MAJOR ARTICLE

Enhanced Allergic Inflammation and Airway Responsiveness in Rats with Chronic *Cryptococcus neoformans* Infection: Potential Role for Fungal Pulmonary Infection in the Pathogenesis of Asthma

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Although *Cryptococcus neoformans* is recognized for its ability to cause meningoencephalitis and pneumonia among immunocompromised persons, subclinical pulmonary infection is also common among immunocompetent persons. The significance of this infection is unknown. Using a rat model, we explored the potential for pulmonary cryptococcosis to modify allergic responses and airway reactivity. Our findings indicate that localized pulmonary cryptococcal infection (but not disseminated infection) can exacerbate allergic responses to respiratory challenge with ovalbumin, as measured by total immunoglobulin E levels, ovalbumin-specific immunoglobulin E titers, and eosinophil content of bronchoalveolar lavage fluid. Infection-associated enhancement of allergic responses was not dependent on cryptococcal encapsulation and was partially ameliorated by the administration of fluconazole. Increases in both the number of goblet cells and airway responsiveness, which are also features of reactive airway disease, were also present with pulmonary infection. An examination of lung cytokine levels in the context of active pulmonary infection revealed increased expression of interleukin (IL)–10, tumor necrosis factor–α, and IL-13, but not IL-12 or interferon-γ. In contrast, systemic infection was associated with higher levels of interferon-γ but lower levels of IL-13. Our studies highlight the potential for localized pulmonary *C. neoformans* infection to potentiate allergic responses and airway reactivity and suggest a potential role for subclinical pulmonary cryptococcosis in the pathogenesis of asthma.

Cryptococcus neoformans is an encapsulated basidiomycete that is recognized for its ability to cause meningitis in immunocompromised persons. Nevertheless, asymptomatic or minimally symptomatic, clinically unrecognized pulmonary infection is believed to be frequent in immunocompetent persons [1–5]. Given the epidemiological association between pigeon guano and *C. neoformans,* along with the high density of pigeons in urban areas, it has been suggested that pulmonary infection with *C. neoformans* is highly prevalent among immunocompetent persons from urban areas [6]. In agreement with this notion, we and others have found that most New York City children >2 years old have serological evidence of *C. neoformans* infection [7, 8]. Of note, this population is also at high risk for asthma [9].

In animal models, *C. neoformans* is remarkable for its ability to evoke Th2-type polarized immune responses. Enhanced virulence among *C. neoformans* strains is associated with the ability to elicit Th2 polarized responses [10]. In addition, susceptibility to experimental cryptococcal infection in certain mouse strains (i.e., BALB/c and C57BL/6) has been linked to increased Th2 and decreased Th1 cytokine production [11, 12]. The pathogenesis of allergic airway diseases and asthma has been associated with Th2 polarized responses in the lung. Given the high likelihood of exposure to this fungus among immunocompetent persons and the propensity for this fungus to promote Th2 polarization, we inves-

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tigated the potential for pulmonary cryptococcal infection to modify both allergic inflammation and airway responsiveness, using a rat model of pulmonary cryptococcal infection.

MATERIALS AND METHODS

Animals. Specific pathogen–free male, brown Norway rats weighing 200–250 g were obtained from Harlan Sprague-Dawley. Rats were housed in the animal facility at our institution, and experiments were performed with the approval of our Animal Use Committee.

C. neoformans. Rats were infected with *C. neoformans* ATCC strain 24067, which is also known as 52D. This is a D serotype strain that has been extensively studied in animal models of cryptococcosis [13–15]. For some experiments, *C. neoformans* was killed by heating to 70°C for 30 min. To determine the effects of the *C. neoformans* capsule on allergic responses, some rats were infected with an acapsular strain of *C. neoformans,* cap 67 [16].

Infection and sensitization. Rats were anesthetized with isoflurane and intratracheally or intravenously inoculated with *C. neoformans* or PBS, as described elsewhere [13]. After infection, rats were sensitized by intraperitoneal injection of 1 mg of grade V ovalbumin (Sigma) in a 1.3% aluminum hydroxide adjuvant (Accurate Chemical and Scientific). A second intraperitoneal injection was given 10 days after the first.

Ovalbumin challenge. Two weeks after the first injection of ovalbumin (4 days after the last injection of ovalbumin), rats were challenged by exposure to a nebulized 1% ovalbumin solution for 12 min on 2 consecutive days. This was done using a compressor nebulizer system (Omron Health Care) that produces a flow from 4 to 8 L/min and particle sizes from 0.5 to 5 μm.

Bronchoalveolar lavage (BAL). An angiocatheter (BD Medical Systems) was inserted into the trachea. Four 10-mL volumes of sterile PBS were instilled and withdrawn. For differential leukocyte counts, cells were cytospun and identified with a Wright stain.

Fungal burden. The right lung was removed and homogenized in 5 mL of PBS containing a protease inhibitor cocktail (Roche Diagnostics), as described elsewhere [17]. Organ homogenates were plated on Sabouraud's dextrose agar. The remainder of the sample was used for cytokine studies.

IgE and ovalbumin-specific IgE ELISA. For determinations of total IgE levels, a capture ELISA using a goat–anti IgE (BD-Pharmingen Biosciences; 2 μg/mL) was done. Serial dilutions of

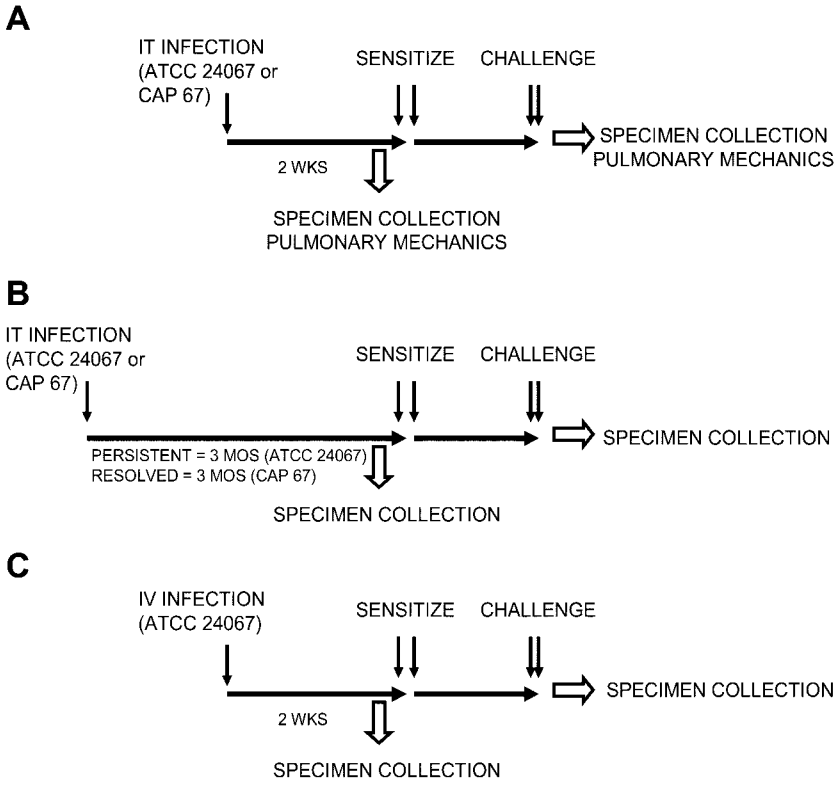

Figure 1. Overview of experimental design. *A,* For short-term pulmonary infection, rats were intratracheally infected with 10^4 *Cryptococcus neoformans* (strain 24067 or cap 67) and sensitized with ovalbumin 2 weeks after infection. *B,* For persistent pulmonary infection, rats were intratracheally infected with 10^7 *C. neoformans.* For resolved infection, rats were intratracheally infected with 10^4 *C. neoformans.* For both forms of infection, rats were sensitized to ovalbumin 3 months after inoculation with *C. neoformans. C,* To test the effects of systemic infection on allergic inflammation, rats were intravenously infected with 10^4 *C. neoformans* organisms and were sensitized 2 weeks later. IT, intratracheal; IV, intravenous; MOS, months; WKS, weeks.

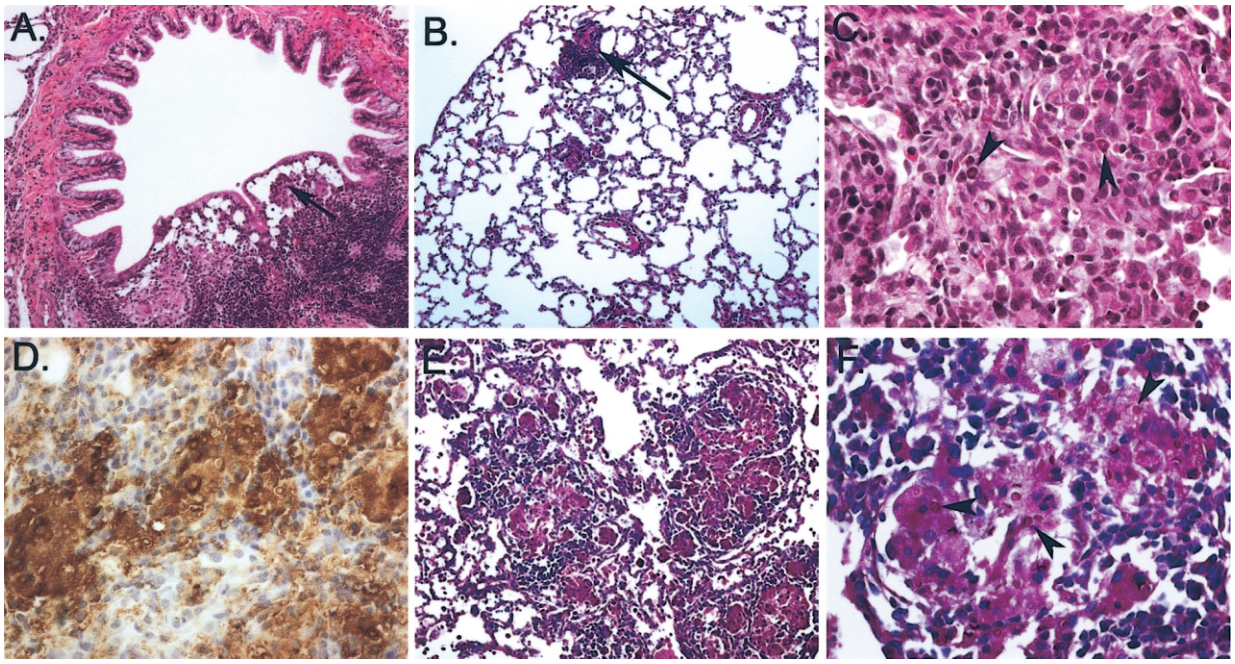

Figure 2. Histology of cryptococcal infection in sensitized and challenged rats. *A,* Endobronchial lesion in the lung of a persistently infected rat (hematoxylin-eosin stain; original magnification, ×50). Arrow, organisms underneath respiratory epithelium. *B,* Peripheral parenchymal lung disease *(arrow)* in rat with short-term infection due to strain 24067 (hematoxylin-eosin stain; original magnification, ×50). *C,* Granulomatous inflammation in the lung of a persistently infected rat (original magnification, ×200). *Arrowheads,* infiltrating eosinophils (hematoxylin-eosin stain; original magnification, ×400). *D,* Granulomatous area containing abundant cryptococcal polysaccharide *(brown)* in lung of rat with persistent pulmonary infection as demonstrated by immunohistochemistry (hematoxylin-eosin counterstain; original magnification, ×200). *E,* Extensive parenchymal disease in lung from a rat with systemic cryptococcal infection (periodic acid–Schiff stain; original magnification, ×50). *F,* Higher magnification (×200) of lung section in panel E shows numerous cryptococci *(arrowheads)* within affected area.

serum and a standard rat IgE (American Research Products) were applied, followed by applications of a biotin-labeled mouse anti–rat IgE (BD-Pharmingen Biosciences; 10 μg/mL) and alkaline phosphatase–labeled avidin (Vector Laboratories). For ovalbumin-specific IgE, sample IgE was captured as described above using goat anti–rat IgE. Biotin-labeled ovalbumin (10 μg/mL) was then applied, followed by alkaline phosphatase–labeled avidin (Vector Laboratories). Color was developed with 4-nitrophenyl phosphate (Southern Biotechnology Associates). Background absorbance at 405 nm from wells lacking serum was subtracted from experimental results. The highest dilution of serum giving an absorbance of 2.5 times the absorbance of serum from normal rats was defined as the titer.

Histological and immunohistochemical analyses. The post caval lung lobe was removed, placed in formalin, and then embedded in paraffin. Sections were cut and stained with hematoxylin-eosin. Periodic acid–Schiff and mucin staining were done to identify goblet cells. Staining for cryptococcal polysaccharide was done using a monoclonal antibody to cryptococcal polysaccharide, as described elsewhere [18].

Pulmonary resistance. Rats were anesthetized by inhalational exposure to isoflurane, and the trachea was intubated. A catheter was inserted into the esophagus to measure intrathoracic pressure. Rats were placed in a plethysmograph chamber (Buxco), ventilated with a tidal volume of 10 mL/kg at a rate of 70–80 breaths/min, and exposed to doubling doses of methacholine (Sigma) via nebulization. Baseline and airway resistance (resistance is defined as $cmH_2O/mL^{-1}s^{-1}$) following exposure to methacholine were measured. The dose of methacholine at which airway resistance doubled was determined by plotting the maximum airway resistance for each dose of methacholine and extrapolating from the curve.

Cytokine levels. Levels of tumor necrosis factor–α, interleukin (IL)-4, IL-10, and interferon-γ (IFN-γ) in supernatants of lung homogenates were determined using commercially available rat ELISA kits from BD-Pharmingen Biosciences. Levels of IL-12 and IL-13 were determined using rat ELISA kits from BioSource International.

Statistics. Cell counts were log-transformed and compared by a Student's *t* test. Total IgE and cytokine and chemokine levels were compared by a Student's *t* test. Ovalbumin-specific IgE titers were compared by Kruskal-Wallis one-way analysis of variance, and individual comparisons made using comparisons with *t* distribution (Unistat 5.5 software). $P < .05$ was considered to be statistically significant.

Experimental design. Rats were inoculated with *C. neofor-*

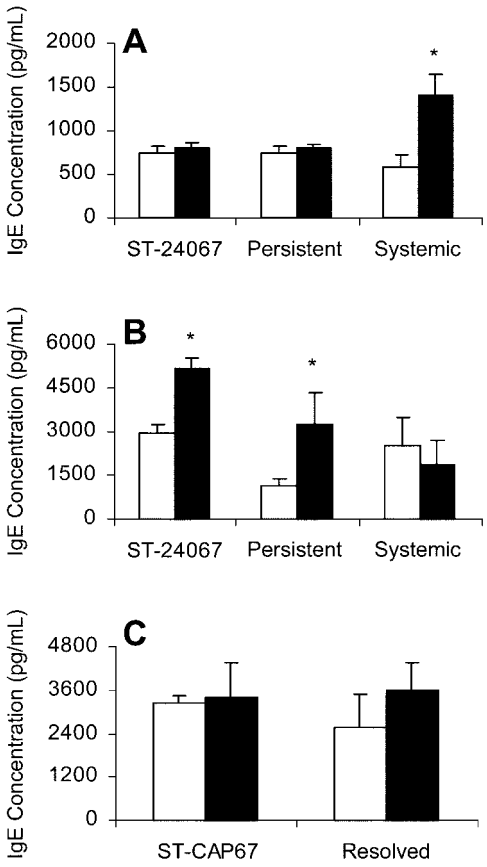

Figure 3. *A,* Serum IgE levels in rats ($n = 5$ rats/group) inoculated with *Cryptococcus neoformans* (strain 24067; *black columns*) or PBS (controls; *white columns*). For short-term infection with strain 24067 (ST-24067), rats were intratracheally infected with *C. neoformans,* and serum IgE measurements were done 2 weeks later. For persistent pulmonary infection, rats were inoculated intratracheally with *C. neoformans* or PBS, and serum IgE was measured 3 months later. For systemic infection, rats were intravenously injected with *C. neoformans* or PBS, and IgE determinations were done 2 weeks of later. *B,* Serum IgE concentrations for infected rats *(black columns)* and PBS-treated rats *(white columns)* ($n = 5$ rats/group) that were sensitized and challenged with ovalbumin. *C,* Serum IgE concentrations for sensitized and challenged rats that were intratracheally infected with strain cap 67 for 2 weeks (ST-CAP67) or 3 months ("Resolved") before sensitization. White columns denote rats inoculated with PBS; black columns denote rats infected with *C. neoformans.* Error bars denote 1 SD. $^*P \leq .05$, vs. PBS-treated control rats.

mans or PBS, sensitized with ovalbumin, and then challenged with ovalbumin. Four modes of infection were studied: short-term pulmonary infection (figure 1*A*), persistent pulmonary infection (figure 1*B*), resolved pulmonary infection (figure 1*B*), and systemic infection (figure 1*C*). For short-term pulmonary infection, rats were intratracheally inoculated with 10^4 *C. neoformans* organisms (either strain 24067 or cap 67) for 2 weeks before sensitization. As an additional control, some rats were inoculated with heat-killed *C. neoformans* 24067. For persistent pulmonary infection, rats were infected with 10^7 organisms (strain 24067), and ovalbumin sensitization was done 3 months later. This inoculum was chosen on the basis of our prior experience with establishing persistent infection with this strain [19]. For resolved pulmonary infection, rats were infected with 10^4 *C. neoformans* cap 67 organisms, and ovalbumin sensitization was done 3 months later. For systemic infection, rats were intravenously infected with 10^7 organisms (strain 24067), and sensitization was done 2 weeks later. For all modes of infection, aerosolized ovalbumin challenge was done 2 weeks after initial sensitization. After challenge, rats were analyzed for evidence of allergic inflammation. In separate experiments, the effects of infection alone (i.e., without sensitization) on allergic inflammation were determined for rats infected with strain 24067. For treatment experiments, rats were intratracheally inoculated with 10^4 *C. neoformans* (strain 24067) or PBS and 2 weeks later given fluconazole (20 mg/kg) or water daily by orogastric inoculation for an additional 2 weeks. Finally, the effects of short-term pulmonary infection with strain 24067 on airway responsiveness were studied.

RESULTS

Lung fungal burden and cryptococcal infection. Cryptococcal pulmonary infection was not associated with mortality or clinical symptoms of disease. In the context of sensitization and challenge, the mean fungal burden per lung at 1 month ($\pm$SD) was $\log_{10}$ 4.99 $\pm$ 0.11 and 3.90 $\pm$ 0.10 organisms for short-term infection with strains 24067 and cap 67, respectively. Fluconazole treatment decreased lung fungal burden by 1.3 $\log_{10}$ at 1.5 months of infection (4.3 $\pm$ 0.56 vs. 5.6 $\pm$ 0.15 organisms; $P < .001$). The mean lung fungal burden ($\pm$SD) of rats with persistent pulmonary infection at 3.5 months of infection was $\log_{10}$ 4.34 $\pm$ 0.12 organisms. Cryptococci were not detected in the lungs of rats with resolved pulmonary infection (limit of detection, $\log_{10}$ 1.7 organisms). After systemic infection, lung fungal burden for sensitized and challenged rats was $\log_{10}$ 5.99 $\pm$ 0.37 organisms at 1 month of infection. These rats also had significant extrapulmonary disease, as manifested by an average spleen fungal burden of $\log_{10}$ 2.74 $\pm$ 0.75 organisms. In contrast, there was no splenic infection in rats following intratracheal infection.

In the context of sensitization and challenge, short-term infection with either strain 24067 or cap 67 was associated with peribronchiolar disease. In some regions, extension of infection into the airway lumen was present (figure 2*A*). More disease and inflammation was present in the lungs of rats infected with strain 24067 than in lungs of rats infected with cap 67. Nevertheless, lung inflammation was qualitatively very similar, consisting of granulomas with scattered eosinophils within both the granuloma center and surrounding lymphocyte mantle (figure 2*C* and 2*D*). For rats with resolved infection, we were unable to observe any cryptococcal granulomas. The lungs of systemically infected rats contained numerous granulomas containing

Table 1. Effects of cryptococcal infection on allergic inflammation for sensitized and challenged rats.

Parameter	Short-term infection		Persistent infection	Systemic infection	Resolved infection
	ATCC 24067	cap 67			
IgE level	↑		↑		
Ovalbumin-specific IgE level	↑	↑	↑		
Eosinophil counts in bronchoalveolar fluid	↑	↑	↑		↑
Eosinophil counts in perivascular cuffs	↑	↑			
Goblet cell counts	↑	↑	↑	↑	
Interleukin-13 levels	↑↑	↑↑	↑↑	↑	
Interferon-γ levels				↑	

NOTE. *Arrow* indicates significant increase in parameter relative to PBS-inoculated control. For interleukin-13, *double arrows* indicates greater magnitude than *single arrow.* For short-term infection, rats were infected intratracheally with strains 24067 or cap 67 for 2 weeks before sensitization. For persistent infection, rats were infected for 3 months before sensitization. For systemic infection, rats were infected intravenously with strain 24067 for 2 weeks before sensitization. For resolved infection, rats were infected with strain cap 67 for 3 months before sensitization.

cryptococcal polysaccharide (figure 2*E* and 2*F*). In some areas, inflammation was so confluent that little air space was present.

IgE responses. Both short-term and persistent pulmonary infection with strain 24067 (in the absence of sensitization) failed to produce an increase in serum IgE, compared with that in rats inoculated with PBS alone (figure 3*A*). In contrast, systemic infection with the same strain resulted in an increase in serum levels of IgE, compared with that seen after PBS inoculation (1396 ± 737 vs. 584 ± 284 ng/mL; $P = .05$). In general, serum IgE levels were higher for sensitized and challenged rats than for nonsensitized rats, regardless of infection status. In the context of sensitization and challenge, certain forms of infection were associated with increased IgE relative to PBS inoculation. This includes short-term pulmonary infection with strain 24067 and persistent pulmonary infection (figure 3*B*; table 1). Fluconazole treatment of infected rats resulted in a small but statistically significant decrease in serum levels of IgE, compared with that in infected rats treated with PBS (4822 ± 156 vs. 5662 ± 255 ng/mL; $P < .001$). In the context of sensitization, short-term infection with cap 67, resolved pulmonary infection, and systemic infection were not associated with increases in serum levels of IgE, compared with that in rats inoculated with PBS (figure 3*C*). No differences in serum IgE levels were observed between rats inoculated with heat-killed *C. neoformans* and those inoculated with PBS (2619 ± 128 vs. 2800 ± 248 ng/mL; $P = .29$).

IgE specific to ovalbumin was not detected in either infected or PBS-treated rats that were not sensitized to ovalbumin. After ovalbumin sensitization and challenge, higher IgE titers to ovalbumin were present in rats with short-term pulmonary infection (both strains 24067 and cap 67) and persistent pulmonary infection than in PBS-inoculated rats (figure 4; table 1). Fluconazole treatment of short-term–infected rats resulted in a small but statistically significant reduction in levels of IgE to ovalbumin, compared with that in infected rats treated with water (median titers, 48 and 96, respectively; $P < .01$). Rats inoculated with heat-killed *C. neoformans* (data not shown) and rats with resolved pulmonary infection did not exhibit higher levels of IgE to ovalbumin than seen in PBS-inoculated rats. Furthermore, systemically infected rats that were sensitized demonstrated no increase in specific IgE, compared with sensitized controls.

Cellular inflammation. In the absence of sensitization and challenge, all forms of localized pulmonary infection with strain 24067 (i.e., short-term and persistent) resulted in a small increase in the total number of cells in BAL fluid relative to PBS-inoculated controls (figure 5*A*). An increase in the percentage of eosinophils among cells in BAL fluid relative to that in PBS controls was also noted for all forms of infection with strain 24067 (i.e., short-term, persistent, and disseminated) (figure 5*B*). Total cell counts in BAL fluid (data not shown) and eosinophil percentages were generally higher for sensitized and challenged rats than for their nonsensitized counterparts. In the context of sensitization, certain forms of infection were associated with increased eosinophil percentage relative to PBS-inoculated rats (table 1; figure 5*C*): short-term pulmonary infection (strains 24067 and cap 67), persistent pulmonary infection, and resolved infection. No increase in number of cells in BAL fluid or eosinophil

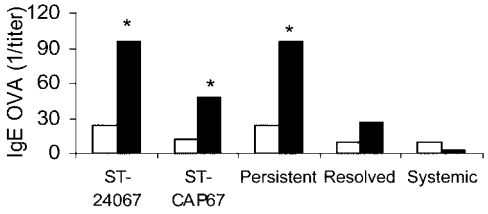

Figure 4. Median IgE ovalbumin (OVA) titers for sensitized and challenged rats in the context of various forms of *Cryptococcus neoformans* infection. White columns denoted infected rats, whereas the black columns denote PBS-treated control rats. "ST-24067" and "ST-CAP67" denote short-term infection with *C. neoformans* strains ATCC 24067 and cap 67, respectively, for 2 weeks before sensitization. "Resolved" denoted infection for 3 months before sensitization. For all experiments, bars represent median titers. Titers were compared with Kruskal-Wallis one-way analysis of variance, and group comparisons were done using comparisons with *t* distribution. *$P < .05$, infected vs. noninfected rats.

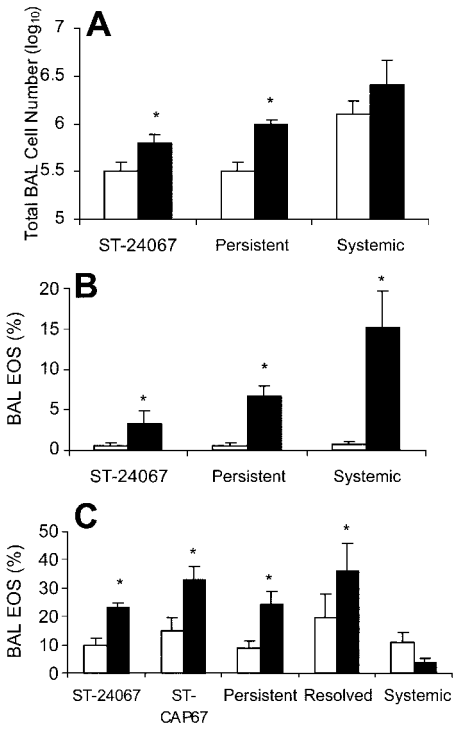

Figure 5. Effects of various forms of *Cryptococcus neoformans* infection on cell and eosinophil (EOS) counts in bronchoalveolar lavage (BAL) fluid. *A,* Total cell count in BAL fluid for rats inoculated with strain 24067 *(black columns)* or PBS *(white columns). B,* Percentage of EOS in total BAL cells for rats inoculated with strain 24067 *(black columns)* or PBS *(white columns). C,* Percentage of EOS in total BAL cells for rats inoculated with *C. neoformans (black columns)* or PBS *(white columns)* that were subsequently sensitized and challenged with ovalbumin. "ST-24067" and "ST-CAP67" denote short-term infection with *C. neoformans* strains ATCC 24067 and cap 67, respectively, for 2 weeks before sensitization. "Resolved' denotes infection for 3 months before sensitization. Five rats per group were used. Mean and SD (error bar) are shown. $*P < .05$, vs. PBS-treated control rats.

percentage was present for rats inoculated with heat-killed *C. neoformans,* compared with that in PBS-inoculated rats (data not shown). Furthermore, sensitized, systemically infected rats failed to demonstrate an increase in the percentage of eosinophils in BAL fluid relative to that seen in PBS-inoculated controls (figure 5*C*), although an increase in total number of cells in BAL fluid was present (data not shown).

In addition to examining BAL fluid for eosinophils, the histology of lung tissues was studied. For ovalbumin-sensitized and challenged rats, an increase in lung inflammation, primarily in the form of perivascular inflammation, was noted relative to that in nonsensitized rats, regardless of infection status. Within these perivascular cuffs, a higher proportion of eosinophils was present. In the context of sensitization, short-term infection with either strain 24067 or cap 67 was associated with an increase in the size and proportion of eosinophils within these perivascular cuffs relative to that seen in PBS-inoculated rats (figure 6*A* and 6*B*; table 1) In contrast, persistent infection, resolved pulmonary infection, systemic infection, and inoculation of heat-killed organisms were not associated with increased cuff size or eosinophil number relative to those parameters in PBS-inoculated controls.

An increase in the number of goblet cells in the large and medium-sized bronchioles of rats with active cryptococcal infection relative to that in control rats was readily apparent (figure 6*C* and 6*D*). This effect was present regardless of ovalbumin sensitization and challenge status and was observed for rats with short-term (both 24067 and cap 67), persistent, and disseminated infection (table 1). No increase in goblet cells was observed in the lungs of rats inoculated with heat-killed *C. neoformans* or in those with resolved pulmonary infection.

Pulmonary resistance. For these studies, rats infected short-term with strain 24067 were exposed to methacholine, with and without sensitization to and challenge with ovalbumin. Baseline airway resistance was not different between infected and noninfected rats. For nonsensitized rats, baseline resistance for noninfected and infected rats was 0.43 ± 0.13 versus 0.38 ± 0.04 $cmH_2O/mL^{-1}s^{-1}$ ($P = .2$). A lower dose of methacholine induced a doubling of airway resistance for infected rats, compared with that in noninfected rats. This effect was observed regardless of ovalbumin sensitization and challenge status (figure 7).

Cytokines. Levels of tumor necrosis factor–α, IL-10, and

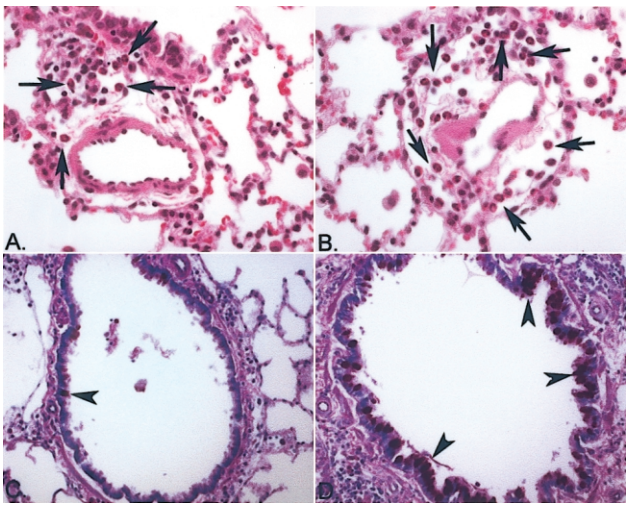

Figure 6. Perivascular inflammation and Goblet cells. *A,* Perivascular cuff in lung from a PBS-treated rat that was also sensitized and challenged with ovalbumin. Scattered eosinophils *(arrows)* are seen. (hematoxylin-eosin stain; original magnification, ×400) *B,* Perivascular cuff in the lung from a rat that was infected short-term with strain 24067 and also sensitized and challenged with ovalbumin. Increased number of eosinophils *(arrows)* are present, compared with PBS control (hematoxylin-eosin stain; original magnification, ×400). *C,* Goblet cells in respiratory epithelium from a PBS-inoculated rat from short-term experiments. *Arrow,* goblet cell (periodic acid–Schiff stain; original magnification, ×100) *D,* Goblet cells in respiratory epithelium from a rat infected short-term with strain cap 67.

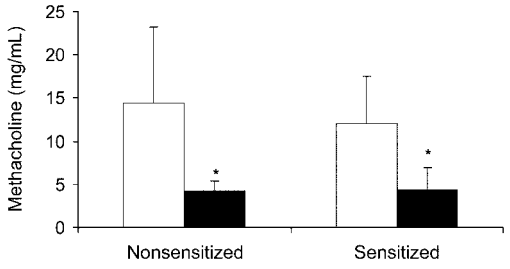

Figure 7. Airway hyperresponsiveness to methacholine for short-term-infected rats *(black columns)* and PBS-inoculated rats *(white columns)* in the absence and presence of sensitization and challenge. Shown are the mean concentrations of methacholine at which baseline airway resistance doubled. Error bars represent 1 SD. $*P < .05$.

IL-13 in lungs were increased with all forms of active infection (i.e., short-term, persistent pulmonary, and disseminated) with strain 24067, compared with levels in uninfected controls (figure 8*A*). However, lower levels of IL-13 in lung were present in disseminated infection compared with local pulmonary infection. Furthermore, disseminated infection was associated with increased levels of IFN-γ in lung, whereas pulmonary infection was not. Neither IL-4 nor IL-12 was detected in lung homogenates, regardless of infection status. The pattern of cytokines in lungs of infected, sensitized, and challenged rats was similar to that observed in rats that were infected but not sensitized (figure 8*B*; table 1). The same pattern was observed for rats infected short-term with either strain 24067 or cap 67 (not shown). Inoculation of heat-killed *C. neoformans* and resolved infection had no detectable effects on measured cytokines in lungs.

DISCUSSION

Many infections have been associated with exacerbation of reactive airway disease in animal models [20, 21], and there is considerable circumstantial evidence suggesting that microbes may contribute to the pathogenesis of human asthma (reviewed in [22, 23]). There is also evidence indicating that infections that elicit Th2-type polarized responses and chronic inflammation may be associated with asthma [24, 25]. Our decision to investigate a potential mechanistic link between *C. neoformans* infection and reactive airway disease was based on 4 observations: serological studies indicate a high prevalence of cryptococcal infection in children in the borough of the Bronx in New York City [7, 8]; *C. neoformans* infections are often chronic, with minimal or no symptoms [26]; there is a remarkably high prevalence of asthma among children in the Bronx [9]; and *C. neoformans* infections in certain hosts elicit Th2 polarized immune responses [27].

Like humans, rats can effectively contain cryptococcal infection within the lung, and this infection can persist for prolonged periods [13]. Thus, rats can be used to study the effects of cryptococcal infection on allergic inflammation in the context of an effective immune response. Infected rats manifested increased allergic inflammation in response to ovalbumin challenge, including increased total serum IgE levels, increased levels of IgE specific to ovalbumin, and increased numbers of eosinophils in BAL fluid. This effect occurred with both short- and long-term pulmonary infection but was present only with active infection and was not present with systemic infection. Pulmonary cryptococcosis also resulted in goblet cell hyperplasia and airway hyperreactivity. Hence, cryptococcal infection in the rat elicited inflammatory responses reminiscent of those found in patients with reactive airway disease.

Our results indicate that the tendency to develop enhanced allergic responses to ovalbumin in association with active pulmonary infection correlates with induction of IL-13 and lack of induction of IL-12 and IFN-γ. In contrast, systemic cryptococcal infection, which did not enhance allergic responses, elicited lower levels of IL-13 and higher levels of IFN-γ in the lung. The tendency of pulmonary cryptococcal infection to elicit Th2 polarization is likely a result of both host and pathogen

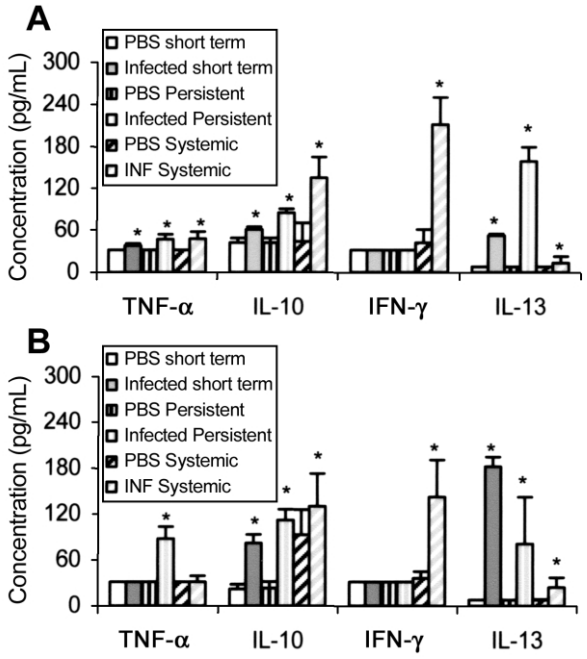

Figure 8. Lung cytokine profiles (IFN-γ, interferon-γ; IL, interleukin; TNF-α, tumor necrosis factor–α) in infected rats. *A,* Average lung cytokine concentrations for various forms of infection with *Cryptococcus neoformans* strain 24067. For "PBS Short Term" and "Infected Short Term," rats were infected intratracheally with PBS or strain 24067 for 2 weeks before lung cytokine determinations were made. For "PBS Persistent" and "Infected Persistent," rats were intratracheally inoculated with PBS or *C. neoformans* 3 months before lung cytokine determinations. For "PBS Systemic" and "Infected Systemic," rats were injected intravenously 2 weeks before lung cytokine determinations. *B,* Average lung cytokine levels in rats that were infected and subsequently sensitized and challenged. All cytokine levels represent the average of 5 rats per group. Error bar represents 1 SD. $*P < .05$, vs. PBS control.

characteristics. In murine studies, the virulence of *C. neoformans* is associated with the ability to promote Th2 polarization in pulmonary immune responses [10]. The precise mechanisms by which this occurs have not been fully defined, but fungal-mediated inhibition of IL-12 p40 production by IFN-γ–stimulated macrophages has been described in vitro [28]. Investigations have also focused on the role of the cryptococcal polysaccharide in this process [29–31]. In particular, *C. neoformans*–mediated suppression of IL-12 production by monocytes appears to be related to encapsulation, which is important in IL-10 induction and inhibition of phagocytosis [32]. Nevertheless, we found that encapsulation was not required for production of a Th2 response in this model. Recently, chitinase has been shown to be an important mediator of IL-13 allergic inflammation and airway hyperresponsiveness [33]. Given that the cell wall of *C. neoformans* contains chitin, it is possible that fungal chitinase induced in the host may be in part responsible for the observed effects of cryptococcal infection on allergic responses.

Although it may be argued that greatly enhanced allergic response of brown Norway rats in the context of cryptococcal infection is nonspecific and merely reflects the underlying Th2 bias of these animals, we note that inoculation of heat-killed *C. neoformans* and resolved pulmonary infection did not enhance allergic inflammation. This finding suggests that active infection is necessary for this phenomenon. We also found that systemic cryptococcosis failed to enhance allergic responses to ovalbumin, indicating that the type of cryptococcal infection is important. Furthermore, Hylkema et al. [34] have shown that not all infections in brown Norway rats enhance allergic inflammation. Specifically, these investigators found that infection with bacille Calmette-Guérin before sensitization and challenge with ovalbumin had no effect on allergic indices, whereas simultaneous administration of bacille Calmette-Guérin with sensitization resulted in decreased allergic inflammation. Together, these findings suggest that active local pulmonary cryptococcal infection is specifically responsible for the enhanced allergic responses within the lung.

Pulmonary cryptococcal infection was also associated with increased airway reactivity. A significantly lower dose of methacholine was required to induce doubling of airway resistance, compared with that in noninfected animals. This effect was present in the absence of sensitization and suggests a direct effect of infection on airway responsiveness. Increased airway responsiveness in association with ongoing infection has been reported in other animal models of infection, including *Mycoplasma pneumoniae* [35], influenza A virus [36], and respiratory syncytial virus [37]. Likewise, airway hyperresponsiveness in humans occurs in association with respiratory infections due to *Pneumocystis carinii* and respiratory syncytial virus [38–40]. Of note, we did not observe an increase in methacholine response in association with ovalbumin sensitization and challenge despite increased eosinophil content of BAL fluid and ovalbumin-specific IgE. Discrepancies between allergic inflammation and airway hyperresponsiveness have been described elsewhere [41]. It is possible that other factors, specifically airway remodeling in association with infection, are an important determinant of the observed increase in airway responsiveness in this model.

In our studies, fluconazole treatment partially ameliorated the enhancement of allergic inflammation associated with cryptococcal infection, as manifested by decreased eosinophil content and lower titers of IgE specific to ovalbumin. This was, in turn, associated with decreased fungal burden in the lung and is consistent with the lack of allergic inflammation in rats with resolved pulmonary infection. Our findings echo reports describing improvement of asthma symptoms in association with antifungal therapy [42,43]. In one study, fluconazole therapy given to asthmatics with overt dermatophyte infection was associated with a clinical improvement in asthma symptoms [43].

In summary, our study shows that pulmonary *C. neoformans* infection may serve as a potent modifier or cofactor of both allergic responses and airway reactivity. On the basis of our data, we hypothesize that cryptococcal infection may enhance an established tendency to Th2 polarization in atopic persons. The ability of pulmonary cryptococcal infection to exacerbate allergic inflammation and airway hyperresponsiveness is particularly intriguing, because it relates to the problem of asthma in urban areas [9, 44], where the prevalence of subclinical cryptococcal infection is high [8]. In light of our findings, we suggest that human epidemiological studies are warranted to explore the potential contribution of subclinical pulmonary *C. neoformans* infection to the high prevalence of urban asthma.

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