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In the Absence of Reactive Oxygen Species, T Cells Default to a Th1 Phenotype and Mediate Protection against Pulmonary Cryptococcus neoformans Infection

Robert J. Snelgrove,* Lorna Edwards,* Andrew E. Williams,* Aaron J. Rae,† and Tracy Hussell**

In recent years, the prevalence of invasive fungal infections has increased, attributed mostly to the rising population of immunocompromised individuals. Cryptococcus neoformans has been one of the most devastating, with an estimated 6–8% of AIDS-infected patients succumbing to Cryptococcus-associated meningitis. Reactive oxygen species (ROS) are potent antimicrobial agents but also play a significant role in regulating immune cell phenotype, but cause immunopathology when produced in excess. We now show that mice lacking phagocyte NADPH oxidase have heightened macrophage and Th1 responses and improved pathogen containment within pulmonary granulomatous lesions. Consequently, dissemination of this fungus to the brain is diminished, an effect that is independent of IL-12. Similar results are described using the metalloporphyrin antioxidant manganese(III) tetrakis(N-ethyl pyridinium-2-yl)porphyrin, which also promoted a protective Th1 response and reduced dissemination to the brain. These findings are in sharp contrast to the protective potential of ROS against other fungal pathogens, and highlight the pivotal role that ROS can fulfill in shaping the profile of the host’s immune response. The Journal of Immunology, 2006, 177: 5509–5516.

The encapsulated budding yeast Cryptococcus neoformans is a significant respiratory pathogen. The primary site of infection is the lung, where it elicits pulmonary eosinophilia in immunocompetent individuals, delayed-type hypersensitivity and summer-type hypersensitivity pneumonitis in Japan (1, 2). However, it is immunocompromised patients who are at greatest risk from this opportunistic fungus, with failure to contain pulmonary cryptococcus culminating in dissemination to the brain with ensuing meningoencephalitis and frequently death (3). The prevalence of C. neoformans infection has been illuminated in the past 10 years in patients with underlying T cell deficiencies, with 6–8% of all AIDS patients developing C. neoformans-associated meningitis.

Murine models of C. neoformans infection provide an excellent insight into comprehending the protective immune response to this pathogen. Protection is dependent upon the genetic background of the mouse, with resistant mice (BALB/c, C.B-17, and cytometric bead array (CBA))2 generating a Th1-driven response, with higher levels of IL-12, IFN-γ, and TNF-α and resolution of infection (4–6). Conversely, susceptible mice (C57BL/6, C3H, and B10.D2) display a more prominent Th2 response, with elevated IL-5 and chronic pulmonary eosinophilia (7). This Th2 response leads to unchecked fungal growth and dissemination to the brain.

The phagocyte NADPH oxidase is a multimeric membrane-associated complex on the surface of professional phagocytes. Activation of this oxidase culminates in an increase in oxygen consumption known as the respiratory burst. The NADPH oxidase synchronously uses the reducing power of NADPH to catalyze the conversion of molecular oxygen to superoxide radicals (O2•–) (8). The superoxide generated by the phagocyte NADPH oxidase exhibits potent antimicrobial activity, and superoxide is converted to hydrogen peroxide (H2O2) in the regulation of immune cell function (9–11). Furthermore, superoxide can subsequently be converted to a vast array of other reactive oxygen species (ROS) that are detrimental to the invading organism (9, 10). The critical role fulfilled by ROS species in the control of pathogen burden is made clearly apparent by the genetic condition chronic granulomatous disease (CGD), characterized by deficiency of a functional phagocytic oxidase, and greater susceptibility to infection with bacterial and fungal pathogens (9, 11). Furthermore, a role of ROS in intracellular signaling and in the regulation of immune cell function is now recognized (12–14). Superoxide and hydrogen peroxide (H2O2) are anticipated to act as secondary messengers through oxidizing redox-sensitive transcription factors such as NFκB. However, ROS may also be the causative agents of bystander tissue damage owing to their high toxicity and lack of specificity and are frequently the underlying determinant of immunopathology and disease (15).

Gene targeting has been used to generate Cybb tm1 mice with a null allele of the gene involved in X-linked CGD, which encodes the 91-kDa subunit of the oxidase cytochrome b of the NADPH oxidase (11). These mice have helped in evaluating the role of phagocyte-derived oxidants in controlling inflammation and protection to an array of pathogenic infections, but not as yet C. neoformans. There are discrepancies regarding the efficacy of superoxide and its derivatives in the eradication of C. neoformans, yet there is cited evidence defining the potential of polymorphonuclear neutrophils and macrophages to kill the organism by oxidative mechanisms (16). Furthermore, this fungus displays an array of protective mechanisms (superoxide dismutase (SOD), mammotin, melanin) to confer resistance to ROS, suggesting that in some instances they may be toxic and...
adverse to its survival. *C. neoformans* deficient in melanin exhibit reduced virulence in vivo (17), and CuSOD lacking mutants are more susceptible to oxygen radicals in macrophages (18).

We now report the role of ROS in resolution of *C. neoformans* infection using the Cybb tm1 mice and a novel antioxidant, manganese(III) tetrakis[N-ethyl pyridinium-2-yl]porphyrin (MnTE-2-PyP). We show that Cybb tm1 mice elicit a heightened macrophage-driven Th1 response with containment of cryptococci within pulmonary granulomatus lesions. Furthermore, we observed improved pathogen clearance in lung and airways and reduced dissemination to the brain. We have used the MnTE-2-PyP antioxidant to validate the phenotype observed in Cybb tm1 mice. MnTE-2-PyP is a cell-permeable manganic-porphyrin that is superlative to previously used endogenous SOD treatments owing to its prolonged half-life and reduced m.w. It displays a broad antioxidant activity, acting as an SOD and catalase mimic and being capable of scavenging lipid peroxides and peroxynitrite (19, 20).

MnTE-2-PyP-treated mice again evoked a Th1-skewed immune response with reduced pathogen load in the brain, but failed to develop the granulomatus lesions observed in the Cybb tm1 mice. MnTE-2-PyP has previously been reported to confer protection in a variety of oxidative stress injuries such as liver ischemia, diabetes, and stroke (19, 21), and could offer a plausible therapeutic to combat *C. neoformans* infection.

## Materials and Methods

### Mice and pathogens

Eight- to 12-wk-old female C57BL/6 (Harlan Olac) were kept in pathogen-free conditions according to Home Office guidelines. Cybb tm1 mice (backcrossed to C57BL/6 background at least 10 times) were purchased from The Jackson Laboratory. *C. neoformans* strain 52 was obtained from the American Type Culture Collection and for infection grown to stationary phase (48–72 h) at room temperature on a shaker in Sabouraud dextrose broth (1% neopentone and 2% dextrose; Difco). The cultures were washed in saline, counted on a hemocytometer, and diluted in sterile non-pyrogenic saline to the required infective dose.

### Mouse infections and treatment

On day 0, wild-type C57BL/6 and Cybb tm1 mice were anesthetized and intranasally (i.n.) infected with 2 × 10⁷ CFU *C. neoformans* in 50 μl of sterile PBS. In some experiments, wild-type C57BL/6 mice were treated with 50 μg of MnTE-2-PyP (Merck Biosciences) or PBS i.n. on day 0, and then every 4 days after infection. In some experiments, mice were treated with 100 μg of anti-IL-12 i.p. (rat mAb C15.6.7) 1 day before and 1, 4, 8, and 11 days following *C. neoformans* infection. Mice were sacrificed various days after infection by injection of 3 mg of pentobarbitone and exsanguinated via the femoral vessels.

### Cell recovery and flow cytometry

Lung lavage (bronchoalveolar lavage (BAL)) and residual lung tissue were sampled as described previously (22), and single-cell suspensions were stained for the following surface markers: anti-CD45RB-FITC, anti-CD4-PerCP, anti-CD8-allophycocyanin, anti-Gr-1-FITC, anti-MHC class II (MHC-II)-PE, anti-CD11b-PerCP, or anti-CD11c-allophycocyanin for 30 min at 4°C and fixed with 2% paraformaldehyde (15 min at room temperature). All Abs were purchased from BD Pharmingen. To detect intracellular cytokines, 10⁶ cells/ml were incubated with 50 ng/ml PMA, 500 ng/ml ionomycin (Calbiochem), and 10 ng/ml brefeldin A for 4 h at 37°C. Cells were then stained with anti-CD4-allophycocyanin and anti-CD8-PerCP and fixed as before. After permeabilization with PBS containing 1% saponin/1% BSA/0.05% azide (saponin buffer) for 10 min, cells were incubated with anti-IFN-γ-FITC and anti-IL-5-PE diluted 1/50 in saponin buffer. Thirty minutes later, cells were washed once in saponin buffer and once in PBS containing 1% BSA and 0.05% azide. All data were acquired on a FACS-Calibur at acquiring at least 30,000 lymphocyte events (BD Biosciences).

### Lung histology

Excised lungs were inflation-fixed with 2% formalin in PBS 12 days after *C. neoformans* infection. The inflated lungs were excised and embedded in paraffin wax by L. Lawrence (Leukocyte Biology, Imperial College, London, U.K.). Four-micrometer sections from four to five mice were stained with H&E.

### Enumeration of eosinophils

Eosinophils were enumerated by flow cytometry based on their distinctive size (forward scatter) and granularity (side scatter). The proportion of eosinophils was confirmed from cytoplasm preparations of BAL fluid following H&E staining, using their characteristic nuclear morphology and presence of acidophilic red granules.

### Enumeration of *C. neoformans*

Lungs and brain were homogenized by passage through 100-μm cell striainers (BD Labware). A total of 100 μl of cell suspension from lung homogenate, brain homogenate, and BAL were diluted in PBS and incubated at room temperature for 48 h on Saouraud dextrose agar plates (Sigma-Aldrich). The total CFU per sample was then determined (number of colonies × dilution factor × original cell suspension volume).

### BAL IgE Ab ELISA

A total of 2 × 10⁵ CFU/ml heat-killed *C. neoformans* in PBS was used to coat 96-well microtiter plates overnight at room temperature on a shaker. After blocking with 3% BSA/PBS for 2 h at 37°C, dilutions of sample sera were added for a further hour at room temperature. Bound Ab was detected using peroxidase-conjugated rabbit anti-mouse Ig and o-phenylenediamine as a substrate. The reaction was stopped with 50 μl of 2.5 M sulfuric acid. ODs were read at 490 nm, and mean blank values (ODs from normal mouse serum) were subtracted from the OD values of test samples.

### BAL LDH detection

The amount of LDH in the BAL was measured using Sigmus in vitro toxicology LDH based assay kit. Samples (tested in triplicate) were added to an equal volume of LDH assay mixture (assay dye, substrate, and enzyme) and incubated at room temperature for 30 min. Absorbance was measured at 490 nm, and protein concentration was calculated by comparison with an albumin standard.

### Lactate dehydrogenase (LDH) detection

The protection afforded by ROS to respiratory fungal pathogens is in vivo. Aspergillus fumigatus. However, their role in the resolution of *C. neoformans* infection remains unclear. In this study, we use Cybb tm1 mice, a model of CGD, to identify the significance of NADPH oxidase-derived superoxide in protection to *C. neoformans*. We first investigated the phenotype of naïve Cybb tm1 mice relative to wild-type controls, before assessing the role of NADPH oxidase during *C. neoformans* infection. The lungs of wild-type and knockout mice were examined histologically by FACs and showed that Cybb tm1 mice exhibited a small but significant increase in total cellular numbers in their lungs (wild type, 2.78 ×
10^5 ± 0.75 SEM; Cybb tm1, 8.2 × 10^5 ± 1.25 SEM). However, there were no alterations in cellular infiltrate into the airways with numbers very low in wild-type and knockout mice.

Following *C. neoformans* infection, both Cybb tm1 mice and C57BL/6 controls exhibited comparable pulmonary cellular infiltrate, but the distribution of the infiltrate within the lung tissue was distinct between groups. Wild-type mice displayed a generalized cellularity with extensive perivascular and peribronchial infiltrate with significant occlusion of alveoli. Conversely, the Cybb tm1 mice exhibited small distinct pockets of infiltrate, granulomatous in nature, surrounding cryptococi (Fig. 1A). It is important to stress the numbers of cells in the lungs of naive mice are at least an order of magnitude lower than that recruited during *C. neoformans* infection (at peak infection, wild type: 8.9 × 10^6 ± 2.9 SEM; Cybb tm1, 11.5 × 10^6 ± 2.5 SEM).

Cybb tm1 mice have previously been demonstrated to develop a granulomatous response to other fungal pathogens, but have been compromised in their ability to resolve infection. In contrast, Cybb tm1 mice showed an enhanced ability to combat *C. neoformans* infection with a significantly reduced fungal load in the lung tissue, the BAL, and, importantly, the brain at days 5, 8, and 12 after infection (Fig. 1B). The reduced pathogen load in Cybb tm1 mice is clearly visible in cytospins of the BAL (Fig. 1A, inset).

Macrophages are central to the development of a granulomatous response orchestrating cellular recruitment and containment of the pathogen. Naive Cybb tm1 mice displayed a significant increase in the number of resident lung macrophages relative to wild-type controls (wild type, 0.5 × 10^6 ± 0.09 SEM; Cybb tm1, 2.87 × 10^6 ± 1.25 SEM) that may indicate a role for NAPDH oxidase in homeostatic regulation of these cells, potentially through regulation of apoptosis. Although the increase in macrophage numbers was significant, it is again important to stress that they are still insignificant compared with those recruited during *C. neoformans* infection. Analysis of H&E-stained lung sections from *C. neoformans*-infected mice (Fig. 1A) demonstrates an accumulation of macrophages and multinucleated giant cells at the center of the granuloma in Cybb tm1 mice. Conversely, the cellularity in the wild-type lung, although containing macrophages, was dominated by lymphocytes and eosinophils. This was confirmed by FACS analysis of lung tissue, which clearly showed a significantly greater number of macrophages in Cybb tm1 mice throughout the course of infection (Fig. 1C).

*C. neoformans* is directly responsible for much of the observed lung pathology observed during the course of infection owing to its considerable size and extensive numbers. To assess whether the reduced pathogen load observed in Cybb tm1 mice reduced lung damage, we assessed the levels of total protein (Fig. 1D) and LDH (Fig. 1E) in the BAL fluid. Both markers of pathology were significantly reduced in Cybb tm1 mice.

The granulomatous response to *C. neoformans* seen in Cybb tm1 mice and the universal reduction in pathogen burden at distal sites may indicate containment of the pathogen within the lung tissue. There was significantly reduced cellularity in the airways of Cybb tm1 mice (Fig. 2A). This, coupled with the considerably lower CFUs at this site may imply that containment within the lung tissue hinders dissemination into the airways with consequently reduced number of cells recruited into the BAL.

![Image](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/images/0/02/FIGURE_1.png)

**FIGURE 1.** Cybb tm1 mice display improved clearance of *C. neoformans* and reduced lung pathology. A. Representative H&E-stained lung sections of *C. neoformans*-infected Cybb tm1 and wild-type C57BL/6 mice 12 days after infection (×200 magnification). Insets are representative H&E-stained cytocentrifuged BAL samples of *C. neoformans*-infected wild-type and Cybb tm1 mice at day 12 after infection (×200 magnification). Arrows indicate cryptococcal bodies. B. *C. neoformans*-infected wild-type and Cybb tm1 mice were sacrificed at multiple time points, and CFUs were determined by plating out lung homogenate, BAL, and brain homogenate. C. Lungs were removed from *C. neoformans*-infected wild-type and Cybb tm1 mice, and single-cell suspension was stained with CD11b-PerCP and CD11c-allophycocyanin. Total numbers of lung CD11b^+ macrophages were determined by the following: percentage of single-positive cells by flow cytometry × total number of viable cells. D and E. At day 12 after *C. neoformans* infection, BAL was removed and total protein (D) and LDH (E) were measured in supernatants. Closed symbols, Wild-type mice; open symbols, Cybb tm1 mice. Results represent mean values ± SEM from five mice per group (*, p < 0.05; **, p < 0.01) and are representative of three experiments.

![Image](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/images/0/02/FIGURE_2.png)

**FIGURE 2.** *C. neoformans*-infected Cybb tm1 mice exhibit reduced eosinophilia and an enhanced CD8^+ T cell response. A. BAL was performed on *C. neoformans*-infected wild-type or Cybb tm1 mice on days 0, 5, 8, and 12, and total viable cells were enumerated. B. The percentage of eosinophils in the BAL 12 days after *C. neoformans* infection was determined from H&E-stained cytospin preparations. Lungs were removed from *C. neoformans*-infected wild-type or Cybb tm1 mice 12 days after infection, and single-cell suspension was stained with CD45RB-FITC, CD4-PerCP, and CD8-allophycocyanin. The percentage of CD4^+ and CD8^+ lymphocytes was determined by flow cytometry. C. The percentage of CD4^+ and CD8^+ T cells that were CD45RB^high was also determined (D). Closed symbols, Wild-type mice; open symbols, Cybb tm1 mice. Results represent mean values ± SEM from five mice per group (*, p < 0.05; **, p < 0.01) and are representative of three experiments.
Protection against *C. neoformans* is dependent on the development of a Th1 immune response. C57BL/6 mice usually exhibit a strong nonprotective Th2 response with chronic eosinophilia in the lung and airways and dissemination of fungus to the brain. Differential cell counts of H&E-stained cytospin preparations (Fig. IA, inset), and FACS analysis (Fig. 2B) revealed a marked reduction in the percentage of eosinophils in the lung and airways, in Cybb tm1 mice. Eosinophilia is dependent on CD4^+^ T cells. In Cybb tm1 mice, however, these were reduced relative to CD8^+^ T cells, which were increased (Fig. 2C). Both CD4^+^ and CD8^+^ T cells exhibited a heightened activation state (CD45RB^low^) in Cybb tm1 mice at days 8 and 12 (Fig. 2D) after infection.

To determine whether a shift in the cytokine profile accounted for reduced eosinophilia in Cybb tm1 mice, we used intracellular cytokine staining. Cytokine profiles of uninfected wild-type and Cybb tm1 mice were compared, but only negligible amounts of cytokines were observed by intracellular cytokine staining of T cells or through detection of soluble cytokine by CBA. During infection, however, both CD4^+^ and CD8^+^ T cells produced increased levels of IFN-γ (Fig. 3A) and TNF-α (data not shown) in Cybb tm1 mice compared with wild-type mice. Conversely, Cybb tm1-derived T cells produced lower levels of IL-5 (data not shown). The ratio of IFN-γ-producing T cells to IL-5-producing T cells was greatly augmented in Cybb tm1 mice (Fig. 3B) showing a significant Th1 bias. Soluble cytokine levels in lung homogenate were also monitored by CBA throughout the course of infection and displayed a similar Th1 bias in Cybb tm1 mice. There were significantly elevated levels of TNF-α at days 8 and 12 after infection in Cybb tm1 mice and reduced levels of IL-5 at days 5 and 8 (Fig. 3C).

Because CD4^+^ T cells assist in antifungal Ab production, we next compared the effect in Cybb tm1 mice. Cybb tm1 mice exhibited significantly reduced B cells in the lungs and airways (Table I). Furthermore, reduced pathogen-specific total Ab and IgG1 isotype in the serum at day 12 postinfection, and IgE isotype in the BAL fluid were observed at day 5 postinfection (Table I).

IL-12 is a critical cytokine in the induction of a Th1 response (23–27). IL-12 is also critical in controlling dissemination of *C. neoformans* to the brain (28, 29). The heightened macrophage response observed in Cybb tm1 mice could presumably affect levels of this cytokine and thus control dissemination with greater aptitude. Neutralizing anti-IL-12 Ab was thus used to infer the role of IL-12 in the enhanced protection observed in Cybb tm1 mice.

Wild-type mice administered anti-IL-12 Ab exhibited a dramatic increase in *C. neoformans* dissemination to the brain (Fig. 4A). However, the enhanced ability of Cybb tm1 mice to resolve cryptococcal infection was not compromised by anti-IL-12 Ab treatment. CFUs in the lung and BAL were not affected by anti-IL-12 treatment in either strain (Fig. 4A). Furthermore, macrophage numbers were augmented in the lungs of both anti-IL-12 Ab-treated and untreated Cybb tm1 mice relative to wild-type controls (data not shown), and eosinophil numbers markedly reduced (Fig. 4B). Anti-IL-12 treatment of wild-type mice evoked heightened IL-5 production and reduced IFN-γ production, but the cytokine profile of Cybb tm1 mice receiving the treatment remained comparable to those of knockout mice that did not receive anti-IL-12 Ab (data not shown).

We have previously noted marked differences in the phenotype of naive wild-type and Cybb tm1 mice as discussed, and appreciate many inherent problems of using knockout mice to evaluate the role of immune components in the outcome of infection. We therefore assessed the role of ROS in *C. neoformans* infection using the metallic manganese porphyrin MnTE-2-PyP, which acts as an SOD mimic and possesses ROS scavenging potential. Mice administered MnTE-2-PyP exhibited a marked reduction in cellular infiltrate into the lung tissue (Fig. 5A) as well as the airways, relative to controls. This is distinct to the scenario in Cybb tm1 mice, which possessed a significant granulomatous lung infiltrate. The reduced cellularity by MnTE-2-PyP treatment was accounted for by a reduction in total numbers of lymphocytes (Fig. 5B) and eosinophils (Fig. 5C) in the lungs and airways. Similar to Cybb tm1 mice, those treated with MnTE-2-PyP showed a marked bias toward a CD8^+^ T cell response relative to CD4^+^ when compared with control mice (Fig. 5D). Furthermore, both CD4^+^ and CD8^+^ T cells from mimetic-treated mice exhibited a Th1 cytokine bias with a greater percentage of cells expressing intracellular IFN-γ relative to PBS-treated mice (Fig. 5E). Finally, MnTE-2-PyP-treated mice showed comparable fungal loads in their lungs and airways, but exhibited reduced dissemination to the brain relative to control mice (Fig. 5F).

The Th1 bias and improved protection seen in Cybb tm1 mice or those administered MnTE-2-PyP suggests a role for ROS in defining the Th1/Th2 paradigm. This may be partly explained by the heightened macrophage numbers in *C. neoformans*-infected Cybb tm1 mice. Because ROS have been implicated in apoptosis (12–14, 30, 31) of cells, we examined the influence of MnTE-2-PyP on the J774 macrophage cell line treated with a variety of stimuli and indeed found reduced apoptosis (data not shown). We have subsequently investigated macrophage apoptosis during *C. neoformans* infection with or without administration of MnTE-2-PyP. Although we found the total number of annexin V^+^ macrophages was reduced, this was a reflection of the total reduction in cellular infiltrate rather than an alteration in the proportion of cells undergoing apoptosis. It would appear that a reduction in macrophage apoptosis is therefore not the sole reason for the elevated Th1 response and improved pathogen clearance.

We finally determined whether administration of the antioxidant MnTE-2-PyP affected clearance of *C. neoformans* at later time
6–8% of AIDS-infected patients succumbing to has been one of the most devastating, with an estimated formans munocompromised individuals (3). The fungal pathogen
In recent years, there has been an increasing prevalence of invasive infection. Therefore, mice were infected with C. neoformans and administered PBS or MnTE-2-PyP at 4-day intervals and were culled at day 45 after infection. At this time point, total cell numbers in the lung (Fig. 6A) and BAL (data not shown) were still significantly lower in MnTE-2-PyP-treated mice, particularly CD4+ and CD8+ T cells (Fig. 6B). The total number of pulmonary macrophages were partially reduced in those mice administered the mimetic, reflecting a general reduction in cellular infiltrate at this site. However, those macrophages remaining expressed elevated levels of C. neoformans CFUs in the brain (Fig. 6E) similar to day 12 after infection, but now also exhibited a significant reduction in pathogen burden in the lungs (Fig. 6D) that had not previously been seen at earlier time points with mimetic treatment.

Discussion
In recent years, there has been an increasing prevalence of invasive fungal infections, attributed mostly to the rising population of immunocompromised individuals (3). The fungal pathogen C. neoformans has been one of the most devastating, with an estimated 6–8% of AIDS-infected patients succumbing to Cryptococcus-associated meningitis. In this study, we have investigated the antimicrobial action of ROS against C. neoformans, using mice that lack a functional phagocyte NADPH oxidase (Cybb tm1 mice) and through administration of a manganic porphyrin with broad antioxidant activity. ROS are believed to fulfill a critical role in conferring protection against fungal infections; the most important evidence provided by patients with CGD, who lack a functional NADPH oxidase, improved clearance of the pathogen is uncharacteristic. Cybb tm1 mice show improved clearance of C. neoformans in the lung tissue and airways with reduced dissemination to the brain. CGD is characterized by recurring life-threatening bacterial and fungal infections such as Staphylococcus aureus and A. fumigatus, with neutrophils from CGD patients demonstrated to display defective killing against certain bacterial and fungal pathogens (9, 11). A. fumigatus is capable of eliciting disease in mice lacking the NADPH oxidase at significantly lower doses than in wild-type controls, and higher doses of this fungal pathogen prove fatal to Cybb tm1 knockout mice (9). Similarly, phagocytes unable to generate superoxide exhibit reduced potential to kill Candida albicans (35, 36), and Sporothrix schenckii infection of CGD mice leads to systemic infection and death (35). Although compromised pathogen clearance is the norm in the absence of a phagocyte NADPH oxidase, improved immunity and reduced bacterial load has been reported for H. pylori (33), and we have reported an improved clearance of influenza virus from the lungs of Cybb tm1 mice (37).

Despite the recurring fungal infections in CGD patients, there are no clinical studies showing enhanced susceptibility to C. neoformans. However, there are studies citing the potential of polymorphonuclear leukocytes and macrophages to kill Cryptococcus by oxidative mechanisms (16). Furthermore, the fungus appears to possess a host of intrinsic mechanisms to protect against an oxidative attack such as an SOD, and radical scavenging mediators melanin and mannitol. This would imply that, in some instances at least, ROS may be toxic to this fungus. Melanin-deficient C. neoformans possess a reduced capacity to kill mice (17), and CuSOD lacking Cryptococcus displaying attenuated growth within macrophages (18). The improved killing of C. neoformans in the absence of ROS has been described previously in vitro, whereby incubation of peritoneal macrophages with SOD and catalase augments anticytotoxic activity owing to a concomitant increase in NO production (38).

Despite evidence signifying a role for ROS in protection to C. neoformans, these host mediators appear less critical than against other fungal pathogens. The differences in susceptibility of NADPH oxidase-deficient mice to C. neoformans relative to other fungal pathogens is probably partially attributable to the fact that protection against A. fumigatus and C. albicans, at least in the early stages of infection, is mediated primarily through ROS of phagocytes, whereas protection against C. neoformans is dependent on Th1 cell-mediated immune response, which we have demonstrated to be augmented in Cybb tm1 mice. It is plausible that other non-oxygen-mediated cytotoxic mechanisms are more important to eradicate C. neoformans, and in an in vivo scenario these may compensate for the absence of a functional NADPH oxidase. The array of protective mechanisms exhibited by C. neoformans may negate the potential of superoxide and other ROS to kill this fungus, and oxidative cytotoxicity will only become apparent if these oxidative defenses are absent. C. neoformans is an encapsulated yeast, with the large high m.w. polysaccharide capsule protecting against different facets of the host’s immune response (3, 39). Such a capsule is not present around A. fumigatus and could explain the

| Table I. Cybb tm1 mice elicit a reduced B cell and Ab response to C. neoformans* |
|---------------------------------|-----------------|-----------------|-----------------|
| % B220 | Total Ab | IgG1 | IgE |
| Wild type | 46.1 ± 2.1 | 0.59 ± 1.7 | 0.11 ± 0.01 | 0.29 ± 0.09 |
| Cybb tm1 | 28.3 ± 4.0 | 0.13 ± 0.02 | 0.09 ± 0.003 | 0.05 ± 0.01 |

* Lungs were removed from naive Cybb tm1 and wild-type C57BL/6 mice at day 12 postinfection, and single-cell suspensions were obtained. Viable cells from each organ were determined by trypan blue exclusion. Single-cell suspensions were stained with anti-B220-PerCP, and the percentage of lymphocytes expressing this marker was determined by flow cytometry. The levels of C. neoformans-specific total Ab and IgG1 in the serum were determined at day 12 postinfection, and the levels of total IgE in the BAL were assessed at day 5. ODs were read at 490 nm, and mean blank values (ODs from normal mouse serum) were subtracted from the OD values of test samples. Results represent the mean ± SD of five individual mice in three independent experiments.
The capsule surrounding the Cryptococcus renders the pathogen less susceptible to phagocytosis and so may deprive ROS accessibility to the fungus (3, 40, 41). C. neoformans also has the potential to elicit deposition of opsonins, which may trigger the phagocyte respiratory burst, at sites below the capsule surface, where they will be incapable of binding complement or FcRs on the surface of myeloid cells (3, 42).

The improved clearance of C. neoformans is probably largely attributable to the enhancement of macrophages and Th1 cytokines in Cybb tm1 mice. In addition, we report elevated CD8+ T cells that have been shown to directly bind C. neoformans and inhibit growth (43). The Th1 phenotype of the recruited T cell population in Cybb tm1 mice is significant because mice lacking Th1 cytokines succumb to cryptococcal infection (5, 28, 44 – 46). IFN-γ will contribute to granuloma formation and the activation of macrophages (47). Furthermore, the chronic eosinophilia normally observed during a C. neoformans infection elicits extensive tissue disruption and damage through degranulation and crystal formation and facilitates replication and spread of Cryptococcus (7). The significant reduction in eosinophilia in Cybb tm1 mice correlates with reduced lung pathology and damage and could also explain the enhanced ability to control this pathogen.

FIGURE 5. The antioxidant MnTE-2-PyP reduces C. neoformans-induced inflammation and reduces dissemination to the brain. C. neoformans-infected mice were treated i.n. with PBS or MnTE-2-PyP on 0, 4, and 8 days after infection. A, Representative H&E-stained lung sections of C. neoformans-infected PBS and MnTE-2-PyP-treated mice 12 days after infection (×200 magnification). B, Lungs were removed from C. neoformans-infected mice after 12 days, and the percentage of lymphocytes was determined by flow cytometry. The number of lymphocytes was calculated by the following: percentage of positive cells by flow cytometry × total number of viable cells. C, BAL eosinophils were enumerated as granulocytes by flow cytometry and confirmed using H&E-stained cytocentrifuge preparations 12 days after infection. Single-cell suspensions of lung tissue were stained with IFN-γ-FITC, CD4-PerCP, and CD8-allophycocyanin. D, Representative plots of CD4+ vs CD8+ in C. neoformans-infected PBS and MnTE-2-PyP-treated mice 12 days after infection. E, The percentage of these T cells expressing IFN-γ was determined by flow cytometry. F, C. neoformans CFUs/brain were determined by plating out brain homogenate on Sabouraud dextrose. Closed symbols, Wild-type mice; open symbols, Cybb tm1 mice. Results represent mean values ± SEM from five mice per group (*, p < 0.05; **, p < 0.01) and are representative of three experiments.
stress in defining human T cell differentiation to Th1/Th2 subsets, with dimethyl naphthoquinone (induces oxidative stress) shown to cause the up-regulation of Th2 cytokines IL-4, IL-5, and IL-13, but not Th1 cytokines. Similarly, it has been demonstrated that exposure of peripheral blood T cells to antioxidant vitamin E reduces IL-4 production, that intake of vitamin E reduces IgE serum levels, and that allergic asthma is linked to a low intake of antioxidant vitamins (51–53). Furthermore, in a model of experimental arthritis, NADPH oxidase knockout mice display increased TNF and IL-1 (54), and we have previously seen an enhancement of Th1 responses in Cybb tm1 mice (37). Considering the pivotal role of IL-12 in the generation of Th1 responses (5), it was surprising that anti-IL-12 treatment had a negligible effect on the immune profile observed in Cybb tm1 mice, especially because similar treatment of wild-type mice enhanced Th2 cytokines and impaired pathogen clearance. Cybb tm1 mice still displayed a potent Th1 response and enhanced protection even when deprived of IL-12. It would appear that the aberrant immune phenotype evoked by the absence of NADPH oxidase and that derived from the NOX family that have been more classically affiliated with a role in signaling. The effects of MnTE-2-PyP reported here are analogous to its effects in a murine model of asthma, where it reduced inflammation and, significantly, eosinophilia (59). Significantly, mice administered the MnTE-2-PyP antioxidant for a longer period were not compromised in their ability to combat C. neoformans infection, with treated mice exhibiting reduced pulmonary inflammation as well as reduced lung and brain fungal titers at day 45 after infection.

In summary, it would appear that the absence of a functional phagocyte NADPH oxidase results in enhanced C. neoformans clearance and, most significantly, reduced dissemination to the brain. This is in contrast to the compromised immunity that such mice show to other fungal pathogens, and is a function of a heightened macrophage infiltration and the ensuing Th1-driven immune response. These findings emphasize a significant role for ROS in defining the Th1/Th2 paradigm and offer a novel mechanism to modulate myeloid immune responses.

Disclosures

The authors have no financial conflict of interest.

References


