the secondary response is enhanced, relative to the primary response, with a substantial contribution from T cells present in the lungs at the time of secondary challenge.

Role of CD4+ T cells in fungal clearance during the secondary immune response to pulmonary C. neoformans infection

A substantial portion of the CD4+ T cells from the lungs of immune mice had a phenotype consistent with persistently activated or effector memory cells. To determine whether CD4+ T cells play a protective role in the secondary immune response to C. neoformans, CD4+ T cells, or CD4+ and CD8+ T cells, were depleted before secondary challenge, using mAbs as described in Materials and Methods.
and Methods. Following secondary challenge, CD4+ mice were significantly more susceptible to *C. neoformans* infection than undepleted controls (Fig. 10). Additional depletion of CD8+ T cells in CD4+ mice did not increase susceptibility to *C. neoformans* growth in the lungs (Fig. 10). No dissemination to the CNS was observed in any of the groups at this time point. It is noteworthy that depletion of CD4+ T cells from mice undergoing a primary response does not lead to increased susceptibility to *C. neoformans* infection during the first week of infection but does increase susceptibility after the first week (18). These data demonstrate that CD4+ T cells are required for the accelerated clearance observed during the secondary response to pulmonary *C. neoformans* infection.

Cell surface exposed *C. neoformans* Ag-specific Ab responses during primary and secondary infection

There is accumulating evidence that humoral immunity may play a role in immunity to *C. neoformans* (27). Furthermore, enhanced clearance during secondary infection could be facilitated by Abs. To determine whether mice generated a specific Ab response to *C. neoformans*, serum *C. neoformans*-specific Abs were assayed at various time points following primary and secondary infection by flow cytometry, as described in Materials and Methods. This assay measures the titer of IgG Abs that bind to whole cryptococci, i.e., Ab titers against cell surface-exposed Ags (does not measure titers vs total cryptococcal proteins). A robust *C. neoformans*-specific IgG response was generated during primary infection (Fig. 11A). *C. neoformans*-specific IgG levels peaked at day 17 postinfection,

**FIGURE 9.** TNF-α and IFN-γ production by freshly isolated (A) and restimulated (B) lung CD4+ and CD8+ T cells from mice at day 4 post-*C. neoformans* infection. The production of TNF-α and IFN-γ was determined by intracellular flow cytometry of cultured lung leukocytes, as described in Materials and Methods. Representative dot plots are shown from a single animal. Numbers in each quadrant represent the mean of 3 animals. Similar results were obtained in two independent experiments. C. The levels of IFN-γ and TNF-α in whole lungs were determined by ELISA of lung homogenates at day 4 postinfection, as described in Materials and Methods. Bars represent mean ± SEM of 5 mice. *, p < 0.05.

**FIGURE 10.** Role of CD4+ and CD8+ T cells in pulmonary fungal clearance during secondary *C. neoformans* infection. Pulmonary fungal burden was assessed at day 7 postinfection as described in Materials and Methods. Data points represent the mean ± SEM of 6–8 mice. Data are from two independent experiments. Note that the y-axis is in log scale. *, p < 0.05.
but tended to wane over time (Fig. 11A). Following secondary infection, there was no increase in *C. neoformans*-specific IgG titer in 5 of 5 mice (Fig. 11B). Thus, primary infection results in the production of *C. neoformans*-specific IgG Abs against cell surface Ags, but IgG levels wane over time and do not increase upon secondary infection.

**Discussion**

In this study, we demonstrated that CBA/J mice harboring a low-grade persistent *C. neoformans* infection mounted a protective cell-mediated immune response upon secondary *C. neoformans* infection. Long-lived CD4+ T cells of effector-memory phenotype were present in the lungs of mice that had controlled the primary infection without sterilizing the lungs. The secondary immune response in these mice had characteristics of protective cell-mediated immunity: 1) the infection was controlled much more rapidly; 2) clearance was concomitant with more rapid recruitment of mononuclear cells to the lungs and resolution of inflammation was also more rapid; 3) the secondary CD4+ T and CD8+ T cell responses were increased in magnitude; 4) as assessed by the production of IFN-γ and TNF-α, T cell effector function in the secondary response was enhanced; 5) depletion of CD4+ T cells during the secondary response adversely affected clearance of *C. neoformans* from the lungs; 6) the secondary response was not associated with increased IgG production against cell surface exposed Ags. Thus, the presence of a chronic, low-level pulmonary *C. neoformans* infection does not prevent the manifestation of a rapid and effective secondary cell-mediated response to re-infection by *C. neoformans*.

Our results demonstrated that a high frequency of CD4+, but not CD8+ T cells expressing the phenotype CD4+highCD69highCD62LlowCD25lowCD154lowCD45RBlow were maintained in the lungs 12 wk post-*C. neoformans* infection. Recent evidence has demonstrated that a large proportion of memory T cells are maintained in nonlymphoid tissues (28, 29). Persistence of memory phenotype CD4+ and CD8+ T cells in the lungs has been previously demonstrated following recovery from respiratory virus infections (30, 31). The phenotypic profile of memory T cells in nonlymphoid tissues differs from that in lymphoid tissues. High expression of CD69 is classically associated with an activated T cell phenotype (24). However, a number of studies have found that memory T cells in the lungs are in a more activated state than memory T cells in lymphoid tissues (25, 26, 30). CD4+ T cells maintained in the lungs of mice that had recovered from primary *C. neoformans* infection displayed an intermediate activation profile, as evidenced by high CD69, but low CD25 and CD154. Low expression of CD25 distinguishes these cells from those found in the lungs at the peak of infection (32). Maintenance of T cells with an effector memory phenotype occurs during persistent *Trypanosoma cruzi* infection in mice, as well (33). Thus, CD4+ (but not CD8+) T cells maintained in the lungs of mice with persistent *C. neoformans* infection have a phenotype consistent with effector memory cells.

Significant numbers of CD4+ T cells in the lungs of secondary mice produced IFN-γ and/or TNF-α. Although low frequencies of TNF-α production were observed by lung CD8+ T cells from uninfected and primary mice following restimulation, no TNF-α production was observed from CD8+ T cells during secondary infection. The lack of TNF-α production by CD8+ T cells during secondary *C. neoformans* infection was unexpected. TNF-α production by CD8+ T cells plays a significant role in immunity to some pathogens (34). Additionally, TNF-α plays a critical role in immunity to *C. neoformans* (35, 36). However, similar functional distinctions in T cells have been reported in other infections (37, 38). In the CD8+ T cell response to *Listeria monocytogenes*, intracellular cytokine staining for IFN-γ detects higher frequencies of tetramer positive (Ag specific) cells than TNF-α (39). It is noteworthy that significant numbers of CD4+ T cells in the lungs of secondary mice produced IFN-γ or TNF-α, but few cells produced both cytokines (Fig. 8). This may be due to the cycling of cytokine production by CD4+ T cells, as has been demonstrated for CD8+ T cells (40, 41), or alternate states of differentiation (42). Our results demonstrate disparate effector cytokine production by CD4+ and CD8+ T cell during secondary pulmonary *C. neoformans* infection.

Depletion of CD4+ T cells before secondary infection resulted in increased growth of *C. neoformans* in the lungs of mice. Similar results were obtained in the other experimental system used to study the secondary response to fungi. In the secondary response to *Histoplasma capsulatum* infection, depletion of CD4+ T cells alone delays clearance of the pathogen, whereas depletion of both CD4+ and CD8+ T cells results in increased mortality via dissemination of the fungus (43). Protective roles for IFN-γ, GM-CSF, and TNF-α have been demonstrated as well (44-46). Together, these studies demonstrate that T cells, and the production of Th1 type cytokines are essential for the secondary response to fungi.
Furthermore, CD8⁺ T cells, in the absence of CD4⁺ T cells, cannot drive the clearance of *C. neoformans* during secondary exposure.

Our results demonstrated that mice with persistent low-level primary *C. neoformans* infection display immunity to subsequent pulmonary challenge. There are discordant views regarding the role of Ag in the establishment and maintenance of memory. A number of studies have demonstrated that mature T cell memory is established only after sterile clearance of the pathogen. Evidence for this view comes predominantly from acute infections (6, 47, 48). Another view (though certainly not mutually exclusive) is that maintenance of protective immunity in some circumstances depends upon the continued presence of Ag. Supporting this point of view, protective immunity to a number of persistent infections, including *Leishmania,* *Plasmodium,* and *Mycobacterium* species, is maintained poorly without the continued presence of the infectious agent (7–10, 49). Evidence suggests that subclinical or misdiagnosed *C. neoformans* infection may be more frequent that previously appreciated, particularly in urban areas (12, 50). Thus, persistent *C. neoformans* infection is a clinically relevant scenario. Altogether, these studies suggest that the loss of anti-cryptococcal cell-mediated immunity to persistent infection represents a novel mechanism of cryptococcal disease pathogenesis.

An emergent view of microbial pathogenesis is the damage-response framework (15–17). As one of its tenets, this model proposes that the host-relevant outcome of a microbial encounter is the amount of damage to the host. The states represent decreasing host benefit and increasing host damage: commensalism, colonization, latency/persistence, and disease. These states are continuous and can change over time. The coexistence of microbial persistence, immunity to that microbe and anti-inflammatory mechanisms evoked by the presence of the microbe suggests that there exists a “homeostasis threshold” for host-microbe interactions. This would be defined as the microbial burden above which inflammatory processes are evoked and predominate over anti-inflammatory processes, thereby decreasing microbial load. When the microbial burden decreases below this threshold, anti-inflammatory processes are strongly up-regulated and predominate over anti-inflammatory processes, shutting down inflammation to prevent damage to the host but also preventing complete elimination of the microbe. This threshold, however, is highly variable depending upon the nature of the pathogen, and its potential for causing damage to the host. The immunomodulatory factors produced by *C. neoformans,* including polysaccharide capsule and prostaglandins, likely decrease inflammation, thus conferring a higher tolerable threshold than other more invasive pathogens (51, 52). This “homeostasis threshold” is most likely an off-shoot of our generally peaceful coexistence with the microflora in our body (which outnumber our own cells 10:1), but may also serve as a source of Ag to maintain strong memory responses.

Our studies demonstrate that primary pulmonary *C. neoformans* infection in mice results in a disease state maintained at the level of latency/persistence, which is defined as a state in which the “microorganism persists in a host and can be associated with damage that can be measured at a cellular or tissue level, but is not associated with disease” (17). Although mice 12 wk postinfection carry low-level persistent *C. neoformans* infection, they are relatively free of overt disease and can respond with a rapid, effective secondary immune response if the burden of *C. neoformans* increases. Thus, persistent low-grade *C. neoformans* infection represents a balance between microbial growth, and an ongoing inflammatory response, either of which would result in further damage to the host if uncontrolled. These data support the concept that immunologically healthy individuals can maintain low numbers of cryptococci that can become a nidus for re-activation disease during immunodeficient states such as AIDS.

**Disclosures**

The authors have no financial conflict of interest.

**References**


