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IL-5 Is Required for Eosinophil Recruitment, Crystal Deposition, and Mononuclear Cell Recruitment During a Pulmonary *Cryptococcus neoformans* Infection in Genetically Susceptible Mice (C57BL/6)¹

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CBA/J (highly resistant), BALB/c (moderately resistant), and C57BL/6 (susceptible) mice displayed three resistance patterns following intratracheal inoculation of *Cryptococcus neoformans* 52. The inability to clear the infection correlated with the duration of the eosinophil infiltrate in the lungs. The role of IL-5 in promoting the pulmonary eosinophilia and subsequent inflammatory damage in susceptible C57BL/6 mice was investigated. C57BL/6 mice developed a chronic alveolar, peribronchiolar, and perivascular eosinophilia following *C. neoformans* infection. This resulted in the accumulation of intracellular Charcot-Leyden-like crystals in alveolar macrophages by wk 4 and the extracellular deposition of these crystals in the bronchioles with associated destruction of airway epithelium by wk 6. IL-5 mRNA was expressed in the lungs, and injections of anti-IL-5 mAb prevented eosinophil recruitment and crystal deposition but did not alter cryptococcal clearance. Depletion of CD4⁺ T cells (but not CD8⁺) ablated IL-5 production by lung leukocytes in vitro and eosinophil recruitment in vivo. Neutralization of IL-5 also inhibited the recruitment of macrophages, CD8⁺ T lymphocytes, and B lymphocytes by 47 to 57%. Anti-IL-5 mAb inhibited CD4⁺ T lymphocyte recruitment by 30% but did not affect neutrophil recruitment. Thus, the development of a chronic eosinophil infiltrate in the lungs of *C. neoformans*-infected C57BL/6 mice is a nonprotective immune response that causes significant lung pathology. Furthermore, IL-5 promotes the recruitment and activation of eosinophils, resulting in the recruitment of additional macrophages and lymphocytes into the lungs. *The Journal of Immunology*, 1998, 160: 2393–2400.

Pulmonary eosinophilia is one manifestation of a host response during pulmonary mycosis but is generally not associated with protective immunity against the infection. Pulmonary eosinophilia commonly occurs following chronic *Aspergillus* infection, a condition known as allergic bronchopulmonary aspergillosis (1). However, eosinophils have also been identified in the bronchoalveolar lavage fluid of patients infected with *Trichosporon*, *Coccidioides*, or *Cryptococcus* (1–6). In Japan, summer-type hypersensitivity pneumonitis with associated pulmonary eosinophilia is the most common type of hypersensitivity pneumonitis and is caused by *Trichosporon* or *Cryptococcus* infection (7, 8). Pulmonary eosinophilia has also been documented in some cases of *cryptococcus neoformans* infection in HIV-negative patients (4–6). Host genetics are probably a determinant in whether pulmonary eosinophilia develops following *C. neoformans* infection, since 1) the demographic distribution of *C. neoformans*-induced summer-type hypersensitivity pneumonitis is relatively restricted even though *C. neoformans* infec-

tions occur worldwide; and 2) pulmonary eosinophilia in HIV-negative cryptococcosis patients occurs in only a subset of these patients (1, 7, 9).

The genetic background of the host is a major determinant of the immune response in murine models of pulmonary *C. neoformans* infection. Inbred immunocompetent mouse strains such as CBA/J, C.B-17, and BALB/c can progressively clear a *C. neoformans* strain 52 infection from the lungs (10–13). In contrast, the immunocompetent mouse strain C57BL/6 develops a nonresolving cryptococcal pneumonia (11, 12). Eosinophils are a major cellular constituent of the inflammatory infiltrate in *C. neoformans* (strain 52)-infected BALB/c and C57BL/6 mice at 2 wk postinfection but only a minor population in CBA/J and C.B-17 mice at the same time point (13–16). Eosinophils are capable of phagocytizing *C. neoformans* in vitro (14). There are also early differences in cytokine production by lung leukocytes from C57BL/6 mice compared with C.B-17 and BALB/c mice, but not in total lung leukocyte numbers (12, 15). Depletion of CD4⁺ and CD8⁺ T lymphocytes attenuates inflammatory cell recruitment and inhibits pulmonary clearance of *C. neoformans* in CBA/J, C.B-17, and BALB/c mice (15, 17–19). TNF- α , monocyte chemoattractant protein-1, macrophage inflammatory protein-1 α , IFN- γ , IL-12, and nitric oxide all play an important role in protective cell-mediated immunity in the lungs of C.B-17 and CBA/J mice (16, 20–23, 45). Granulocyte-macrophage CSF, TNF- α , and IL-12 also play a role in systemic immunity (24–27). Cultured lung leukocytes from *C. neoformans*-infected C57BL/6 mice produce higher levels of IL-5 and lower levels of IFN- γ or IL-2 at wk 1–2 compared with lung leukocytes from *C. neoformans*-infected C.B-17 or BALB/c mice (12, 15). There is some association between pulmonary clearance of *C. neoformans* and

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the genetically determined ability of the host to produce Th1-type cytokines in response to *C. neoformans* infection, but the mechanism underlying resistance differences between strains such as CBA/J and C57BL/6 has not been elucidated. It remains to be determined whether there is a relationship between the inability to clear *C. neoformans* from the lungs and the duration of the *C. neoformans*-induced eosinophil infiltrate in the lungs.

The role of IL-5 in host defense and leukocyte recruitment during a pulmonary *C. neoformans* infection has not been previously investigated. Isolated lung leukocytes from BALB/c and C57BL/6 mice produce IL-5 following *in vitro* stimulation (12, 15). IL-5 is an eosinophil differentiation and activation factor that plays a critical role in tissue eosinophilia such as in allergic bronchopulmonary aspergillosis (28, 29). Persisting tissue eosinophilia results in tissue destruction due to release of eosinophil-derived mediators at the site of inflammation including reactive oxygen metabolites, major basic protein, and eosinophil cationic protein. The presence of Charcot-Leyden crystals (eosinophil lysophospholipase/galectin) is often used as a diagnostic tool for the identification of eosinophil-mediated inflammatory diseases in humans (30–35). Rodent eosinophils possess significant lysophospholipase activity and have germ-line DNA sequences that are homologous to those encoding Charcot-Leyden crystal protein in humans (32, 33). IL-5 and other T cell-derived cytokines play an important role in eosinophil development, activation, and (human) Charcot-Leyden crystal formation (28, 36, 37). However, IL-5-driven Charcot-Leyden crystal deposition has not previously been identified in eosinophil-mediated pathology in rodents (30).

Our objectives were to 1) determine whether there is an association between the development of a persisting eosinophil infiltrate in the lungs and susceptibility to pulmonary *C. neoformans* infection in immunocompetent mice and 2) determine the role of IL-5 in the development of pulmonary eosinophilia, tissue pathology, and leukocyte recruitment in the lungs of *C. neoformans*-infected C57BL/6 mice.

Materials and Methods

C. neoformans

C. neoformans strain 52 was obtained from the American Type Culture Collection (no. 24067, Rockville, MD). For infection, yeast were grown to stationary phase (48–72 h) at room temperature in Sabouraud dextrose broth (1% neopeptone and 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 3.3×10^5 CFU/ml in sterile nonpyrogenic saline.

Mice

Female C57BL/6, BALB/c, and CBA/J mice (6–8 wk old) were obtained from The Jackson Laboratory (Bar Harbor, ME) or Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Mice were housed under specific pathogen-free conditions in enclosed filter-top cages. Clean food and water were given *ad libitum*. The mice were handled and maintained using microisolator techniques with daily veterinarian 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 *M. pulmonis*.

Intratracheal inoculation of *C. neoformans*

Mice were anesthetized by *i.p.* injection of pentobarbital (0.074 mg/g weight of mouse; Butler, Columbus, OH) and were restrained on a small board. A small incision was made through the skin over the trachea, and the underlying tissue was separated. A 30-gauge needle (Becton Dickinson, Rutherford, NJ) was bent and attached to a tuberculin syringe (Monoject, St. Louis, MO) filled with the diluted *C. neoformans* culture. The needle was inserted into the trachea, and 30 μ l of inoculum was dispensed into the lungs (10^4 CFU). The needle was removed, and the skin was closed with a cyanoacrylate adhesive. The mice recovered with minimal visible trauma.

Aliquots of the inoculum were collected periodically to monitor the number of CFU delivered.

Preparation of lung leukocytes

The lungs were perfused free of blood with PBS, excised, minced, and enzymatically digested for 30 min using 15 ml/lung of digestion buffer (RPMI, 10% FCS, antibiotics, 1 mg/ml collagenase (Boehringer Mannheim Biochemical, Chicago, IL), and 30 μ g/ml DNase (Sigma Chemical Co., St. Louis, MO)). The cell suspension and undigested fragments were further dispersed by drawing them up and down through the bore of a 10-ml syringe. The total cell suspension was then pelleted, and erythrocytes were lysed by resuspending them in ice-cold NH_4Cl buffer (0.829% NH_4Cl , 0.1% KHCO_3 , and 0.0372% Na_2EDTA , pH 7.4). A 10-fold excess of HBSS (Sigma) was added to make the solution isotonic, and the cells were repelleted and resuspended in complete medium. Total leukocytes were enumerated by hemocytometer counting in trypan blue.

Eosinophils, neutrophils, macrophages, and lymphocytes were visually counted in Wright-Giemsa-stained samples of lung cell suspensions cytopun onto glass slides (Shandon Cytospin, Pittsburgh, PA). For Wright-Giemsa staining, the slides were fixed/prestained for 2 min with a one-step methanol-based Wright-Giemsa stain (Harleco, EM Diagnostics, Gibbstown, NJ) followed by steps 2 and 3 of the Diff-Quik whole blood stain (Diff-Quik, Baxter Scientific, Miami, FL). This modification of the Diff-Quik stain procedure improves the resolution of eosinophils from neutrophils in the mouse. A total of 200 to 400 cells were counted from randomly chosen high power microscope fields for each sample. The percentage of a leukocyte type was multiplied by the total number of leukocytes to give the absolute number of that type of leukocyte in the sample.

Cell staining and two-color flow cytometry

Lung cells (5×10^5) were incubated for 30 min on ice in a total volume of 120 μ l of staining buffer (FA Buffer, Difco), 0.1% NaN_3 , and 1% FCS. Each sample was incubated with 1 μ g of one of the following FITC-labeled mAbs (PharMingen, San Diego, CA): RM4-5 (anti-CD4), 53-6.7 (anti-CD8), RA3-6B2 (anti-B220, B lymphocyte marker), or isotype-matched rat IgG. The samples were washed in staining buffer and fixed in 1% paraformaldehyde (Sigma) in buffered saline. Stained samples were stored in the dark at 4°C until analyzed on a flow cytometer (C, Coulter Corp., Hialeah, FL). The percentage of a lymphocyte subset was multiplied by the total number of leukocytes to give the absolute number of that type of lymphocyte in the sample.

In vivo depletion of CD4^+ and CD8^+ T cells

Mice were given an *i.p.* injection of 300 μ g of mAb GK1.5 (anti-CD4), mAb YTS169.4 (anti-CD8), or rat IgG at the time of infection, and a second dose (100 μ g of the same mAb) was given 7 days later. T cell depletion was confirmed by flow cytometry (staining for CD4 and CD8) of draining pulmonary lymph node cells or spleen cells.

Histology

Following euthanasia and before removal, the lungs were fixed by inflation 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 paraffin embedded. Five-micrometer sections were cut, deparaffinized, stained with hematoxylin and eosin, and viewed by light microscopy.

Detection of IL-5 mRNA by RT-PCR

Whole lungs were removed, homogenized in TRIzol reagent (Life Technologies, Gaithersburg, MD), extracted as outlined in the TRIzol protocol, and precipitated with isopropanol. The RNA was washed with 70% ethanol, dissolved in distilled H_2O , reprecipitated with 100% ethanol, and quantified by UV spectrophotometry using absorbance at 260 nm. To confirm the integrity of the RNA and assure equal loading for the RT reaction, 10 μ g of total RNA was electrophoresed, and 18S and 28S ribosomal bands were visualized by ethidium bromide staining. For conversion of mRNA into cDNA (RT reaction), excess amounts of trinucleotides, Moloney murine leukemia virus RT (Life Technologies), and oligo(dT) (18-mer) primer (University of Michigan core facility, Ann Arbor, MI) were used. Equal aliquots of RT products from the same RT reaction were transferred to the PCR reaction mixtures for the various cytokines along with Taq DNA polymerase (Life Technologies), nucleotides, and oligonucleotide primers for the specific cytokine amplified (University of Michigan Core Facility). Two 10-fold dilutions of the RT product (0.2 and 0.02 μ g) were used for the PCR reaction to control for possible overamplification of cDNA in the

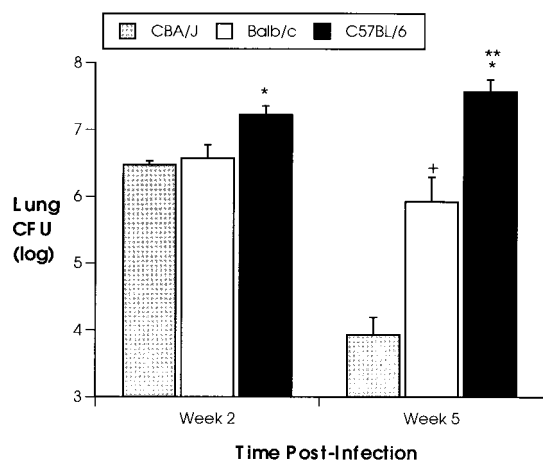


FIGURE 1. Pulmonary clearance of *C. neoformans* strain 52 in BALB/c, CBA/J, and C57BL/6 mice following intratracheal inoculation (10^4 CFU). * indicates $p < 0.05$, comparing C57BL/6 to CBA/J mice at the same time point. ** indicates $p < 0.05$, comparing C57BL/6 to BALB/c mice at the same time point. + indicates $p < 0.05$, comparing BALB/c to CBA/J mice at the same time point.

samples. The oligonucleotide primers for β -actin were 5'-ATG-GAT-GAC-GAT-ATC-GCT-G-3' (sense) and 5'-GAT-TCC-ATA-CCC-AGG-AAG-G-3' (antisense). The oligonucleotide primers for IL-5 were 5'-GCA-ATG-GAA-GGC-TGA-GGC-TG-3' (sense) and 5'-GGG-TAT-GTG-ATC-CTC-CTG-CGT-C-3' (antisense). The PCR reaction parameters for β -actin were 31 cycles with an annealing temperature of 50°C, and those for IL-5 were 35 cycles with an annealing temperature of 61°C. RT-PCR reaction products were electrophoresed and visualized by ethidium bromide staining, and the sizes of the RT-PCR products were confirmed by comparison with a 100-bp ladder run in parallel on the same gel.

In vivo neutralization of IL-5

To determine whether IL-5 was required for pulmonary eosinophilia, mice were injected i.p. with 1 mg of either TRFK-5 mAb (anti-IL-5) or rat IgG on days 0 and 4 of infection and were analyzed on days 14 to 15. To determine whether IL-5 was required for Charcot-Leyden-like crystal formation, mice were injected i.p. with 1 mg of either TRFK-5 (anti-IL-5) or rat IgG on days 0, 4, 14, and 28 of infection and were examined on day 42. The TRFK-5 hybridoma was provided by Robert Coffman at DNAX Corp. (Palo Alto, CA).

Lung leukocyte culture and IL-5 ELISA

Lung leukocytes (2×10^6) were cocultured with 5 μ g of Con A (Sigma) in 1 ml of complete medium supplemented with 2 μ g of amphotericin B (Sigma; to inhibit yeast growth), 250 U of catalase (Worthington Biochemicals, Freehold, NJ; to inhibit H_2O_2 formation), and 1 μ g of indomethacin (Sigma; to inhibit PG synthesis) in 24-well plates (Corning, Corning, NY) at 37°C in 5% CO_2 . Supernatants were harvested after 24 h and assayed for IL-5 by two-site sandwich ELISA as previously described (15) using the anti-IL-5 mAbs TRFK-5 and TRFK-4 (PharMingen). IL-5 in unknown samples was quantified by comparison with standard curves, using either linear or four-parameter curve-fitting programs. The limit of sensitivity for IL-5 detection was 10 pg/ml.

Calculations and statistics

Statistical significance was calculated using a *t* test, with a significance of $p < 0.05$ for a single comparison. Significance for multiple comparisons was calculated using a *t* test modified by the Bonferroni correction: a modified *p* value (p_m)³ was generated by multiplying the *p* value by the number of comparisons, with significance being $p_m < 0.05$. The *p* values given in each figure are the values (p_m) after applying the Bonferroni correction. Data points in Figures 1, 2, 5, 6, 7, and 8 are the mean \pm SEM for each group of pooled data derived from two or three experiments, and the *n* values for the data in each figure are as follows: Figure 1, 7 to 12; Figure

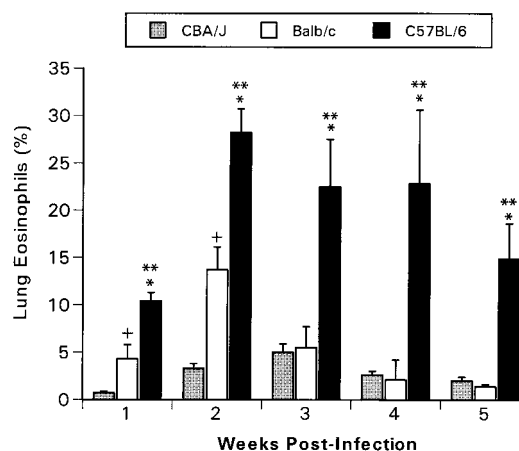


FIGURE 2. Pulmonary eosinophilia in *C. neoformans*-infected mice. BALB/c, CBA/J, and C57BL/6 mice were inoculated intratracheally with *C. neoformans* strain 52. Eosinophils were enumerated by Wright-Giemsa staining from enzymatically digested lungs at weekly intervals as outlined in *Materials and Methods*. The results shown are the percentage of eosinophils in the total leukocyte infiltrate in the lungs. The total number of leukocytes in the lungs was also greater in C57BL/6 mice than in CBA/J or BALB/c mice at wk 1 to 5 (G. B. Huffnagle et al., manuscript in preparation). The absolute number of eosinophils in C57BL/6 mice ranged from 10^7 (at wk 1) to 4×10^7 (peak, at wk 2–3). * indicates $p < 0.05$, comparing C57BL/6 to CBA/J mice at the same time point. ** indicates $p < 0.05$, comparing C57BL/6 to BALB/c mice at the same time point. + indicates $p < 0.05$, comparing BALB/c to CBA/J mice at the same time point.

2, 10 to 33 mice/group/time point for wk 1 to 3 and 4 to 9 mice/group/time point for wk 4 to 5; Figures 5 to 8, 4 to 9 mice/group/time point.

Results

Pulmonary eosinophilia in C. neoformans-infected mice is genetically controlled and correlates with decreased pulmonary clearance of the infection

The inability to clear *C. neoformans* from the lungs was associated with the development of a chronic eosinophil infiltrate in the lungs. At 2 wk postinfection, the pulmonary burden of *C. neoformans* strain 52 was 5-fold higher in C57BL/6 mice compared with that in CBA/J and BALB/c mice (Fig. 1). At 5 wk postinfection, differences in the pulmonary cryptococcal burden among the three strains were more pronounced. The number of lung CFU in C57BL/6 mice was 5000-fold higher than that in CBA/J mice and 100-fold higher than that in BALB/c mice (Fig. 1). Therefore, based on pulmonary clearance patterns, CBA/J mice could be classified as highly resistant, BALB/c mice as moderately resistant, and C57BL/6 mice as susceptible to *C. neoformans* strain 52 infection.

The level of resistance to infection correlated with the duration of pulmonary eosinophilia that developed following *C. neoformans* infection in these three mouse strains. There were few eosinophils in the lungs of highly resistant CBA/J mice at any time point between wk 1 and 5 (<5% of the total lung leukocytes; Fig. 2). There was a transient influx of eosinophils into the lungs of moderately resistant BALB/c mice at wk 1 to 2. Eosinophils comprised almost 15% of the lung leukocytes at wk 2, but decreased to <5% by wk 3 to 5 (Fig. 2). In contrast, one of the most prominent features of the inflammatory response in the lungs of susceptible C57BL/6 mice was the large number of eosinophils from wk 1 to 5 (Fig. 2). Eosinophils ranged from 10 to 25% of the inflammatory cells over wk 1 to 5, peaking at wk 2 to 3. The total number of leukocytes recruited into the lungs of C57BL/6 mice was comparable to or greater than that in CBA/J and BALB/c mice at wk 1 to

³ Abbreviation used in this paper: p_m , modified *p* value.

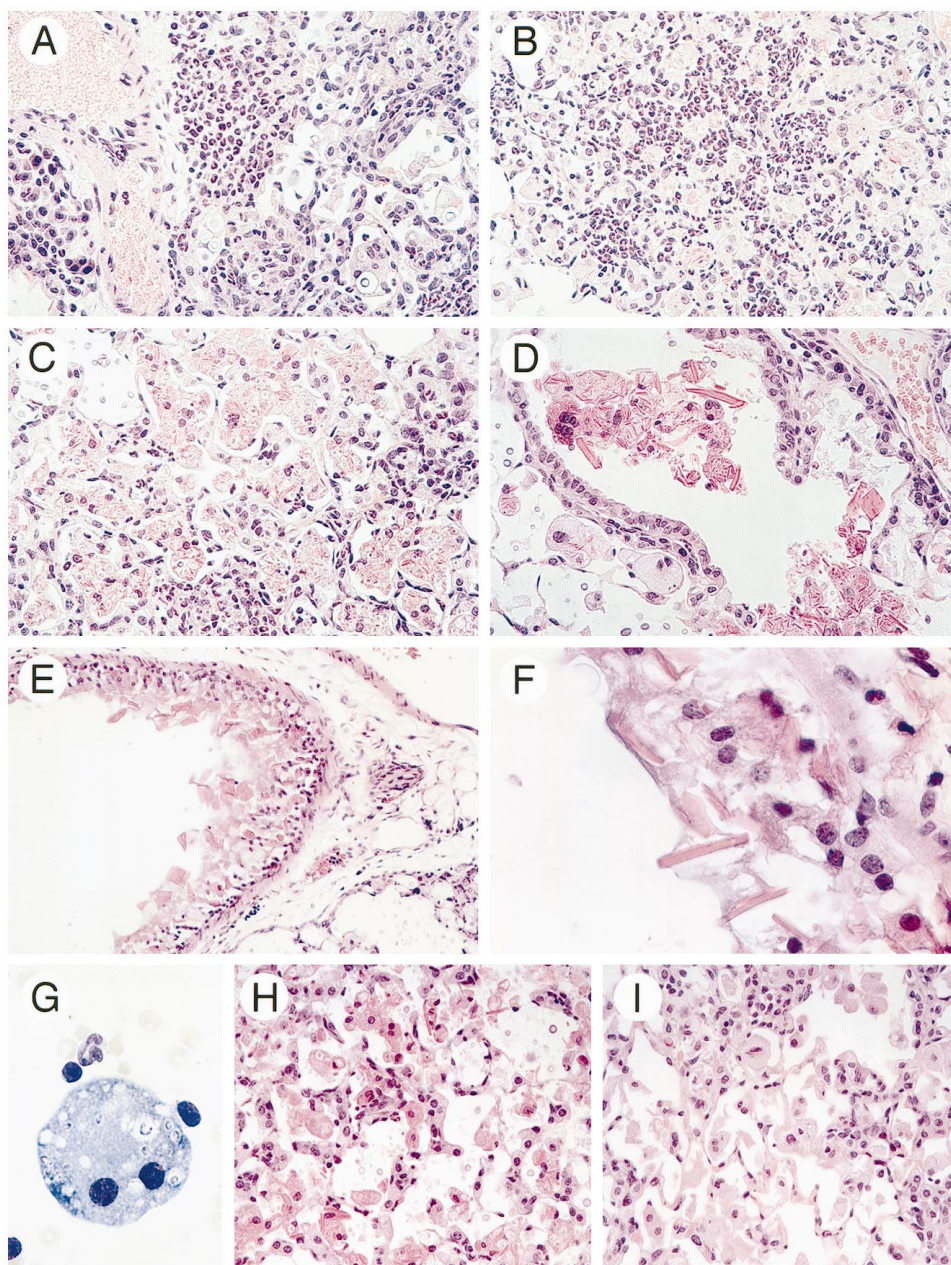


FIGURE 3. Histologic analysis of the pathogenesis of *C. neoformans* infection in the lungs of C57BL/6 mice. Photomicrographs of sections from the lungs of *C. neoformans*-infected C57BL/6 mice at 2 wk (A), 4 wk (B), and 6 wk (C–F). Note the numerous eosinophils (A and B) early in the infection, the Charcot-Leyden-like crystals later in the infection (B–F), and the subsequent damage to the airway epithelium (E and F). G, An isolated multinucleated giant cell at wk 2 with numerous phagocytized eosinophils at various stages of degradation. H and I, Photomicrographs of sections from the lungs of *C. neoformans*-infected C57BL/6 mice at 6 wk following treatment with either irrelevant IgG (H) or anti-IL-5 mAb (I) on days 0, 4, 14, and 28. Note the numerous intracytoplasmic pink crystalline inclusions in the macrophages in irrelevant IgG-treated mice (H) that are absent in anti-IL-5-treated mice (I), demonstrating the requirement for IL-5 in crystal deposition late in the infection. A, B, C and E, $\times 50$, hematoxylin-eosin stain; D, $\times 100$, hematoxylin-eosin stain; F and G, $\times 500$, hematoxylin-eosin stain; H and I, $\times 200$, hematoxylin-eosin stain.

5 (G. B. Huffnagle, M. B. Boyd, L. K. McNeil, L. E. Pray, R. A. McDonald, and G. B. Toews, manuscript in preparation). Therefore, the absolute number of eosinophils was also dramatically greater in C57BL/6 mice at all time points and ranged from a low of 10^7 (at wk 1) to a peak of 4×10^7 (at wk 2–3). In summary, these data demonstrated that the development of a chronic eosinophil infiltrate in the lungs was associated with an inability to clear a pulmonary *C. neoformans* infection (i.e., susceptibility). Furthermore, inbred strains of mice displayed marked differences in pulmonary clearance and associated pulmonary eosinophilia.

Chronic pulmonary eosinophilia in C. neoformans-infected C57BL/6 mice results in the deposition of Charcot-Leyden-like crystals in the lungs and epithelial cell damage

The chronic cryptococcal pneumonia and eosinophilia in C57BL/6 mice was analyzed histologically. At wk 2, there were numerous eosinophils both in the perivascular cuffs of the inflammatory infiltrate and in the alveoli, often in contact with cryptococci (Fig. 3A). There were also numerous monocyte/macrophages and some neutrophils in the alveoli and/or perivascular regions. At wk 4,

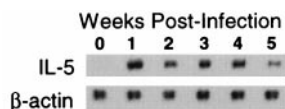


FIGURE 4. Kinetics of IL-5 mRNA expression in the lungs of *C. neoformans*-infected C57BL/6 mice. IL-5 mRNA expression in whole lung was analyzed by RT-PCR, and a representative gel is shown. Aliquots of the same lung cDNA samples were also analyzed for expression of the constitutive housekeeping gene β -actin to demonstrate relative equal loading of the RT product in the PCR reactions for all samples.

there was still a marked inflammatory infiltrate of eosinophils and macrophages, and there were large numbers of cryptococci (Fig. 3B). A prominent histologic feature at this time point was the appearance of intracellular eosinophilic crystals in macrophages. In addition, the proportion of eosinophils with few cytoplasmic granules had increased (data not shown), suggesting that the eosinophils were being activated to degranulate.

The amount of crystal deposition in the lungs by wk 6 was striking and gave the lung sections an eosinophilic appearance (Fig. 3C). The majority of these crystals were intracellular inclusions within macrophages. However, numerous extracellular flat, square crystals were also present in the bronchioles and alveoli (Fig. 3D). These crystals fluoresced when the sections were illuminated under UV light (data not shown), a physical property reported for Charcot-Leyden crystals. There was also evidence of increased mucous secretion in the bronchioles. Marked destruction of the normal airway epithelium occurred in some of the bronchioles and correlated with the presence of extracellular crystals in the disrupted epithelium (Fig. 3, E and F). In some samples there were fibrotic foci, indicating additional inflammatory-mediated pulmonary damage (histology not shown). Another interesting histologic feature of the response was the presence of macrophages (and multinucleated giant cells) that had phagocytized one or more eosinophils (Fig. 3G). Macrophage phagocytosis of dying eosinophils or free eosinophil granules has been documented in humans as a necessary step in Charcot-Leyden crystal formation (31). Overall, the three prominent histologic features of the pulmonary inflammatory response in *C. neoformans*-infected C57BL/6 mice were 1) the chronic alveolar and perivascular eosinophilia, 2) the formation of intracellular crystals in macrophages within the alveoli, and 3) the deposition of extracellular crystals in the bronchioles and associated destruction of airway epithelium.

Activation of eosinophils by IL-5 has been demonstrated to enhance degranulation of eosinophils and lead to Charcot-Leyden crystal formation (36). Thus, we examined whether IL-5 was expressed in vivo during a *C. neoformans* infection in the lungs of C57BL/6 mice. Whole lung RNA was isolated at wk 1, 2, 3, 4, and 5 and analyzed by RT-PCR for IL-5 expression. IL-5 mRNA was not detectable in the lungs of uninfected mice, but was detectable by wk 1 in *C. neoformans*-infected mice (Fig. 4). IL-5 mRNA continued to be expressed from wk 2 to 5. Treatment of mice with anti-IL-5 mAb on days 0, 4, 14, and 28 completely ablated crystal deposition in the lungs on day 42 (Fig. 3, H and I). In addition, eosinophils could not be identified in tissue sections of the lungs from anti-IL-5-treated mice, providing further evidence that the crystals were eosinophil derived. Overall, these data demonstrated that IL-5 was required for crystal formation in the lungs of *C. neoformans*-infected C57BL/6 mice, similar to the mechanism described for human Charcot-Leyden crystal formation (31).

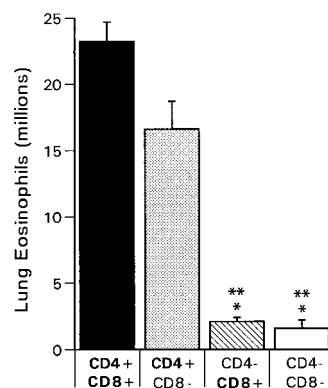


FIGURE 5. Effect of T cell subset depletion on pulmonary eosinophilia in *C. neoformans*-infected C57BL/6 mice at 2 wk postinfection (the peak of eosinophilia in control mice; Fig. 2). The data shown are the absolute number of eosinophils in whole lung after perfusing the lungs free of blood. Mice were treated with irrelevant IgG ($CD4^+CD8^+$), anti-CD8 mAb ($CD4^+CD8^-$), anti-CD4 mAb ($CD4^-CD8^+$), or both anti-CD4 and anti-CD8 mAb ($CD4^-CD8^-$) on days 0 and 7 of infection. Eosinophils were identified and quantified by Wright-Giemsa staining of leukocytes isolated from enzymatically digested lungs as outlined in *Materials and Methods*. * indicates $p < 0.05$ compared with $CD4^+CD8^+$ mice. ** indicates $p < 0.05$ compared with $CD4^+CD8^-$ mice.

Eosinophil recruitment into the lungs of C. neoformans-infected C57BL/6 mice requires CD4⁺ T lymphocytes but not CD8⁺ T lymphocytes

We investigated whether $CD4^+$ and/or $CD8^+$ T cells were required for eosinophil recruitment into the lungs of *C. neoformans*-infected C57BL/6 mice. *C. neoformans*-infected C57BL/6 mice were treated with anti-CD4 mAb, anti-CD8 mAb, both anti-CD4 and anti-CD8 mAb, or rat IgG. Anti-CD4 and anti-CD8 treatment resulted in a $>95\%$ reduction in the number of $CD4^+$ and $CD8^+$ T cells, respectively (data not shown). Eosinophil recruitment was not attenuated by CD8 depletion compared with that in control mice (Fig. 5). However, pulmonary eosinophilia was ablated at wk 2 in both CD4-deficient and CD4/CD8-deficient mice (Fig. 5). Thus, $CD4^+$ T cells (but not $CD8^+$ T cells) were required for eosinophil recruitment into the lungs of *C. neoformans*-infected C57BL/6 mice.

We also measured IL-5 levels in mitogen-stimulated lung leukocyte cultures from anti-CD4- or anti-CD8-treated mice to determine whether the decrease in eosinophil recruitment correlated with a decrease in IL-5 production by these cells. Lung leukocytes from *C. neoformans*-infected, anti-CD8-treated mice at wk 2 produced as much IL-5 as lung leukocytes from infected control Ig-treated mice (Fig. 6). However, lung leukocytes from *C. neoformans*-infected, anti-CD4-treated mice at wk 2 produced significantly less IL-5 than that from control mice. Thus, $CD4^+$ T cells, but not $CD8^+$ T cells, isolated from the lungs of *C. neoformans*-infected C57BL/6 mice could produce IL-5 upon activation and implicated IL-5 as a mediator of $CD4^+$ T cell-dependent pulmonary eosinophilia in *C. neoformans*-infected C57BL/6 mice.

IL-5 is required for pulmonary eosinophilia in C. neoformans-infected C57BL/6 mice

To determine whether IL-5 was required for pulmonary eosinophilia, C57BL/6 mice were treated with either rat IgG or rat anti-mouse IL-5 mAb (TRFK-5) on days 0 and 4 of infection, and eosinophilia was measured on day 14 (the peak of pulmonary eosinophilia). Neutralization of IL-5 decreased the number of eosinophils in the lungs by $>98\%$ (Fig. 7). While eosinophils have been

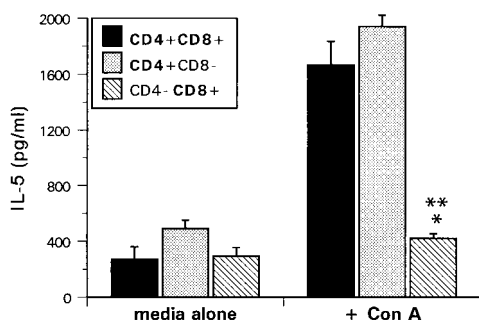


FIGURE 6. Effect of T cell subset depletion on IL-5 production by lung leukocytes from *C. neoformans*-infected C57BL/6 mice at 2 wk postinfection (the peak of eosinophilia in control mice; Fig. 2). Mice were treated with irrelevant IgG (CD4⁺CD8⁺), anti-CD8 mAb (CD4⁺CD8⁻), or anti-CD4 mAb (CD4⁻CD8⁺) on days 0 and 7 of infection. Leukocytes from enzymatically digested lungs were cultured with or without Con A for 24 h, and culture supernatants were assayed by ELISA for IL-5. * indicates $p < 0.05$ compared with CD4⁺CD8⁺ mice. ** indicates $p < 0.05$ compared with CD4⁺CD8⁻ mice.

implicated as a potential effector cell against *C. neoformans* (14), the elimination of eosinophils in the lungs by anti-IL-5 treatment did not increase the already high cryptococcal burden in the lungs (7.21 ± 0.41 vs 7.30 ± 0.29 log CFU on day 14). These results demonstrated that IL-5 production was required for pulmonary eosinophilia in *C. neoformans*-infected C57BL/6 mice, and pulmonary eosinophilia was a nonprotective CD4⁺ T cell-mediated response to the infection.

IL-5 promotes mononuclear cell recruitment into the lungs of C. neoformans-infected C57BL/6 mice

Further analysis of the leukocytic infiltrate in the lungs revealed that IL-5 was also required for the recruitment of leukocytes other than eosinophils. Total leukocytes decreased by 59% following anti-IL-5 treatment (control mice, $115.2 \pm 13.5 \times 10^6$; anti-IL-5-treated mice, $47.9 \pm 6.2 \times 10^6$; $p < 0.05$). The reduction in eosinophils accounted for over half of the decrease in total lung leukocytes (Fig. 7). The remainder of the decrease was in the number of macrophages and lymphocytes (Fig. 8). Neutralization of IL-5

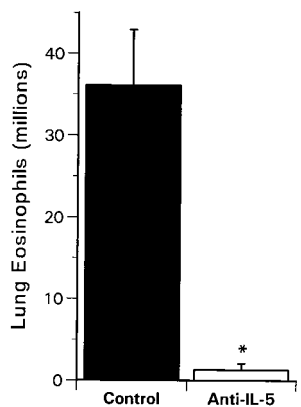


FIGURE 7. Effect of IL-5 neutralization on eosinophil recruitment into the lungs of *C. neoformans*-infected C57BL/6 mice at 2 wk postinfection. The data shown are the absolute number of eosinophils in whole lung after perfusing the lungs free of blood. Mice were treated with either irrelevant IgG (control) or anti-IL-5 mAb on days 0 and 4 of infection. Eosinophils were identified and quantified by Wright-Giemsa staining of leukocytes isolated from enzymatically digested lungs as outlined in *Materials and Methods*. * indicates $p < 0.05$ compared with control mice.

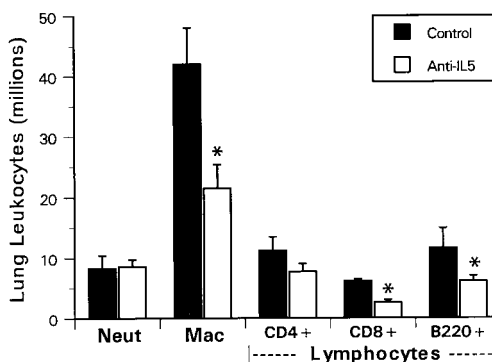


FIGURE 8. Effect of IL-5 neutralization on neutrophil and mononuclear cell recruitment into the lungs of *C. neoformans*-infected C57BL/6 mice at 2 wk postinfection. The data shown are the absolute number of a leukocyte subset in whole lung (after perfusing the lungs free of blood). The groups are the same as those in Figure 7. Leukocytes were identified by Wright-Giemsa staining and by flow cytometry. * indicates $p < 0.05$ compared with control mice.

did not affect neutrophil recruitment (Fig. 8). Neutralization of IL-5 reduced the number of macrophages by 49%. In addition, the numbers of CD8⁺ T lymphocytes and B lymphocytes were 57 and 47% lower, respectively, in anti-IL-5-treated *C. neoformans*-infected mice. The number of CD4⁺ T lymphocytes in the lungs was slightly lower following anti-IL-5 treatment, but the differences were variable, and the means were not statistically significant ($p = 0.21$; Fig. 8). Thus, IL-5 also played an important role in the recruitment of macrophages, CD8⁺ T lymphocytes, and B lymphocytes into the lungs of *C. neoformans*-infected C57BL/6 mice.

Discussion

There are four major points addressed in these studies. 1) The development of a chronic eosinophil infiltrate in the lungs is associated with an inability to clear pulmonary *C. neoformans* infection (genetic susceptibility). The duration of the pulmonary eosinophilia correlates with relative susceptibility to the infection. 2) Eosinophil recruitment into the lungs during *C. neoformans* infection requires IL-5 from CD4⁺ T cells. 3) This is a murine model of a chronic microbe-initiated inflammatory disease in the lungs that results in eosinophil-mediated tissue damage in the lungs, including deposition of crystals morphologically similar to Charcot-Leyden crystals. While this pathologic process is well documented in a number of human diseases, Charcot-Leyden crystal deposition has never been demonstrated in a rodent model. 4) These studies demonstrate that IL-5 and/or eosinophils can play a major role in the recruitment of macrophages and CD4⁺ T, CD8⁺ T, and B lymphocytes into the lungs. In summary, these studies demonstrate that IL-5 and eosinophils play an important role in mediating the nonprotective T cell-dependent response in the lungs of *C. neoformans*-infected C57BL/6 mice.

The development of chronic pulmonary eosinophilia in response to *C. neoformans* infection is genetically determined and is associated with an inability to clear the infection from the lungs. Intratracheal inoculation of moderately virulent *C. neoformans* 52 into C57BL/6 mice results in a chronic infection. A vigorous inflammatory response develops in the lungs that is marked by large numbers of eosinophils throughout the response. The end result is eosinophil degranulation, crystal deposition, and tissue damage. In contrast, intratracheal inoculation of *C. neoformans* 52 into CBA/J or BALB/c mice generates an infection that is progressively cleared. A vigorous inflammatory response also develops in these

strains of mice (13, 15). However, few eosinophils are recruited into the lungs of CBA/J mice, while there is a transient influx of eosinophils in BALB/c mice. C.B-17, 129/J, and A/JCr mice are all able to control the growth of *C. neoformans* strain 52 in the lungs and develop either a minimal or transient eosinophilia (16, 38) (G. B. Huffnagle, unpublished observations). C.B-17 mice will develop an eosinophilia if pretreated with Abs to IFN- γ or IL-12 (45). Taken in the context of our previous findings, our current data demonstrate that T cell-mediated immune responses associated with a Th2-type T cell response (chronic eosinophilia, lack of delayed-type hypersensitivity, and minimal IFN- γ production) are detrimental for clearing a *C. neoformans* infection and support the concept that protective T cell-mediated immunity is mediated by a Th1-type response.

IL-5 and CD4⁺ T cells are required for eosinophil recruitment into the lungs of *C. neoformans*-infected C57BL/6 mice. Three different treatments prevent pulmonary eosinophilia in these mice: neutralization of IL-5, depletion of CD4⁺ T cells, or depletion of both CD4⁺ and CD8⁺ T cells. Depletion of CD4⁺ T cells also eliminates IL-5 production by lung leukocyte cultures. IL-5 is a major cytokine produced by Th0- and Th2-type CD4⁺ T cells (28, 37, 39), and in gelatin-sponge delayed-type hypersensitivity reactions to *C. neoformans* in immunized mice, CD4⁺ T cells produce IL-5 along with IL-2 and IFN- γ (40). Depletion of CD4⁺ T cells in BALB/c mice ablates the ability of lung leukocytes to produce IL-5 (15). There is increasing evidence that CD8⁺ T cells can be an important source of IL-5; however, depletion of CD8⁺ T cells does not block eosinophil recruitment into the lungs or decrease IL-5 production by lung leukocytes in *C. neoformans*-infected C57BL/6 mice. Together, our studies demonstrate that IL-5 and CD4⁺ T cells are required for pulmonary eosinophilia and that CD4⁺ T cells are the major producers of IL-5 in the lungs.

The histology reported here also demonstrates crystals resembling Charcot-Leyden crystals in rodents. Charcot-Leyden crystals are crystallized formations of eosinophil lysophospholipase/galectin found at sites of eosinophil accumulation in human disease (30–35, 41). Eosinophils from both rodents and humans contain high levels of lysophospholipase (32). Activation of rodent or human eosinophils results in the formation of electron-dense elongated intracellular structures (30). These intracellular structures are also found in rodent eosinophils following phagocytosis of cryptococci (14). Physical disruption of human eosinophils results in Charcot-Leyden crystal formation; however, disruption of rodent eosinophils does not (30). The crystals in our studies are four-sided, whereas in humans the crystals are hexagonal (30). It is not clear why there is a species difference in Charcot-Leyden crystal formation; however, pulmonary *C. neoformans* infection in C57BL/6 mice results in the deposition of crystals morphologically similar to human Charcot-Leyden crystals.

Our studies demonstrate that IL-5 and macrophages play a role in crystal accumulation in the lungs. Most of the crystals are concentrated in macrophages. The interaction between macrophages and eosinophils has been identified as an important step in Charcot-Leyden crystal formation in humans (31, 42). Macrophages can phagocytize and degrade dying eosinophils, resulting in the release of lysophospholipase from the eosinophil and the formation of Charcot-Leyden crystals in the macrophage phagosome (31, 42). Before the appearance of crystals histologically in C57BL/6 mice, macrophages can be identified that had phagosomes containing eosinophils at various stages of degradation. These macrophages are not found in *C. neoformans*-infected mice when eosinophils are absent (43), e.g., these eosinophil-containing macrophages are not found in CBA/J mice, anti-IL-5-treated C57BL/6 mice, or anti-CD4-treated C57BL/6 mice (43) (Fig. 3I

and histology not shown). Addition of IL-5 to human cord blood cultures enhances piecemeal degranulation of eosinophils and Charcot-Leyden crystal formation (36). Continuous neutralization of IL-5 also blocks crystal formation in the lungs of *C. neoformans*-infected C57BL/6 mice (Fig. 3I). In summary, all these stages in Charcot-Leyden crystal formation could be identified in the lungs of *C. neoformans*-infected C57BL/6 mice: macrophages containing phagocytized eosinophils, eosinophil degranulation, IL-5 expression at the tissue site of degranulation and crystal formation (the lungs), requirement for IL-5 in crystal formation, macrophages containing intracellular crystals, and extracellular crystals in the tissue. Thus, it is extremely likely that the crystals in the lungs of *C. neoformans*-infected C57BL/6 mice are murine Charcot-Leyden crystals.

The evidence of eosinophil activation (crystal deposition) at sites of airway epithelium damage in *C. neoformans*-infected C57BL/6 mice is consistent with the epithelial cell cytotoxicity of other eosinophil-derived mediators. Eosinophils are found throughout the inflammatory infiltrate, often in juxtaposition to cryptococci. As the inflammatory response progresses, there is an increase in the proportion of eosinophils with few cytoplasmic granules, indicating that the eosinophils are degranulating. In *C. neoformans*-infected BALB/c mice, eosinophils are almost exclusively compartmentalized to the perivascular cuffs of the inflamed lung (15). The perivascular cuffs are the sites of eosinophil extravasation into the lungs, and this highly compartmentalized location of the eosinophils is consistent with the transient nature of the response in BALB/c mice. However, eosinophils are specifically recruited into the lungs of *C. neoformans*-infected C57BL/6 mice and are activated to degranulate during the inflammatory response, resulting in airway epithelium damage.

Our studies also demonstrated that IL-5 and eosinophils play a role in the recruitment of macrophages and CD4⁺ T, CD8⁺ T, and B lymphocytes into the lungs during *C. neoformans* infection. Eosinophils are also capable of producing a number of proinflammatory cytokines or chemokines following activation (44). The >95% reduction in eosinophils and the 60% reduction in mononuclear cells by anti-IL-5 Abs are comparable to the effect of anti-T cell Abs (G. B. Huffnagle, unpublished observation), raising the possibility that the production of IL-5 by CD4⁺ T cells is required for leukocyte recruitment into the lungs of *C. neoformans*-infected C57BL/6 mice. Taken together, our data support the model that IL-5 production by CD4⁺ T cells in *C. neoformans*-infected C57BL/6 mice promotes the recruitment and activation of eosinophils and the recruitment of additional macrophages and CD4⁺ T, CD8⁺ T, and B lymphocytes.

Compared with other IL-5/eosinophil-mediated responses in mice, the pulmonary inflammatory response in *C. neoformans*-infected C57BL/6 mice is unique in the magnitude of eosinophil activation, degranulation, crystal deposition, and tissue damage. Eosinophils have recently been demonstrated to phagocytize *C. neoformans* (14). Our in vivo studies demonstrate an inverse correlation between the duration of pulmonary eosinophilia and the pulmonary clearance of *C. neoformans*. Thus, eosinophils are not a major fungicidal effector cell in the inflammatory Th0/Th2-like milieu generated during *C. neoformans* infection in C57BL/6 mice. The phagocytic activity of eosinophils in vitro leaves open the possibility, however, that these leukocytes could be activated for fungicidal activity in vivo by an immunotherapeutic regimen. This model of a microbe-initiated, T cell-dependent, pulmonary inflammatory disease provides an opportunity for dissecting the in vivo factors necessary for eosinophil differentiation, recruitment, activation/degranulation, crystal formation, and associated tissue

damage during the course of eosinophilic inflammation in the lungs.

Note Added in Proof. Crystals have also been identified in bone marrow macrophages of adult mice (46) and likely arise from ingestion and degradation of eosinophils by macrophages (47). Crystals have also been reported in alveolar macrophages of motheaten mice and mice with acidophilic macrophage pneumonia (48, 49).

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