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Robust Th1 and Th17 Immunity Supports Pulmonary Clearance but Cannot Prevent Systemic Dissemination of Highly Virulent *Cryptococcus neoformans* H99

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The present study dissected the role of a Th2 bias in pathogenesis of *Cryptococcus neoformans* H99 infection by comparing inhalational H99 infections in wild-type BALB/c and IL-4/IL-13 double knockout mice. H99-infected wild-type mice showed all major hallmarks of Th2 but not Th1/Th17 immunity in the lungs and lung-associated lymph nodes. In contrast, the IL-4/13$^{-/-}$ mice developed robust hallmarks of Th1 and Th17 but not Th2 polarization. The IL-4/IL-13 deletion prevented pulmonary eosinophilia, goblet cell metaplasia in the airways and resulted in elevated serum IgE, and a switch from alternative to classical activation of macrophages. The development of a robust Th1/Th17 response and classical activation of macrophages resulted in significant containment of H99 in the lungs of IL-4/13$^{-/-}$ mice compared with unopposed growth of H99 in the lungs of wild-type mice. However, IL-4/13$^{-/-}$ mice showed only 1-week longer survival compared with wild-type mice. The comparison of brain and spleen cryptococcal loads at weeks 2, 3, and 4 postinfection revealed that the systemic dissemination in IL-4/13$^{-/-}$ mice occurred with an approximate 1-week delay but subsequently progressed with similar rate as in the wild-type mice. Furthermore, wild-type and IL-4/13$^{-/-}$ mice developed equivalently severe meningitis/encephalitis at the time of death. These data indicate that the Th2 immune bias is a crucial mechanism for pulmonary virulence of H99, whereas other mechanisms are largely responsible for its central nervous system tropism and systemic dissemination. ***(Am J Pathol 2009, 175:2489–2500; DOI: 10.2353/ajpath.2009.090530)***

Cryptococcus neoformans is a leading cause of fatal mycosis in HIV-positive individuals in numerous countries around the globe.[1] *C. neoformans* is also a problem in organ transplant recipients, patients with hematological malignancies, and those undergoing immunosuppressive therapies.[2] Interestingly, over 50% of *C. neoformans* infections in the United States are reported in HIV negative patients, of which many express no apparent immune deficiencies.[3] This may be attributed to the emergence of new high-virulence strains of *C. neoformans* and a high potential of the organism to adapt to extreme environmental conditions and a variety of hosts.[3–6] The invasion of an immunocompetent host is possible when the microbes develop mechanisms that allow them to evade and/or modulate the immune responses to cause "immune deviation." Reports from *C. neoformans* infection models suggest that *C. neoformans* may exploit both of these mechanisms to achieve its virulence.[7,8]

Dissemination into the central nervous system (CNS) and the subsequent development of meningitis/encephalitis is the major cause of mortality in uncontrolled cryptococcosis.[5,9,10] The CNS infection is caused by secondary dissemination of *C. neoformans* from primary sites of infection (lung) and it occurs readily when pulmonary growth of *C. neoformans* is not controlled by the infected host. In mouse models, highly virulent *C. neoformans* strains such as H99, 145A, and NU2 pulmonary infections are associated with nonprotective immune responses and lethal dissemination/CNS pathology.[7,11–13] Therefore, it is generally accepted that the development of a protective response in the lungs is both

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sufficient and necessary to prevent systemic/CNS dissemination of *C. neoformans*.[14,15]

Successful clearance of *C. neoformans* is associated with the development of a Th1 immune response and subsequent classical activation of macrophages (CAM) in both human patients and mouse models.[11,16,17] The CAM is thought to be the major effector cell that destroys ingested cryptococci. Recently, Th17 immune response was also shown to play a protective role in *C. neoformans* infection.[18,19] In contrast, defects in the immune responses [T cell, tumor necrosis factor (TNF)-α, or interferon (IFN)-γ deficiencies][7,11,20–22] and/or deviation to a Th2 immunity promote alternative activation of macrophages (AAM).[17,23,24] The alternatively activated macrophages have been demonstrated to harbor *C. neoformans* and their presence is associated with uncontrolled growth of *C. neoformans* and severe lung pathology.[8,17,19] Therefore promotion of AAM has been postulated to be the mechanism by which Th2 bias promotes uncontrolled growth and persistence of *C. neoformans* in the lungs.[8,16,17,23,25–30]

C. neoformans H99 is a human isolate that expresses one of the highest virulence levels among *C. neoformans* strains used for experimental infections. This strain grows in the lungs in an uncontrolled fashion, readily disseminates into CNS, and causes 100% mortality in a variety of immunocompetent mouse strains such as C57BL/6, BALB/c, CB6129F2, and CBA/J mice.[8,13,31–33] The infections with H99 were previously shown to result in upregulation of several hallmarks of Th2 immunity in the lungs. This was associated with the expression of virulence factors by H99 including urease and PLB1.[8,32] These previous studies have suggested that promoting a shift to a non-protective Th2 immune response could be a mechanism of H99-induced virulence.

The goal of the present study was to test the hypothesis that inducing a Th2 immune bias is an important mechanism for H99 virulence. Using the double deletion of Th2-driving cytokines interleukin (IL)-4 and IL-13 in mice, we demonstrate that in the absence of IL-4 and IL-13, H99-infected mice switch from a robust immune response with the Th2 phenotype and AAM to that of a mixed Th1/Th17 phenotype and CAM. This response significantly improves control of pulmonary cryptococcal growth in the lungs. However, it does not protect against brain dissemination of *C. neoformans* and the high mortality associated with an H99 infection. This is the first report that a strong Th1/Th17 immune response and CAM is insufficient for significant protection against CNS invasion and high lethality associated with inhalation of a highly virulent *C. neoformans* H99.

Materials and Methods

Mice

IL-4/13$^{-/-}$ mice were generated and originally provided by Dr. Andrew McKenzie, Medical Research Council, London, UK[34] and were bred at the University of Michigan Animal Research Facility in specific pathogen-free conditions. Age matched wild-type control BALB/c mice were purchased from Charles River Laboratories, (Wilmington, MA). Following at least 1-week acclimatization period at the Arbor VA Medical Center Mice Veterinary Medical Unit, wild-type and IL-4/13$^{-/-}$ mice were infected and subsequently housed in pressurized BSL2 cubicles in microisolator cages covered with filter tops. Food/water was provided *ad libitum* and mice were monitored daily for the period of infection. At the time of data collection mice were humanely euthanized by CO_2 inhalation. All experimental procedures were approved by the University Committee on the Use and Care of Animals and VA Institutional Animal Care and Use and Committee.

C. neoformans

C. neoformans strain H99 (ATCC 208821) was recovered from 10% glycerol frozen stocks stored at −80°C and grown to stationary phase (at least 72 hours.) at 36°C in Sabouraud dextrose broth (1% neopeptone, 2% dextrose; Difco, Detroit, MI) on a shaker. The cultures were then washed in non-pyrogenic saline (Travenol, Deerfield, IL), counted on a hemocytometer, and diluted to 3.3×10^5 yeast cells/ml in sterile non-pyrogenic saline.[8]

Intratracheal Inoculation of C. neoformans

Mice were anesthetized via intraperitoneal injection of ketamine/xylazine (ketamine/xylazine 100/6.8 mg/kg/bw) and were restrained on a foam plate. A small incision was made through the skin covering the trachea. The underlying salivary glands and muscles were separated. Infection was performed by intratracheal injection of 30 μl (10^4 CFU) via 30-gauge needle actuated from a 1-ml tuberculin syringe with *C. neoformans* suspension (3.3×10^5/ml). After inoculation, the skin was closed with cyanoacrylate adhesive.[8,26]

Organ CFU assay

For determination of microbial burden in the lungs, small aliquots of dispersed lungs were collected following the digest procedure. For determination of brain and spleen CFU, the brains and spleens were dissected using sterile instruments, placed in 2 ml of sterile water, and homogenized. Series of 10-fold dilutions of the lung, spleen, and brain samples were plated on Sabouraud dextrose agar plates in duplicates in 10-μl aliquots and incubated at room temperature. *C. neoformans* colonies were counted 2 days later and the number of CFU were calculated on a per-organ basis.[8,26]

Lung Leukocyte Isolation

The lungs from each mouse were excised, washed in RPMI, minced with scissors, and digested enzymatically at 37°C for 30 minutes in 15 ml of digestion buffer per mouse [RPMI, 5% fetal calf serum, antibiotics, 1 mg/ml collagenaseA (Roche Diagnostics, Indianapolis, IN), and 30 μg/ml DNase (Sigma)] and processed as previously

described.[11,29] The cell suspension and tissue fragments were further dispersed by repeated aspiration through the bore of a 10-ml syringe and were centrifuged. Erythrocytes in the cell pellets were lysed by addition of 3 ml of NH_4Cl buffer (0.829% NH_4Cl, 0.1% $KHCO_3$, and 0.0372% Na_2-EDTA, pH 7.4) for 3 minutes followed by a 10-fold excess of RPMI. Cells were resuspended and a second cycle of syringe dispersion and filtration through a sterile 100-μm nylon screen (Nitex, Kansas City, MO) was performed. The filtrate was centrifuged for 25 minutes at 1500 $\times$ *g* in the presence of 20% Percoll (Sigma) to separate leukocytes from cell debris and epithelial cells. Leukocyte pellets were resuspended in 5 ml of complete RPMI media and enumerated on a hemocytometer following dilution in Trypan Blue (Sigma).

Lung-Associated Lymph Node Leukocyte Isolation

Individual lung-associated lymph nodes (LALNs) were excised. To collect LALN leukocytes, nodes were dispersed using a 3-ml sterile syringe plunger and flushed through a 70-μm cell strainer (BD Falcon) with complete media into a sterile tube, as described previously.[8] After being centrifuged at 12,000 rpm/min for 10 minutes, the supernatant was removed and the cell pellets were saved at −70°C for gene expression analysis by real-time reverse transcription polymerase chain reaction (PCR).

Lung Macrophage Isolation

Isolated pulmonary leukocytes (10×10^6 cells/ml) were seeded in six-well plates and cultured at 37°C for 1.5 hours. Plates were washed twice using phosphate-buffered saline to remove non-macrophage cells. Total RNA was collected from adherent cells and used for real-time reverse transcription PCR analysis.

Visual Identification of Leukocyte Populations

Macrophages, neutrophils, eosinophils, monocytes, and lymphocytes were visually counted in Wright-Giemsa-stained samples of lung cell suspensions cytospun onto glass slides. Samples were fixed/pre-stained for 2 minutes in a one-step methanol based Wright-Giemsa stain (Harleco, EM Diagnostics, Gibbstown, NJ) and stained using steps two and three of the Diff-Quik stain. This modification of the Diff-Quik stain procedure improves the resolution of eosinophils from neutrophils in the mouse. A total of 300 cells were counted for each sample from randomly chosen high-power microscope fields. The percentages of leukocyte subsets were multiplied by the total number of leukocytes to give the absolute number of specific leukocyte subsets in the sample.

Cytokine Production

Isolated lung leukocytes were diluted to 5×10^6 cells/ml and were cultured in 24-well plates with 2 ml of complete RPMI medium at 37°C and 5% CO_2 for 24 hours. Supernatants were separated from cells by centrifugation, collected, and frozen until tested. The cytokines TNF-α, IFN-γ, IL-12p35, IL-4, and IL-10 were quantified by enzyme-linked immunosorbent assay (ELISA) using DuoSet kits (R&D Systems, Minneapolis, MN) and OPT-EIA kits (BD Biosciences, San Jose, CA) following the manufacturer's specifications. All plates were read on a Versamax plate reader (Molecular Devices, Sunnyvale, CA).[8,26]

Real-Time PCR

Total RNA was prepared using RNeasy Plus Mini Kit (Qiagen) and first-strand cDNA was synthesized using SuperScriptIII (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cytokine mRNA was quantified with SYBR Green-based detection using an MX 3000P system (Stratagene, La Jolla, CA) according to the manufacturer's protocols. Forty cycles of PCR (94°C for 15 seconds followed by 60°C for 30 seconds and 72°C for 30 seconds) were performed on a cDNA template. The mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels and expressed as fold induction compared with uninfected mouse expression level assigned as 1.

Total Serum IgE Analysis

Serum was obtained from the blood samples collected by severing the vena cava of the mice during lung excision. Following centrifugation to separate cells, serum was removed and total IgE concentrations were assessed using an IgE-specific sandwich ELISA (BD PharMingen).[8,26]

Histology

Lungs were fixed by inflation with 1 ml of 10% neutral buffered formalin, excised *en bloc,* and immersed in neutral buffered formalin. After paraffin embedding, 5-μm sections were cut and stained with hematoxylin and eosin ± counterstained with mucicarmine. Sections were analyzed with light microscopy and microphotographs taken using Digital Microphotography system DFX1200 with ACT-1 software (Nikon Co, Tokyo, Japan).[8,26,29]

Calculations and Statistics

Statistical significance was calculated using Student's *t*-test for individual paired comparisons or *t*-test with Bonferroni adjustment whenever multiple groups were compared. Means with P values of <0.05 were considered significantly different. The survival was analyzed using Kaplan-Mayer analysis. All values are reported as means with their standard errors (SEM). Calculations were performed using Primer of Biostatistics software (McGraw-Hill, NY).

Table 1. Effect of Combined Deletion of IL-4 and IL-13 on Cytokine mRNA Expression Pattern in Leukocytes in *C. neoformans*-Infected Lungs

Gene	Strain	Week 2	Week 3
IL-4	Wild-type	80.63 ± 19.04	238.69 ± 87.39
	IL-4/13$^{-/-}$	ND*	ND*
IL-13	Wild-type	29.2 ± 7.27	46.84 ± 8.34
	IL-4/13$^{-/-}$	ND†	ND*
IL-5	Wild-type	4.47 ± 1.95	7.83 ± 1.93
	IL-4/13$^{-/-}$	2.16 ± 0.80	0.21 ± 0.09*
IL-10	Wild-type	5.60 ± 1.30	9.23 ± 0.96
	IL-4/13$^{-/-}$	9.36 ± 1.81	5.18 ± 0.09†
IFN-γ	Wild-type	1.11 ± 0.13	0.96 ± 0.32
	IL-4/13$^{-/-}$	45.06 ± 12.26†	70.93 ± 12.35*
TNF-α	Wild-type	0.41 ± 0.11	0.15 ± 0.03
	IL-4/13$^{-/-}$	0.81 ± 0.16‡	2.13 ± 0.44*
IL-12p35	Wild-type	1.70 ± 0.50	2.11 ± 0.50
	IL-4/13$^{-/-}$	2.78 ± 1.15	15.58 ± 4.97†
IL-17	Wild-type	1.43 ± 0.31	1.53 ± 0.37
	IL-4/13$^{-/-}$	105.66 ± 15.06*	109.50 ± 43.57†

Mice (IL-4/13$^{-/-}$ and wild-type) were infected with 10^4 CFU via the intratracheal route with *C. neoformans* H99 and analyzed at weeks 2 and 3 postinfection. Total RNA was extracted from lung leukocyte isolates, converted to cDNA, and analyzed by q-PCR for the expression of major "polarizing" cytokines. The data were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels, compared with baseline expression level in the uninfected mice (Calibrator), and expressed as fold induction. Data, pooled from separate matched experiments, are shown as the means ± SEM (n = 6 and above for each of the analyzed time points). ND, not detectable.

*$P < 0.001$.
†$P < 0.01$.
‡$P < 0.05$.

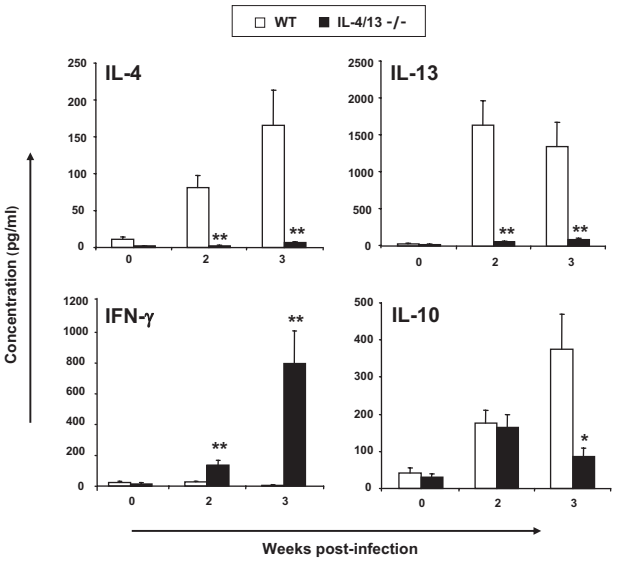

Figure 1. Effect of combined deletion of IL-4 and IL-13 on cytokine protein production by leukocytes isolated from *C. neoformans*-infected lungs. Mice, wild-type (WT) and IL-4/13$^{-/-}$, were infected with 10^4 CFU of *C. neoformans* H99 via the intratracheal route and were analyzed at weeks 2 and 3 postinfection. Lung leukocytes were isolated from infected and uninfected mice and cultured for 24 hours at 5×10^6 cells/ml. Cytokine levels were evaluated by ELISA in cell culture supernatants. Bars represent mean cytokine concentration ± SEM (pg/ml). *$P < 0.01$ and **$P < 0.001$ in comparison with the respective wild-type result.

Results

Combined Deletion of IL-4 and IL-13 Prevented Th2 and Promoted Th1 and Th17 Cytokine Responses in Lung Leukocytes

We hypothesize that promoting a non-protective Th2 immune response is a mechanism for H99-mediated virulence in the lungs.[8] IL-4 and IL-13 are known to be the major factors directing the development of Th2 immunity. Our first goal was to determine whether the combined deletion of IL-4 and IL-13 would prevent the expression of other cytokines associated with non-protective response/lung pathology (Th2) and promote induction of cytokines associated with protective responses (Th1 and Th17). We analyzed profiles of mRNA expression for cytokines using pulmonary leukocytes at weeks 2 and 3 postinfection with H99 including: IL-4, IL-5, IL-10, and IL-13 (associated with non-protective Th2 and/or regulatory responses); IFN-γ, TNF-α, and IL-12p35 (associated with protective Th1 response); and IL-17 (associated with beneficial Th17 response). The Th2 cytokine mRNA for IL-4 and IL-13 was highly up-regulated in the wild-type mice infected with H99 throughout the observed time course of infection, whereas induction of these cytokines was absent in the IL-4/13$^{-/-}$ mice (Table 1). Furthermore, IL-5 and IL-10 became increasingly elevated in the wild-type mice and were expressed at a significantly lower level by IL-4/13$^{-/-}$ mice at week 3. In contrast with the predominantly Th2/regulatory cytokine pattern observed in the wild-type mice infected with H99, the protective cytokine pattern was strongly enhanced in IL-4/13$^{-/-}$ mice (Table 1). IL-4/13$^{-/-}$ mice showed a robust induction of IFN-γ mRNA from week 2 onward, consistent with the development of a Th1 response. Furthermore, IL-12p35 mRNA was significantly elevated at week 3 in leukocytes from IL-4/13$^{-/-}$ mice compared with wild-type mice. TNF-α expression was somewhat up-regulated in IL-4/13$^{-/-}$ mice in contrast with the strong down-regulation observed in the wild-type mice infected with H99. In addition to the major shift from Th2 to Th1 cytokine profile observed in H99-infected lungs in the absence of IL-4 and IL-13, a significant increase in IL-17 expression in H99-infected IL-4/IL-13$^{-/-}$ lungs was evident, suggesting that IL-4/IL-13 gene deletion also resulted in an expansion of the Th17 response.

To confirm that these changes in cytokine gene expression mirrored changes in secreted cytokine protein, we evaluated cytokine production by leukocytes isolated from infected lungs. The levels of IL-4, IL-10, IL-13, and IFN-γ proteins in 24 hours lung-leukocyte culture supernatants were measured by ELISA (Figure 1). The Th2 cytokines IL-4, IL-13, and IL-10 were up-regulated in the leukocytes from wild-type mice infected with H99, while the major Th1 cytokine IFN-γ was produced by leukocytes from the infected IL-4/13$^{-/-}$ mice. The early changes in cytokine expression direct subsequent immune polarization in *C. neoformans*- infected mice.[7,11,21,26] We found that mRNA induction levels for pro-Th1 IL-12p35 and pro-Th17 IL-23p19 were significantly higher in IL-4/13$^{-/-}$ compared with wild-type mice at week 1 (1.64 ± 0.53 versus 0.37 ± 0.04 and 1.36 ± 0.22 versus 0.46 ± 0.01, respectively). In contrast, p40 shared by IL-12 and IL-23, but also forming inhibitory p40(2) homodimer, was

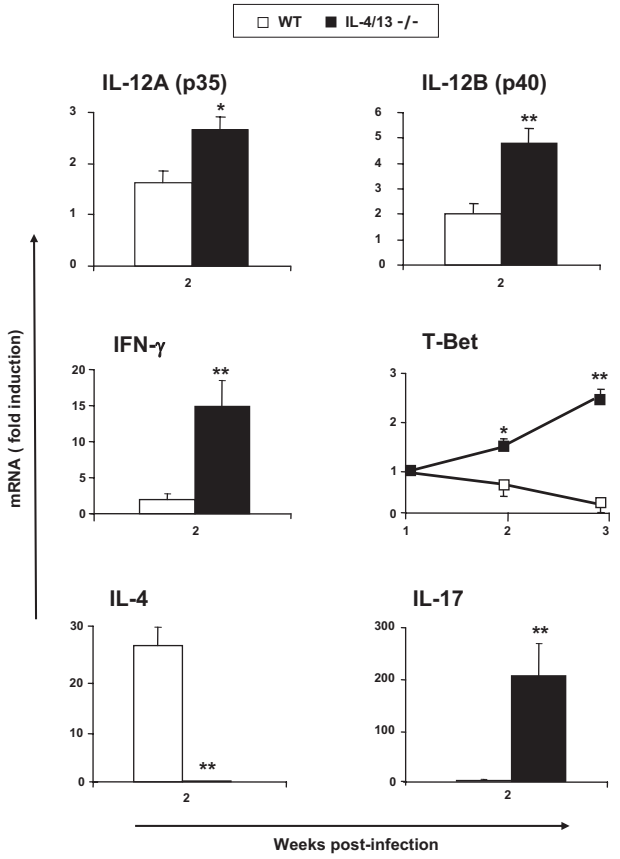

Figure 2. Effect of combined deletion of IL-4 and IL-13 on pulmonary lymph node polarization. Pulmonary lymph nodes were collected from uninfected and H99-infected WT and IL-4/13$^{-/-}$ mice and leukocytes were isolated. Total RNA was extracted, converted to cDNA, and analyzed by real-time RT-PCR for the expression of selected "polarizing" cytokines and the Tbet transcription factor. The data were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels, compared with baseline expression level in the uninfected mice (Calibrator), and expressed as fold induction. Data represent mean ± SEM (n = 6 and above for each of the analyzed time points). *$P < 0.01$, and **$P < 0.001$ in comparison with the respective wild-type result.

up-regulated in both infected groups (3.33± versus 3.32 ± 1.17 fold). Taken together, these data indicate that the combined deletion of IL-4 and IL-13 resulted in a major shift in the polarizing cytokine pattern in H99-infected lungs from the polarized Th2/regulatory cytokine profile to the robust Th1/Th17 cytokine profile.

Combined Deletion of IL-4 and IL-13 Promoted Th1 and Th17 Polarization in the Pulmonary Draining Nodes after H99 Infection

Our next objective was to evaluate if the changes that occurred in the lung immune response were paralleled by the changes of immune polarization in LALNs. We completed this analysis at the mRNA level. Consistent with the changes observed in the lungs, the cytokine profile showed reverse polarization patterns in the presence and absence of IL-4/IL-13 following infection with H99 (Figure 2); IL-12 (p35 and p40), IFN-γ, and IL-17 were significantly up-regulated in the LALNs of H99-infected IL-4/13$^{-/-}$ mice but not in those of the wild-type

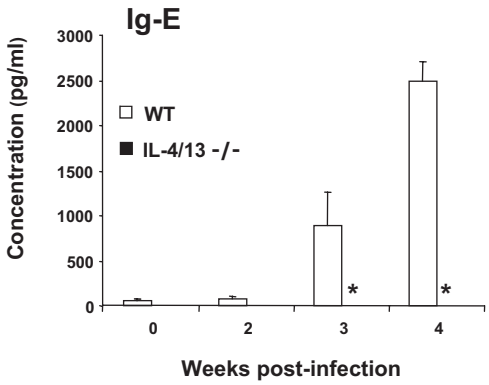

Figure 3. Effect of combined deletion of IL-4 and IL-13 on the production of serum IgE. Blood was collected from uninfected and H99-infected WT and IL-4/13$^{-/-}$ mice (weeks 2–4), sera separated, and analyzed for IgE content by ELISA. Bars represent mean serum IgE concentration ± SEM (pg/ml) (n = 6 and above for each of the analyzed time points). *$P < 0.001$ in comparison with the respective wild-type result.

mice. Furthermore, expression of the Th1 transcription factor Tbet was increasingly up-regulated over time in LALNs of infected IL-4/13$^{-/-}$ mice. In contrast, the wild-type mice showed dramatic up-regulation of IL-4 in the LALNs with a concurrent decrease in Tbet expression. These finding in LALNs are consistent with the cytokine mRNA and protein expression in infected lungs, providing strong evidence that concurrent deletion of IL-4 and IL-13 not only prevented Th2 development but also led to a strong Th1 and Th17 skewing in the lungs and LALNs post-H99 infection.

Combined Deletion of IL-4 and IL-13 Prevented Systemic IgE Accumulation

Our data have documented that H99 infection in mice results in accumulation of serum IgE (a systemic hallmark of a Th2 response).[26,29] To determine whether the IL-4 and IL-13 double deletion would prevent systemic IgE accumulation, we compared serum IgE levels between wild-type and IL-4/13$^{-/-}$ mice infected with H99 (Figure 3). The wild-type mice showed a cumulative increase in serum IgE levels, which is consistent with a Th2 response. In contrast, IgE production was completely abolished by the deletion of IL-4 and IL-13. These data provide further evidence that deletion of IL-4 and IL-13 results in a fundamental change in the adaptive response to H99, including the absence of the major Th2 hallmark (IgE antibody class switching) following the infection with H99.

Combined Deletion of IL-4 and IL-13 Increased the Pulmonary Leukocyte Recruitment in C. neoformans*-Infected Mice*

The deletion of IL-4 and IL-13 combined with the H99 infection resulted in a shift from the undesirable Th2 response to the Th1 and Th17 responses that play an important role in anti-cryptococcal protection. Our next goal was to evaluate whether these protective responses

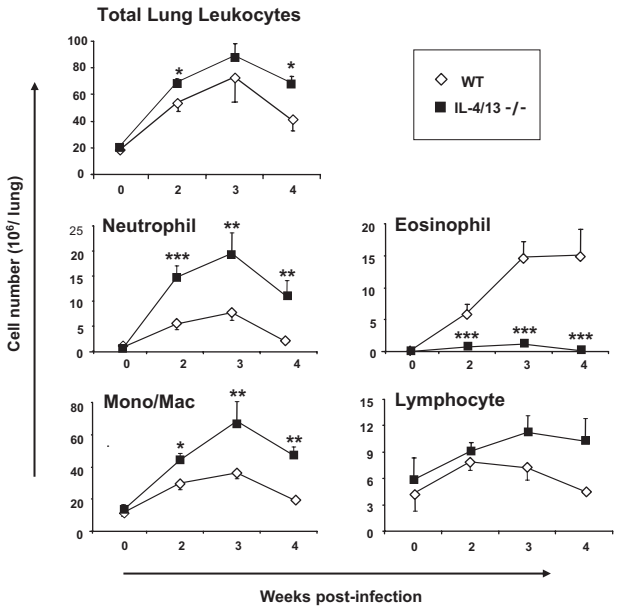

Figure 4. Effect of combined deletion of IL-4 and IL-13 on magnitude of inflammatory response and recruitment of leukocyte subsets into the *C. neoformans*-infected lungs. Lungs were collected from uninfected and H99-infected WT and IL-4/13$^{-/-}$ mice (weeks 2, 3 and 4) and dispersed enzymatically. Leukocytes were isolated from individual mice and enumerated by a hemacytometer. Lung leukocyte subsets were assessed by microscopic evaluation of relative frequencies of each subset on stained cytospin slides and calculated (frequency × total cell count). Values represent mean number ± SEM. Data were pooled from five parallel experiments ($n = 6$ and above for each of the analyzed time points). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ in comparison with the respective wild-type result.

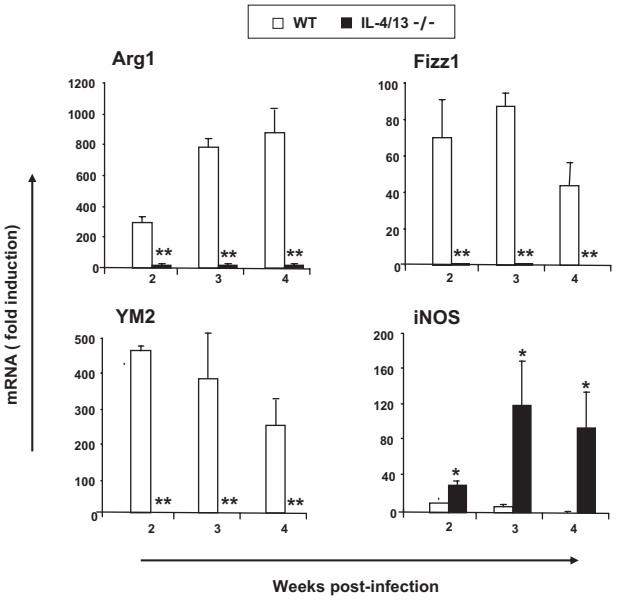

Figure 5. Effect of combined deletion of IL-4 and IL-13 on pulmonary macrophage activation profile. Lung leukocytes were isolated from uninfected and H99-infected WT and IL-4/13$^{-/-}$ mice. The macrophage population was enriched by 90-minute adherence and removal of nonadherent cells. RNA was extracted and analyzed as described above to quantify alternative (Arg1, YM2, and FIZZ1) versus classical activation (iNOS) gene expression. The data were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels and are expressed as fold-induction in comparison with baseline expression in macrophages from uninfected animals. Bars represent mean fold expression ±SEM ($n = 6$ and above for each of the analyzed time points). *$P < 0.01$ and **$P < 0.001$ in comparison with the respective wild-type result.

were robust enough to afford protective effects. To address this question, we performed a kinetic analysis of pulmonary leukocyte numbers at weeks 2, 3, and 4 postinfection from mice infected with *C. neoformans* strain H99. Leukocyte subsets were evaluated in enzymatically digested lungs at weeks 2, 3, and 4 postinfection. Both wild-type and IL4/13$^{-/-}$ mice developed significant inflammatory responses in the lungs following H99 infection. However, significantly greater numbers of leukocytes were recruited into the lungs of IL-4/13$^{-/-}$ mice compared with wild-type mice (Figure 4), indicating that the inflammatory response in the absence of IL-4 and IL-13 was more robust. Subsequently, the leukocyte subsets were evaluated by morphological analysis of cell subsets by microscopy (Figure 4). Significant differences in the composition of the inflammatory cells in the lungs were detected between wild-type and IL4/13$^{-/-}$ mice. The wild-type mice infected with H99 demonstrated significant pulmonary eosinophilia, a marker of Th2 response/pathology.[29,35] The IL-4/IL-13 deletion abrogated pulmonary eosinophilia, which was "replaced" by significant recruitment of neutrophils (one of the hallmarks of Th17 adaptive response), and increased numbers of mononuclear myeloid cells, which serve as effector cells in cryptococcal clearance during Th1 response.[36] The IL-4/IL-13 deletion also resulted in enhanced recruitment of lymphocytes, suggesting that the response that developed in the absence of these cytokines was robust. These cellular data are not only consistent with the replacement of Th2 response in wild-type mice with a mixture of Th1/Th17 response in IL-4/IL-13$^{-/-}$ mice but also demonstrate that these responses were vigorous.

Combined Deletion of IL-4 and IL-13 Prevented AAM and Promoted CAM in the Lungs

The non-protective Th2 response to H99-infected lungs has been associated with evidence of AAM, while protective responses to *C. neoformans* are thought to be performed by CAM. Our next goal was to compare macrophage phenotypes between wild-type and IL-4/IL-13$^{-/-}$ mice infected with H99. We performed analysis of AAM and CAM hallmark gene expression in adherence purified macrophages isolated from H99 infected lungs at weeks 2, 3, and 4 postinfection. The arginase (Arg1), chitinase family protein YM2, found in inflammatory zone 1 protein (FIZZ1), and inducible nitric oxide synthase (iNOS) were compared. Macrophages from wild-type mice infected with H99 showed the following: 1) up to 900-fold up-regulation of macrophage Arg1; 2) 450-fold up-regulation of YM2; 3) 85-fold up-regulation of FIZZ1; and 4) minimal or no induction of iNOS (Figure 5), consistent with a polarized AAM phenotype throughout the analyzed time course of infection in wild-type mice. In contrast, the dramatic up-regulation of iNOS gene expression (up to 140-fold) with AAM gene expression showing near-baseline levels was found in the infected IL-4/IL-13$^{-/-}$ mice (Figure 5) consistent with a CAM phenotype developing in the IL-4/13$^{-/-}$ mice. Thus, the com-

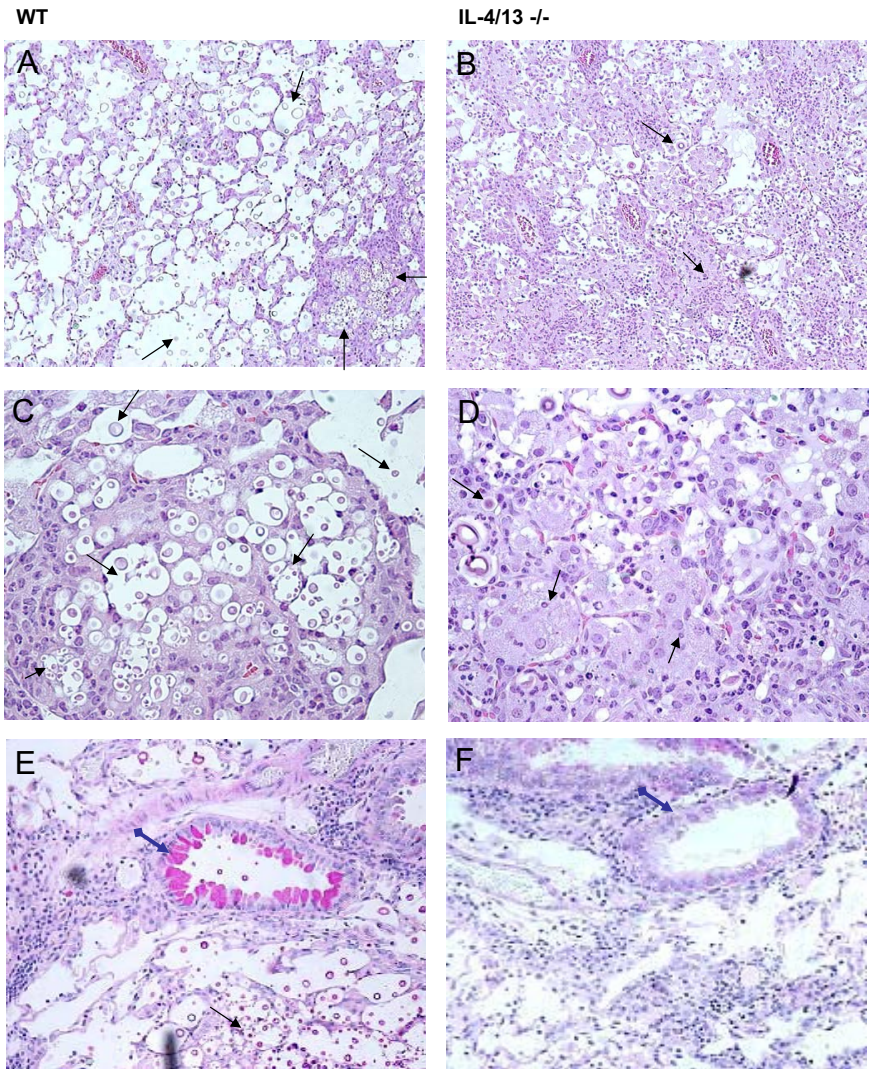

Figure 6. Effect of combined deletion of IL-4 and IL-13 on morphological pattern of pulmonary inflammation and pathological lesions in *C. neoformans* infected lungs. Lungs from H99-infected WT (**A**, **C**, **E**) and IL-4/13$^{-/-}$ (**B**, **D**, **F**) mice were perfused with buffered formalin, fixed, and processed for histology at week 3 postinfection. Representative photomicrographs of H&E + mucicarmine-stained slides taken at 10× (**A**, **B**) and 40× (**C**, **D**) objective power. Slides stained with H&E with PAS (**E**, **F**) were photographed with 20× objective. Note that numerous *C. neoformans* organisms (**black arrows**) are widespread in the lungs of wild-type mice (**A**, **C**, **E**) in both inflamed and inflammation-free portions of the lung. Evidence of cryptococcal growth clusters can be found within the extended macrophages (**C**); numerous PAS-positive goblet cells protrude into the lumen of the airways (**E** and **F**, **blue arrow**). In contrast, IL-4/13$^{-/-}$ lungs (**B, D, F**) show a few singular *C. neoformans* organisms contained in dense inflammatory infiltrates (**D**), foamy macrophages contain partially destroyed ingested yeasts (**D**), and the goblet cells are absent from the airways (**F**).

bined deletion of IL-4 and IL-13 promoted classical rather than alternative activation of macrophages following infection with H99.

Histology Shows Evidence of Improved Host Responses in the Lungs of IL-4/IL-13$^{-/-}$ Mice

Characterization of the immune phenotype strongly suggested that the deletion of IL-4/IL-13 may result in improved host responses and protection from Th2-driven pathology. To determine the effect of combined deletion of IL-4 and IL-13 on lung pathology, we performed histopathological examination of H99-infected lungs at week 3 postinfection (Figure 6).

Wild-type mice showed widespread *C. neoformans* presence throughout a significant portion of their lungs (Figure 6 A, C, E). In many instances yeast cells were not accompanied by leukocytes, which suggest that H99 effectively evaded the immune response. Furthermore, in the inflamed areas of the lungs, H99 formed clusters, which are indicators of rapid cryptococcal growth in the presence of host leukocytes (predominantly eosinophils and macrophages). Large extended macrophages containing multiple cryptococci with large capsular "halos" provide evidence of decreased intracellular killing and increased intracellular growth of *C. neoformans* within macrophages (Figure 6, A and C), which is consistent with their AAM phenotype (Figure 5). In contrast, the lungs of IL-4/13$^{-/-}$ mice showed a robust inflammatory response with much fewer *C. neoformans* visible within the lung field (Figure 6 B, D, F). Furthermore, visible yeast cells are contained by leukocyte infiltrates and do not form cystoid clusters in the lungs of IL-4/13$^{-/-}$ mice. Most of the yeast cells were found as individual cells, suggesting that the growth of H99 was efficiently controlled in these mice (Figure 6D). Furthermore, many *C. neoformans* appeared to be within activated macrophages with morphology, suggesting intracellular killing of digested organism by macrophages. Lack of evidence of intracellular parasitism within macrophages and macrophage morphology in the IL-4/13$^{-/-}$lungs (Figure 6, B and D) is consistent with their CAM molecular profiles (Figure 5). In addition to differences in the magnitude, composition, and appearance of inflammatory infiltrates,

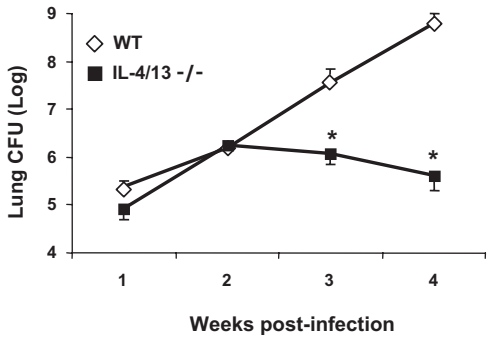

Figure 7. Effect of combined deletion of IL-4 and IL-13 on fungal clearance in the lungs. Mice, WT and IL-4/13$^{-/-}$), were infected intratracheally with *C. neoformans* H99, as described above, and lungs were harvested at 1, 2, 3, and 4 weeks postinfection and dispersed for analysis of fungal burden. *C. neoformans* loads were evaluated via CFU assays performed on serially diluted samples. Data, pooled from four separate matched experiments, are expressed as the mean CFU per lung ± SEM ($n = 12$ and above for each of the analyzed time points). *$P < 0.001$ in comparison with the respective wild-type result.

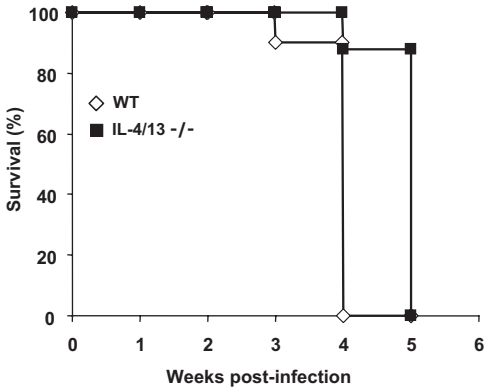

Figure 8. Effect of combined deletion of IL-4 and IL-13 on mouse survival following pulmonary infection with *C. neoformans* H99. Mice, WT and IL-4/13$^{-/-}$, were intratracheally infected with10^4 *C. neoformans* and survival time was analyzed for 5 weeks. Moribund animals were humanely euthanized and deaths were recorded weekly.

we observed differences in airway pathology between wild-type and IL-4/13$^{-/-}$ mice. The wild-type lungs showed enhanced mucus production with strong PAS-positive staining, and swelling of the airway epithelium providing evidence of increased goblet cell metaplasia, a marker of Th2 pathology (Figure 6E). In contrast, the bronchial epithelium appeared less distended and was free of mucus producing goblet cells in the infected IL-4/13$^{-/-}$ mice (Figure 6F). Collectively, these data indicate that the deletion of IL-4 and IL-13 facilitated protective responses that defended the lungs from rapidly spreading infection and prevented the development of Th2-driven pathology.

Combined Deletion of IL-4 and IL-13 Decreased the Pulmonary Microbial Load

Our data showed that IL-4/13$^{-/-}$ mice were not only protected from the development of detrimental Th2 response and AAM development, but also that they developed a robust response that appeared protective. Our next goal was to determine whether all these changes in the immunophenotype were sufficient to promote clearance of H99 infection from the lungs. The pulmonary microbial loads of infected wild-type and IL-4/13$^{-/-}$ animals were compared at weeks 1, 2, 3 and 4 postinfection. The wild-type mice succumbed to logarithmic growth of H99 in the lungs (Figure 7), demonstrating that both early innate and the adaptive Th2 immune responses in the wild-type mice could not control H99 growth. The IL-4/13$^{-/-}$ mice demonstrated similar increases in the microbial burden during the initial 2 weeks postinfection (the innate phase of the immune response); however, significant containment of H99 growth in the lungs was observed at weeks 3 to 4 postinfection (Figure 7). This time frame from week 2 to week 4 is consistent with the efferent phase of the immune response during which IL-4/13$^{-/-}$ mice eliminated on average 75% of the microbes from their lungs compared with the peak burden at week 2 postinfection. The comparison of microbial burdens between wild-type and IL-4/13$^{-/-}$ mice at weeks 3 and 4 postinfection show 1.5 log and 3.5 log differences, respectively, indicating that the IL-4/IL-13 deletion resulted in significant improvements in the antimicrobial responses. Thus, the double deletion of IL-4/13 and the subsequent changes of the adaptive immune response to H99 infection resulted in marked improvement in the control of pulmonary growth of H99 by the host.

Combined Deletion of IL-4 and IL-13 Did Not Provide a Long-Term Protection

Having determined that the double deletion of IL-4/IL-13 resulted in marked improvement in cryptococcal containment and protection of the lungs from Th2-driven pathology, we sought to determine whether IL-4 and IL-13 double deletion would provide a long-term survival benefit to the infected mice. We compared the survival time of wild-type and IL-4/13$^{-/-}$ mice in our experimental H99 infection. The wild-type mice demonstrated 100% mortality with sudden onset between weeks 3 and 4 postinfection (Figure 8). By the end of the fourth week postinfection, all wild-type mice showed severe neurological and respiratory symptoms, which resulted either in spontaneous death or the need for euthanasia. At this time virtually all IL-4/13$^{-/-}$ mice did not present severe symptoms and only 10% mortality was observed. However, during week 5, IL-4/13$^{-/-}$ mice demonstrated rapid worsening and increasing mortality. Thus, the combined deletion of IL-4 and IL-13 did not prevent the eventual mortality in H99-infected mice and extended maximum survival time for only 1 week despite significant protection from pulmonary microbial growth.

Combined Deletion of IL-4 and IL-13 Could Not Prevent CNS Dissemination of C. neoformans *after Pulmonary Infection with H99*

The finding that combined deletion of IL-4 and IL-13 did not provide long-term survival benefit could not be pre-

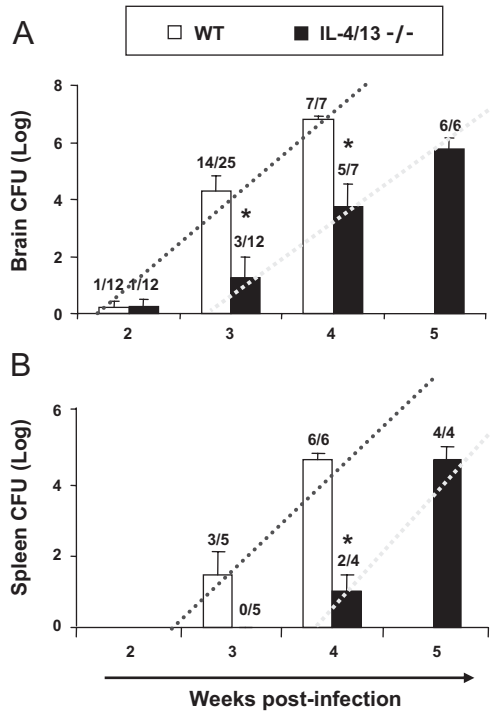

Figure 9. Effect of combined deletion of IL-4 and IL-13 on fungal dissemination and growth in the brains and spleens following pulmonary infection with *C. neoformans* H99. Mice, WT and IL-4/13$^{-/-}$, were infected intratracheally with10^4 *C. neoformans*, and brains and spleens were collected at 2, 3, 4, and 5 weeks postinfection for analysis of fungal burden. Data, pooled from four separate matched experiments, are expressed as the mean CFU per organ ± SEM ($n = 4$ and above for each of the analyzed time points). *$P <$ 0.01 in comparison with the respective wild-type result.

dicted based on the pulmonary microbial load at adaptive immune stage. Since CNS dissemination is the major cause of mortality in both experimental and clinical infections with *C. neoformans*, we compared *C. neoformans* burdens within the brains of infected mice at weeks 2, 3, and 4 post-intratracheal infections (Figure 9A). Additionally, spleen CFUs were evaluated as a surrogate of systemic dissemination of *C. neoformans* (Figure 9B). The wild-type mice demonstrated significant brain and spleen dissemination with a significant fraction of animals showing positive brain and spleen cultures at week 3 (at the onset of massive mortality). At week 4, 100% of infected wild-type animals demonstrated positive brain cultures with the load exceeding 10^6 CFU and spleen cultures with the load exceeding 10^5 CFU, which was consistent with lethal brain infection/morbidity (Figure 9, A and B). In contrast with the strong effect on the H99 growth rate in the lungs, IL-4/IL-13 deletion did not change uncontrolled nature of CNS and systemic (spleen) growth in our infection model (Figure 9, A and B). Although the dissemination was delayed by 1 week in IL-4/13$^{-/-}$ mice, the unopposed increase in microbial growth dynamics was similar to that observed in wild-type mice (illustrated by the parallel trend lines in Figure 9). Thus IL-4/13$^{-/-}$ mice exhibited a significantly lower microbial burden and infection rate compared with wild-type mice at any given time point, but the IL-4/13$^{-/-}$ mice still succumbed to the infection approximately 1 week later.

IL-4/13$^{-/-}$ Mice Developed Severe Meningo-Encephalitis after H99 Infection

Our data showed that IL-4/IL-13 double deletion did not protect the mice from brain invasion with H99 while previous studies with less virulent strains of *C. neoformans* demonstrated that disruption of IL-4 and IL-13 signaling prevented cryptococcal CNS dissemination and *C. neoformans*-inflicted brain pathology.[30] To determine whether IL-4/IL-13 deletion had an effect on the development of brain pathology, the wild-type and IL-4/13$^{-/-}$ mice were examined histologically postmortem. Histological analysis demonstrated that wild-type and IL-4/13$^{-/-}$ mice that died from the H99 infection developed equivalent lesions in the brains (Figure 10). We observed multiple cryptococcal cysts within the brains and diffuse *C. neoformans* infiltration on the meningeal surface indicating that wild-type and IL-4/13$^{-/-}$ mice had succumbed to severe meningo-encephalitis. Thus, IL-4/IL-13 deletion was neither sufficient to prevent CNS dissemination nor protective against severe brain pathology.

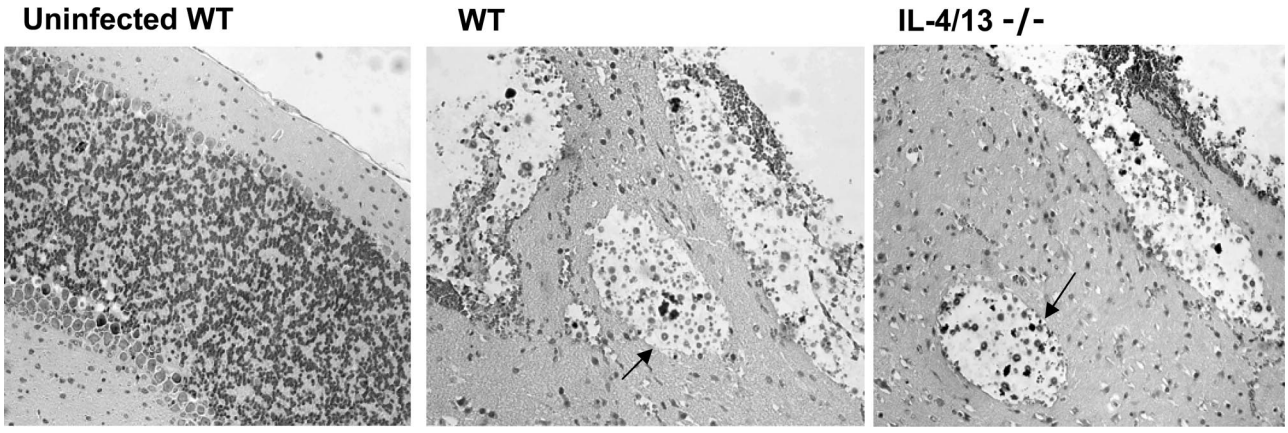

Figure 10. Effect of combined deletion of IL-4 and IL-13 on the development of brain pathology. Brains of uninfected WT and H99-infected wild-type and IL-4/13$^{-/-}$ mice were collected, formalin fixed, and stained with H&E. Left, uninfected brain sections; center, brain sections of moribund wild-type mice; right, brain sections of moribund IL-4/13$^{-/-}$ mice. Note cerebral foci of cryptococcal growth (**black arrows**) and evidence of meningitis in both wild-type and IL-4/13$^{-/-}$ mice infected with H99. Representative photographs were taken at 20× objective power.

Discussion

Our previous study has demonstrated that *C. neoformans* H99 can promote non-protective Th2 response in the lungs of infected mice, and suggested that this is a likely mechanism by which this strain achieves its high virulence.[8] In the present study, we analyzed an H99 infection in IL-4/IL-13 double knockout mice to determine whether eliminating both of these Th2-driving cytokines would counterbalance H99 virulence and promote microbial clearance. The absence of both IL-4 and IL-13 during H99 infection resulted in: 1) elimination of all major elements of Th2 response in the H99-infected lungs and lung-associated lymph nodes; 2) development of robust responses consistent with Th1 and Th17; 3) a reversal from non-protective AAM phenotype to a protective CAM phenotype expression by pulmonary macrophages; and 4) prevention of uncontrolled pulmonary growth of *C. neoformans* between weeks 2 and 4 after infection and protection from pathology associated with the rapid growth of *C. neoformans* and the Th2 response. However, IL-4/IL-13 double deletion failed to provide a long-term survival benefit to the H99-infected mice due to the inability to prevent CNS dissemination and the severe meningo-encephalitis. To our knowledge this is the first report that the robust Th1/Th17 immune responses and CAM are insufficient to provide protection against lethal dissemination of *C. neoformans* into the CNS.

To test if skewing of the immune response to Th2 is an important mechanism for H99 virulence, we compared the outcomes of *C. neoformans* H99 infection in the wild-type and IL-4/13$^{-/-}$ mice. The infection of wild-type (BALB/c) mice with H99 resulted in a strong Th2 response similar to that previously seen in C57BL6 mice.[8] All hallmarks of a robust Th2 response with all molecular and pathological consequences such as: 1) strong induction of IL-4 and IL-13 in the lungs (Table 1 and Figure 1) and in the pulmonary nodes (Figure 2); 2) absence or minimal induction of IFN-γ, TNF-α, and IL-12p35 (Table 1; Figures 1 and 2); 3) accumulation of serum IgE (Figure 3); 4) pulmonary eosinophilia (Figure 4); and 5) alternative activation of macrophages in the lungs (Figure 5) were observed. These attributes of the Th2 response have been accompanied by uncontrolled/logarithmic expansion of *C. neoformans* in the lungs (Figures 6 and 7); rapid dissemination of H99 to the CNS (Figure 9); severe lung and brain pathology (Figures 6 and 10); and 100% mortality by the end of week 4 postinfection (Figure 8). Together these data indicate that the expression of high virulence by H99 is associated with the development of the polarized Th2 response, which is detrimental.[16,17,23,25–30]

Apart from the expected absence of IL-4 and IL-13 in leukocytes from IL-4/13$^{-/-}$ mice, we find reduced induction of IL-5 and IL-10 and the absence of all analyzed Th2 hallmarks such as pulmonary eosinophilia, goblet cell hyperplasia in the airways, and systemic elevation of IgE class antibodies. The effect of IL-4/13 double deletion preventing Th2 immune response in *C. neoformans* H99-infected animals bears many similarities to the effects reported in studies of single IL-4 or IL-13 deletion with other *C. neoformans* strains.[19,37] We also show very robust up-regulation of IFN-γ in lung leukocytes and in the LALNs beginning from week 2 post-H99 infection in IL-4/13$^{-/-}$ (45- and 70-fold increase compared with uninfected mice). The previously reported effects of single deletion of IL-4 or IL-13 on IFN-γ expression were less uniform across different studies.[19,23,37] The differential outcomes of Th2 cytokine deletion on IFN-γ production in *C. neoformans* infected lungs are not surprising. In these models different strains of mice and different *C. neoformans* strains are used and a deletion of just one of the Th2-driving cytokines might not have completely blocked the Th2 pathway. This study demonstrates the effect of double deletion of IL-4 and IL-13 preventing them from possible replacement/compensation. The double deletion of IL-4/13 has a profound stimulatory effect on the induction of IFN-γ, along with the absence of other Th2 hallmarks, increase of other type-1 cytokines and Tbet up-regulation in LALNs. Collectively, this indicates that the switch to Th1 immune response in this model is complete and robust (Table 1; Figures 1 and 2).

Another effect of IL-4/13 deletion was significant up-regulation of IL-17 expression in both lungs and LALNs of IL4/13$^{-/-}$ mice (Table 1 and Figure 2). IL-17 is a potent cytokine directing chronic inflammatory responses and stimulating neutrophilia.[38,39] Although we have not identified the cellular sources of IL-17, our current and previously published data strongly suggest that T cells are the source of the polarizing cytokines in this response.[8,28,40] Additional support for this hypothesis comes from significantly increased levels of IL-23p19 at week 1 post-H99 infection when compared with wild-type controls. However, we cannot rule out the possibility that neutrophils may be an additional source of IL-17 in the lungs of IL-4/13$^{-/-}$ mice[41] and future studies will be needed to clarify this point. Regardless of the cellular sources, a protective role for the IL-23/IL-17 axis has been defined in acute *Klebsiella pneumoniae* infection.[18] Our studies show increased induction of IL-17 is associated with improved control of *C. neoformans* growth in the lungs. Furthermore, we are the first to demonstrate strong counter-regulatory relationship between Th2 cytokines and IL-17 in the lungs and LALNs during *C. neoformans* infection. This observation is consistent with previous reports of *ex vivo* re-stimulated splenocytes from *C. neoformans* 1841D-infected IL-13$^{-/-}$ and IL-4R$\alpha^{-/-}$ mice.[28] Our studies by us and the Muller's et al[28] suggest that induction of IL-17 is inhibited by Th2 cytokines and that IL-17 has a potential protective role in pulmonary clearance of *C. neoformans*.

The phenotype of the inflammatory responses in the lungs further confirms that IL-4 and IL-13 deletion resulted in change of the immune response from Th2 to a robust Th1 and Th17. Pulmonary eosinophilia observed in the wild-type mice (Figure 3) is replaced by enhanced recruitment of macrophages and neutrophils. This conclusion is further strengthened by the shift in the activation status of pulmonary macrophages. The CAM is a crucial element for anti-cryptococcal protection,[11,19] while AAM observed in the context of a Th2 response promote intracellular grow and persistence of *C. neoformans*. We show strong up-regulation of Arg1, FIZZ1, and YM2 but not iNOS genes in pulmonary macrophages

from H99-infected wild-type mice, which evidences their alternative activation.[8,17,19] In contrast, macrophages from H99-infected IL-4/13$^{-/-}$ mice show up-regulation of iNOS but not the AAM genes, consistent with classical activation that occurs in context of Th1 response.[42–44] These results demonstrate AAM induction driven by Th2 immunity is an important element of the strain H99 virulence and that its reversal can be achieved by deletion of IL-4 and IL-13.

The shift of the Th1/Th17 immunophenotype resulted in changes on cryptococcal lung burden. The wild-type mice demonstrated rapid unopposed growth of H99 at all time points in contrast with gradual decrease in *C. neoformans* load in IL-4/13$^{-/-}$ mice from weeks 2 to 4 postinfection, timing consistent with the efferent adaptive immune response.[26,29,40,45,46] This improved containment of H99 resulted in a 1500-fold difference in pulmonary load of H99 between the wild-type and IL-4/13$^{-/-}$ mice at week 4. Interestingly, the marked improvement of pulmonary control of H99 in IL-4/13$^{-/-}$ did not provide long-term survival benefit. The deletion of IL-4 and IL-13 extended the life span of the infected mice only by about 1 week, and this difference was not statistically significant. Furthermore, comparison of brain and spleen cryptococcal loads at weeks 2, 3, and 4 postinfection revealed that the systemic dissemination in IL-4/13$^{-/-}$ mice occurred with approximately 1-week delay, but subsequently progressed with the same rate as in the wild-type mice (Figure 9). Thus, IL-4/13$^{-/-}$ mice exhibited a significantly lower microbial burden in brains and spleens at any given time point postinfection but reached approximately the same level 1 week later (Figure 9). The 1-week delay in systemic spread mirrors the 1-week delay in the IL-4/13$^{-/-}$ mice mortality compared with wild-type mice. Furthermore, pathological examination demonstrated widespread lesions including presence of *C. neoformans* on the meningeal surface and forming intracerebral cysts; these pathological changes were equivalently severe in wild-type and IL-4/13$^{-/-}$ mice at the time of death (Figure 10). Taken together, these data indicate that although IL-4/IL-13 deletion resulted in development of protective Th1/Th17 response in the lungs, it did not protect the mice from CNS dissemination and severe brain pathology.

In terms of the role of Th2 skewing in H99-induced virulence, our data show a divergence between these effects in the lungs and in the systemic phase of the infection. We demonstrate that Th2 skewing is an important mechanism for H99 virulence in the pulmonary phase of H99 infection. However, IL-4 and IL-13 expression in our model has limited effect on the systemic dissemination and CNS invasion by H99, suggesting that H99 exploits different virulence mechanisms to invade the brain. Our observation that Th2 cytokine signaling has no major effect on H99 dissemination differs from a previous study with the less virulent strain of *C. neoformans* 1841D. In that study IL-4Rα signaling (IL-4 and IL-13 receptor subunit) was required for successful CNS invasion by 1841D.[30] This group also demonstrated that deletion of IL-4, IL-13, or IL-4Rα provided significant protection during the pulmonary phase of *C. neoformans* infection.[19,28] The differential effects of IL-4 and IL-13 between our models can be explained by different mechanisms of CNS invasion caused by H99 and some less virulent strains that disseminate into CNS less rapidly and may require macrophages to cross the blood-brain barrier.[11,13,30,47] Our previous study demonstrated that monocytes/macrophages were not required for H99 entry to the brain, and therefore, the change in macrophage activation profile may not be crucial for H99 dissemination.[13]

In summary, our studies demonstrate that highly virulent strain H99 is capable of both inducing a Th2 immune response in the lungs and evading host defenses in the brain regardless of a Th1/Th17 polarized immune environment. We conclude that promoting Th2 bias is a mechanism for pulmonary virulence of H99; however, other mechanisms of virulence are responsible for its strong CNS tropism.

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References

1. Kovacs JA, Kovacs AA, Polis M, Wright WC, Gill VJ, Tuazon CU, Gelmann EP, Lane HC, Longfield R, Overturf G, Macher AM, Fauci AS, Parrillo JE, Bennett JE, Masur H: Cryptococcosis in the acquired immunodeficiency syndrome. Ann Intern Med 1985, 103:533–538
2. Pappas PG, Perfect JR, Cloud GA, Larsen RA, Pankey GA, Lancaster DJ, Henderson H, Kauffman CA, Haas DW, Saccente M, Hamill RJ, Holloway MS, Warren RM, Dismukes WE: Cryptococcosis in human immunodeficiency virus-negative patients in the era of effective azole therapy. Clin Infect Dis 2001, 33:690–699
3. Baddley JW, Perfect JR, Oster RA, Larsen RA, Pankey GA, Henderson H, Haas DW, Kauffman CA, Patel R, Zaas AK, Pappas PG: Pulmonary cryptococcosis in patients without HIV infection: factors associated with disseminated disease. Eur J Clin Microbiol Infect Dis 2008, 27:937–943
4. Hoang LM, Maguire JA, Doyle P, Fyfe M, Roscoe DL: Cryptococcus neoformans infections at Vancouver Hospital and Health Sciences Centre (1997–2002): epidemiology, microbiology and histopathology. J Med Microbiol 2004, 53:935–940
5. Zahra LV, Azzopardi CM, Scott G: Cryptococcal meningitis in two apparently immunocompetent Maltese patients. Mycoses 2004, 47:168–173
6. Hofman V, Venissac N, Mouroux C, Butori C, Mouroux J, Hofman P: Disseminated pulmonary infection due to Cryptococcus neoformans in a non immunocompromised patient. Ann Pathol 2004, 24:187–191
7. Huffnagle GB, Chen GH, Curtis JL, McDonald RA, Strieter RM, Toews GB: Down-regulation of the afferent phase of T cell-mediated pulmonary inflammation and immunity by a high melanin-producing strain of Cryptococcus neoformans. J Immunol 1995, 155:3507–3516
8. Osterholzer JJ, Surana R, Milam JE, Montano GT, Chen GH, Sonstein J, Curtis JL, Huffnagle GB, Toews GB, Olszewski MA: Cryptococcal urease promotes the accumulation of immature dendritic cells and a non-protective T2 immune response within the lung. Am J Pathol 2009, 174:932–943
9. Chretien F, Lortholary O, Kansau I, Neuville S, Gray F, Dromer F: Pathogenesis of cerebral Cryptococcus neoformans infection after fungemia. J Infect Dis 2002, 186:522–530
10. Lortholary O, Improvisi L, Nicolas M, Provost F, Dupont B, Dromer F:

Fungemia during murine cryptococcosis sheds some light on pathophysiology. Med Mycol 1999, 37:169–174
11. Olszewski MA, Huffnagle GB, Traynor TR, McDonald RA, Cook DN, Toews GB: Regulatory effects of macrophage inflammatory protein 1alpha/CCL3 on the development of immunity to Cryptococcus neoformans depend on expression of early inflammatory cytokines. Infect Immun 2001, 69:6256–6263
12. Blackstock R, Buchanan KL, Adesina AM, Murphy JW: Differential regulation of immune responses by highly and weakly virulent Cryptococcus neoformans isolates. Infect Immun 1999, 67:3601–3609
13. Olszewski MA, Noverr MC, Chen GH, Toews GB, Cox GM, Perfect JR, Huffnagle GB: Urease expression by Cryptococcus neoformans promotes microvascular sequestration, thereby enhancing central nervous system invasion. Am J Pathol 2004, 164:1761–1771
14. Blanco JL, Garcia ME: Immune response to fungal infections. Vet Immunol Immunopathol 2008, 125:47–70
15. Ma H, May RC: Virulence in Cryptococcus species. Adv Appl Microbiol 2009, 67:131–190
16. Traynor TR, Kuziel WA, Toews GB, Huffnagle GB: CCR2 expression determines T1 versus T2 polarization during pulmonary Cryptococcus neoformans infection. J Immunol 2000, 164:2021–2027
17. Arora S, Hernandez Y, Erb-Downward JR, McDonald RA, Toews GB, Huffnagle GB: Role of IFN-gamma in regulating T2 immunity and the development of alternatively activated macrophages during allergic bronchopulmonary mycosis. J Immunol 2005, 174:6346–6356
18. Kleinschek MA, Muller U, Brodie SJ, Stenzel W, Kohler G, Blumenschein WM, Straubinger RK, McClanahan T, Kastelein RA, Alber G: IL-23 enhances the inflammatory cell response in Cryptococcus neoformans infection and induces a cytokine pattern distinct from IL-12. J Immunol 2006, 176:1098–1106
19. Muller U, Stenzel W, Kohler G, Werner C, Polte T, Hansen G, Schutze N, Straubinger RK, Blessing M, McKenzie AN, Brombacher F, Alber G: IL-13 induces disease-promoting type 2 cytokines, alternatively activated macrophages and allergic inflammation during pulmonary infection of mice with Cryptococcus neoformans. J Immunol 2007, 179:5367–5377
20. Huffnagle GB, Lipscomb MF, Lovchik JA, Hoag KA, Street NE: The role of CD4+ and CD8+ T cells in the protective inflammatory response to a pulmonary cryptococcal infection. J Leukoc Biol 1994, 55:35–42
21. Herring AC, Falkowski NR, Chen GH, McDonald RA, Toews GB, Huffnagle GB: Transient neutralization of tumor necrosis factor alpha can produce a chronic fungal infection in an immunocompetent host: potential role of immature dendritic cells. Infect Immun 2005, 73:39–49
22. Chen GH, McDonald RA, Wells JC, Huffnagle GB, Lukacs NW, Toews GB: The gamma interferon receptor is required for the protective pulmonary inflammatory response to Cryptococcus neoformans. Infect Immun 2005, 73:1788–1796
23. Hernandez Y, Arora S, Erb-Downward JR, McDonald RA, Toews GB, Huffnagle GB: Distinct roles for IL-4 and IL-10 in regulating T2 immunity during allergic bronchopulmonary mycosis. J Immunol 2005, 174:1027–1036
24. Mowen KA, Glimcher LH: Signaling pathways in Th2 development. Immunol Rev 2004, 202:203–222
25. Blackstock R, Buchanan KL, Cherniak R, Mitchell TG, Wong B, Bartiss A, Jackson L, Murphy JW: Pathogenesis of Cryptococcus neoformans is associated with quantitative differences in multiple virulence factors. Mycopathologia 1999, 147:1–11
26. Chen GH, McNamara DA, Hernandez Y, Huffnagle GB, Toews GB, Olszewski MA: Inheritance of immune polarization patterns is linked to resistance versus susceptibility to Cryptococcus neoformans in a mouse model. Infect Immun 2008, 76:2379–2391
27. Kawakami K, Hossain Qureshi M, Zhang T, Koguchi Y, Xie Q, Kurimoto M, Saito A: Interleukin-4 weakens host resistance to pulmonary and disseminated cryptococcal infection caused by combined treatment with interferon-gamma-inducing cytokines. Cell Immunol 1999, 197:55–61
28. Muller U, Stenzel W, Kohler G, Polte T, Blessing M, Mann A, Piehler D, Brombacher F, Alber G: A gene-dosage effect for interleukin-4 receptor alpha-chain expression has an impact on Th2-mediated allergic inflammation during bronchopulmonary mycosis. J Infect Dis 2008, 198:1714–1721
29. Olszewski MA, Huffnagle GB, McDonald RA, Lindell DM, Moore BB, Cook DN, Toews GB: The role of macrophage inflammatory protein-1alpha/CCL3 in regulation of T cell-mediated immunity to Cryptococcus neoformans infection. J Immunol 2000, 165:6429–6436
30. Stenzel W, Muller U, Kohler G, Heppner FL, Blessing M, McKenzie AN, Brombacher F, Alber G: IL-4/IL-13-dependent alternative activation of macrophages but not microglial cells is associated with uncontrolled cerebral cryptococcosis. Am J Pathol 2009, 174:486–496
31. Cox GM, Mukherjee J, Cole GT, Casadevall A, Perfect JR: Urease as a virulence factor in experimental cryptococcosis. Infect Immun 2000, 68:443–448
32. Noverr MC, Cox GM, Perfect JR, Huffnagle GB: Role of PLB1 in pulmonary inflammation and cryptococcal eicosanoid production. Infect Immun 2003, 71:1538–1547
33. Wormley FL Jr, Perfect JR, Steele C, Cox GM: Protection against cryptococcosis by using a murine gamma interferon-producing Cryptococcus neoformans strain. Infect Immun 2007, 75:1453–1462
34. McKenzie GJ, Fallon PG, Emson CL, Grencis RK, McKenzie AN: Simultaneous disruption of interleukin (IL)-4 and IL-13 defines individual roles in T helper cell type 2-mediated responses. J Exp Med 1999, 189:1565–1572
35. Huffnagle GB, Boyd MB, Street NE, Lipscomb MF: 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). J Immunol 1998, 160:2393–2400
36. Osterholzer JJ, Curtis JL, Polak T, Ames T, Chen GH, McDonald R, Huffnagle GB, Toews GB: CCR2 mediates conventional dendritic cell recruitment and the formation of bronchovascular mononuclear cell infiltrates in the lungs of mice infected with Cryptococcus neoformans. J Immunol 2008, 181:610–620
37. Blackstock R, Murphy JW: Role of interleukin-4 in resistance to Cryptococcus neoformans infection. Am J Respir Cell Mol Biol 2004, 30:109–117
38. Happel KI, Zheng M, Young E, Quinton LJ, Lockhart E, Ramsay AJ, Shellito JE, Schurr JR, Bagby GJ, Nelson S, Kolls JK: Cutting edge: roles of Toll-like receptor 4 and IL-23 in IL-17 expression in response to Klebsiella pneumoniae infection. J Immunol 2003, 170:4432–4436
39. Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P, Oliver P, Huang W, Zhang P, Zhang J, Shellito JE, Bagby GJ, Nelson S, Charrier K, Peschon JJ, Kolls JK: Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. J Exp Med 2001, 194:519–527
40. Lindell DM, Moore TA, McDonald RA, Toews GB, Huffnagle GB: Generation of antifungal effector CD8+ T cells in the absence of CD4+ T cells during Cryptococcus neoformans infection. J Immunol 2005, 174:7920–7928
41. Ferretti S, Bonneau O, Dubois GR, Jones CE, Trifilieff A: IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: iL-15 as a possible trigger. J Immunol 2003, 170:2106–2112
42. Granger DL, Perfect JR, Durack DT: Macrophage-mediated fungistasis in vitro: requirements for intracellular and extracellular cytotoxicity. J Immunol 1986, 136:672–680
43. Lovchik JA, Lyons CR, Lipscomb MF: A role for gamma interferon-induced nitric oxide in pulmonary clearance of Cryptococcus neoformans. Am J Respir Cell Mol Biol 1995, 13:116–124
44. Vecchiarelli A, Pietrella D, Dottorini M, Monari C, Retini C, Todisco T, Bistoni F: Encapsulation of Cryptococcus neoformans regulates fungicidal activity and the antigen presentation process in human alveolar macrophages. Clin Exp Immunol 1994, 98:217–223
45. Huffnagle GB, Strieter RM, McNeil LK, McDonald RA, Burdick MD, Kunkel SL, Toews GB: Macrophage inflammatory protein-1alpha (MIP-1alpha) is required for the efferent phase of pulmonary cell-mediated immunity to a Cryptococcus neoformans infection. J Immunol 1997, 159:318–327
46. Lindell DM, Ballinger MN, McDonald RA, Toews GB, Huffnagle GB: Diversity of the T-cell response to pulmonary Cryptococcus neoformans infection. Infect Immun 2006, 74:4538–4548
47. Charlier C, Nielsen K, Daou S, Brigitte M, Chretien F, Dromer F: Evidence of a role for monocytes in dissemination and brain invasion by Cryptococcus neoformans. Infect Immun 2009, 77:120–127