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RESEARCH ARTICLE

CD4$^+$ T Cells Orchestrate Lethal Immune Pathology despite Fungal Clearance during *Cryptococcus neoformans* Meningoencephalitis

Lori M. Neal,[a,b] Enze Xing,[b] Jintao Xu,[a,b] Jessica L. Kolbe,[b] John J. Osterholzer,[a,b] Benjamin M. Segal,[c] Peter R. Williamson,[d] Michal A. Olszewski[a,b]

Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Michigan Health System, Ann Arbor, Michigan, USA[a]; Research Service, Ann Arbor VA Health System, Department of Veterans Affairs Health System, Ann Arbor, Michigan, USA[b]; Department of Neurology, University of Michigan, Ann Arbor, Michigan, USA[c]; Laboratory of Clinical Immunology and Microbiology, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, Maryland, USA[d]

ABSTRACT *Cryptococcus neoformans* is a major fungal pathogen that disseminates to the central nervous system (CNS) to cause fatal meningoencephalitis, but little is known about immune responses within this immune-privileged site. CD4$^+$ T cells have demonstrated roles in anticryptococcal defenses, but increasing evidence suggests that they may contribute to clinical deterioration and pathology in both HIV-positive (HIV+) and non-HIV patients who develop immune reconstitution inflammatory syndrome (IRIS) and post-infectious inflammatory response syndrome (PIIRS), respectively. Here we report a novel murine model of cryptococcal meningoencephalitis and a potential damaging role of T cells in disseminated cryptococcal CNS infection. In this model, fungal burdens plateaued in the infected brain by day 7 postinfection, but activation of microglia and accumulation of CD45^{hi} leukocytes was significantly delayed relative to fungal growth and did not peak until day 21. The inflammatory leukocyte infiltrate consisted predominantly of gamma interferon (IFN-γ)-producing CD4$^+$ T cells, conventionally believed to promote fungal clearance and recovery. However, more than 50% of mice succumbed to infection and neurological dysfunction between days 21 and 35 despite a 100-fold reduction in fungal burdens. Depletion of CD4$^+$ cells significantly impaired IFN-γ production, CD8$^+$ T cell and myeloid cell accumulation, and fungal clearance from the CNS but prevented the development of clinical symptoms and mortality. These findings conclusively demonstrate that although CD4$^+$ T cells are necessary to control fungal growth, they can also promote significant immunopathology and mortality during CNS infection. The results from this model may provide important guidance for development and use of anti-inflammatory therapies to minimize CNS injury in patients with severe cryptococcal infections.

IMPORTANCE CNS infection with the fungal pathogen *Cryptococcus neoformans* often results in debilitating brain injury and has a high mortality rate despite antifungal treatment. Treatment is complicated by the fact that immune responses needed to eliminate infection are also thought to drive CNS damage in a subset of both HIV+ and non-HIV patients. Thus, physicians need to balance efforts to enhance patients' immune responses and promote microbiological control with anti-inflammatory therapy to protect the CNS. Here we report a novel model of cryptococcal meningoencephalitis demonstrating that fungal growth within the CNS does not immediately cause symptomatic disease. Rather, accumulation of antifungal immune cells critically mediates CNS injury and mortality. This model demonstrates that antifungal immune responses in the CNS can cause detrimental pathology and

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Address correspondence to Michal A. Olszewski, olszewsm@umich.edu.

addresses the urgent need for animal models to investigate the specific cellular and molecular mechanisms underlying cryptococcal disease in order to better treat patients with CNS infections.

KEYWORDS *Cryptococcus*, *Cryptococcus neoformans*, IRIS, PIIRS, T cells, central nervous system infections, encephalitis, fungi, immunopathology, meningitis, opportunistic infections

Cryptococcus neoformans is an environmental fungal pathogen that causes substantial morbidity and mortality worldwide, accounting for an estimated 1 million infections and 200,000 to 600,000 deaths per year (1–3). Although inhalation is considered to be a primary route of infection, the majority of symptomatic disease and deaths are due to fungal dissemination to the central nervous system (CNS) and subsequent meningoencephalitis. Accordingly, the majority of patients do not display overt pulmonary symptoms but present with neurological sequelae. Treating cryptococcal CNS infections is challenging due to the high toxicity and poor blood-brain barrier (BBB) penetration of antifungal drugs in addition to increased development of drug resistance (4–6). Hence, therapeutic failures are common. Acute mortality for CNS infections is up to 30 to 50% despite treatment in both HIV-positive (HIV+) and -negative patients, and relapse is common (6–11). Many surviving patients require life-long therapy and may be left with lasting disabilities, including memory loss, vision deficiencies, hearing and speech impairments, and motor deficits. Thus, there is an urgent need for studies to elucidate the cellular and molecular processes underlying the pathogenesis of cryptococcal CNS disease to develop more effective therapeutic strategies.

Prior studies have established a central role for $CD4^+$ and $CD8^+$ T cells in fungal clearance from the lungs in mice (12–19) and suggest that similar Th1-biased cellular immunity is beneficial for fungal clearance in the CNS (20–24). Similarly, the susceptibility to cryptococcal disease in T cell-deficient human patients, such as due to HIV/AIDS or T cell depletion therapies, including transplant conditioning, supports a paradigm that clinical failures are predominantly due to a deficiency in microbiological control. This paradigm has led to recommendations for T cell adjunctive therapies, such as recombinant gamma interferon (IFN-γ), to treat clinical failures of the disease in both HIV-positive and HIV-negative individuals (6, 25). However, accumulating evidence supports that control of fungal growth is only one aspect of disease, while immune inflammatory factors can significantly contribute to CNS pathology in certain subsets of patients with complicated CNS disease (26–28). Up to one-third of cryptococcus-infected HIV+ patients initiating antiretroviral therapy develop immune reconstitution inflammatory syndrome (IRIS) upon restoration of T cell counts (29). These patients develop severe neurological sequela and morbidity, associated with high levels of proinflammatory markers, often despite fungal eradication (30). These findings were reproduced in a *C. neoformans*-related IRIS model, in which transfer of $CD4^+$ T cells into the infected Rag-$1^{-/-}$ mice produced inflammatory reactions, including systemic increases in IFN-γ and inflammatory lesions in the lungs, liver, and brain (31).

A similar phenomenon occurs among non-HIV patients with severe cryptococcal CNS disease in the setting of microbiological control, termed post-infectious immune response syndrome (PIIRS) (32–35). Studies confirm that cryptococcal PIIRS and IRIS share many characteristic immunological features, including elevated $CD4^+$ and $CD8^+$ T cell counts in cerebrospinal fluid (CSF) and elevated IFN-γ (32, 33, 36–40), suggesting that the Th1 immune responses necessary for fungal clearance simultaneously contribute to clinical disease. A potential detrimental role for host inflammation in these patients is further supported by the benefits of selective and judicious use of corticosteroid therapy in ameliorating IRIS and PIIRS symptoms and by observations that premature or abrupt steroid weaning potentiates relapse in CNS lesion formation and redeterioration in clinical symptoms (6, 11, 27, 32–34, 41, 42). Thus, traditionally recognized hallmarks of the protective immune response can be important contribu-

tors to pathogenesis of CNS injury and its lasting consequences during CNS cryptococcosis.

While several models of cryptococcal CNS infection have been described, these studies have not focused on the role of CNS inflammation in pathogenesis of cryptococcal CNS disease, such as the cryptococcal CNS disease that occurs in IRIS and PIIRS patients (20–24, 28, 43–47), or report on only a few selected inflammatory parameters for the CNS (31). The goal of the present study was to develop a robust, physiologically relevant murine model of *C. neoformans* CNS infection that reproduces aspects of human cryptococcal disease pathology and that is suitable for detailed immunological analysis. Here, using this newly established model of disseminated CNS infection in C57BL/6 mice, we demonstrate that Th1-biased CD4+ T cell-driven inflammation in the CNS can be the primary cause of detrimental pathology at least in a subset of patients with severe or refractory cryptococcal meningoencephalitis. These studies provide new insights regarding clinical outcomes observed in IRIS and PIIRS patients and support the development of therapeutic strategies that limit aspects of inflammation to prevent CNS damage in suitably selected patients with CNS cryptococcosis.

RESULTS

Intravenous infection with *C. neoformans* 52D causes fatal meningoencephalitis, but symptomatic disease is independent of peak fungal burden. In order to establish a reproducible, controlled-onset mouse model of cryptococcal meningoencephalitis, we infected C57BL/6 mice with 10^6 CFU of *C. neoformans* strain 52D via intravenous (i.v.) injection. This strain was chosen for these studies because other highly virulent strains (such as H99) induce rapid 100% mortality by 4 to 14 days postinfection before a measurable adaptive immunological response can develop (21, 23, 24, 43, 45, 48) and thus are less suited to study adaptive responses within the CNS. At selected time points, *C. neoformans* 52D-infected mice were euthanized, and fungal growth was assessed in perfused, homogenized brains. In separate experiments, animals were monitored and evaluated for symptoms of neurological dysfunction and then euthanized when endpoint symptoms of disease were present. Using this model, we found that abundant fungal organisms were present in the brains by 24 h postinfection, indicating that *C. neoformans* rapidly crossed from circulation into the CNS (Fig. 1A). Fungal burdens continued to rise sharply through day 7, after which they remained relatively stable through day 21 postinfection. From days 21 to 35 postinfection, fungal burdens progressively declined, decreasing by an average of 2 orders of magnitude on day 35 compared to the earlier peak. Throughout this time, mice were systematically examined for symptoms of disease and multiple measurements of body condition, behavior, and neurological status were quantified using a murine coma and behavior score (MCBS) (see Table S1 in the supplemental material) modified from the MCBS of Carroll et al. (49). Despite extremely high CNS fungal burdens, which peaked at 7 to 14 days postinfection, mice remained relatively asymptomatic as assessed by MCBS at these time points (Fig. 1B). In fact, MCBS scores of infected mice did not reach a nadir indicative of highly symptomatic disease until day 21 to 28 postinfection, which corresponded with the onset of fungal clearance and mortality. During the same period, mice began displaying overt and severe clinical symptoms of disease, including significant weight loss despite dietary supplementation and/or neurological sequelae, such as cranial swelling, seizures, ataxia, and limb paralysis. Overall, 50% animals succumbed to infection and required euthanasia between days 21 and 35 postinfection (Fig. 1C). Cranial swelling and tissue edema suggestive of raised intracranial pressure (11) and gross injury were frequently evident (Fig. 1D). However, even animals that succumbed to infection had substantially decreased fungal burdens (Fig. 1A), suggesting that clinical deterioration occurred despite the onset of fungal clearance. Thus, these data demonstrate that the magnitude of CNS fungal burden does not directly correlate with the intensity of clinical symptoms or mortality during cryptococcal meningoencephalitis.

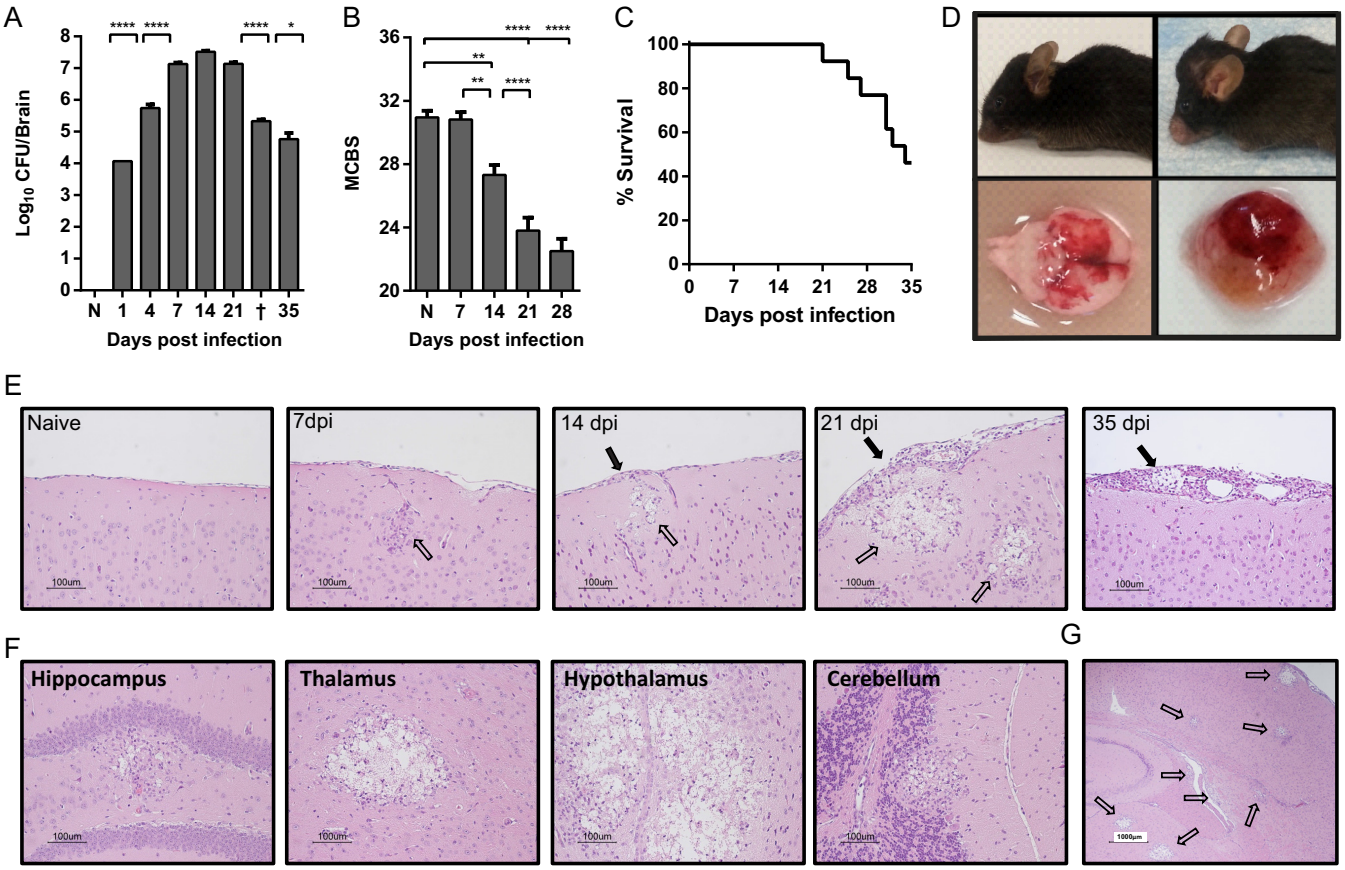

FIG 1 Infection with *Cryptococcus neoformans* 52D causes a highly lethal meningoencephalitis in C57BL/6 mice despite fungal clearance. C57BL/6 mice were infected with 10^6 CFU of *C. neoformans* 52D via retro-orbital intravenous inoculation. (A) Fungal burdens were measured in whole-brain homogenates at the indicated time points postinfection. Naive mice (N) and animals that succumbed to infection and were euthanized between days 31 and 33 postinfection (†) are indicated. (B) Overall health and neurological status was assessed using a modified murine coma and behavioral scale (MCBS), out of a maximum score of 36 points. Data shown in panels A and B are the means plus standard errors of the means (SEM) (error bars) from a representative experiment of 2 to 5 independent experiments with 3 to 8 mice per time point. Values that are statistically significantly different are indicated by brackets and asterisks as follows: *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$. (C) In separate experiments, survival was monitored through day 35 postinfection. Infection was considered fatal, and animals were euthanized when they lost 20% body weight, had persistent cranial swelling, and/or developed neurological sequelae such as seizures, tremors, limb weakness or paralysis. (D) Representative images of severe cranial swelling and CNS tissue injury in infected mice. (E to G) Brains from perfused mice were paraffin embedded, coronally sectioned, and stained with hematoxylin and eosin (H&E). Note that stained *C. neoformans* organisms occupy a relatively small area compared to areas of displaced cerebral tissue by accumulating polysaccharide capsule material, leaving behind a characteristic "Swiss cheese" pattern. Solid black arrows indicate inflammation and cryptococcal organisms in the meninges; clear arrows indicate inflammation and cryptococcal organisms in the parenchyma. (E to G) Representative images shown are from the cortex at various time points postinfection (in days postinfection [dpi]) in panel E (20×), various anatomical structures at 21 days postinfection (20×) (F), and whole brain at 21 days postinfection (4×) (G).

Fungal growth and inflammation are found throughout the brains of mice with cryptococcal meningoencephalitis. Clinical evidence increasingly suggests that the host inflammatory response, rather than pathogen burden, may lead to the development of pathology, neuronal injury, and deteriorating status, especially in IRIS and PIIRS patients (28, 30, 32, 33, 36–40). Thus, our next goal was to evaluate CNS pathology during the progression of *C. neoformans* infection and to determine a possible link to the accumulation of a cellular inflammatory response within the CNS. Histological analysis of brains from infected mice on day 7 postinfection showed that cryptococci were found in meninges, perivascular spaces, and brain parenchyma proper, confirming that *C. neoformans* crossed the blood-brain barrier to colonize the CNS relatively early and in multiple locations (Fig. 1E and data not shown). However, an inflammatory response around areas of cryptococcal growth did not develop immediately. Chronological analysis of infected brains (Fig. 1E) showed minimal inflammation through day 14 postinfection despite the presence of numerous microfocal areas of cryptococcal growth and overall high fungal burdens within the CNS (Fig. 1A). However, on day 21 postinfection, there were marked increases in cellular inflammation surrounding areas

of cryptococci in the parenchyma and in the meninges, signaling the development of fulminant meningoencephalitis. At this time and thereafter, cryptococcal organisms continued to be identified in foci throughout all regions of the brains (Fig. 1F and G), including the cortex, hippocampus, hypothalamus, pons, and cerebellum. Fungal growth was evident throughout the brains of all infected mice with no discernible predilection for specific areas and appeared as larger areas with centrally abundant fungi, resembling characteristic mucoid pseudocysts (32, 43). Notably, the timing of cellular inflammation was synchronized with the onset of mortality and neurological deficits we measured in animals between days 21 and 35 postinfection (Fig. 1B to D). Together, these results suggest that fungal invasion and growth in the CNS alone were not significant factors mediating severe neuronal injury. Rather, progressive cellular inflammation, responsible for driving fungal clearance, correlated with the onset of clinical symptoms and mortality.

Brain-infiltrating leukocyte accumulation is significantly delayed relative to fungal burden but is synchronized with mortality in mice with cryptococcal meningoencephalitis. To quantify and determine the phenotypes of the immune cells in the brains of *C. neoformans*-infected mice, we isolated the leukocyte-enriched brain cell fraction, consisting predominantly of brain-infiltrating leukocytes (BIL) and microglia, containing few structural cells, from brains that had been generously perfused and processed as described in Materials and Methods. These total leukocyte-enriched brain cells were then enumerated and their phenotype was determined using flow cytometry. To correlate the dynamics of resident versus recruited immune cell subsets within the infected CNS and their roles in fungal clearance versus pathology, we extensively analyzed these cells by flow cytometry using the expression level of CD45 to differentiate between microglia ($CD45^{lo}$) and BIL ($CD45^{hi}$) at various times throughout the course of infection. Approximately 1×10^5 cells were isolated from the brains of naive mice, and consistent with the appearance of histological sections, the total number of cells remained stable in the brains of *C. neoformans*-infected mice through day 14 postinfection (Fig. 2A). On day 21, however, the total number of leukocyte-enriched cells recovered from the CNS rose sharply, with a 25-fold increase in cell numbers. The majority of this increase was attributable to an influx of $CD45^{hi}$ recruited BIL (Fig. 2B) consisting primarily of T cells (Fig. 2C) and $CD11b^+$ $Ly6C^+$ myeloid cells (Fig. 2D), which continued to accumulate through day 35 postinfection. Consistent with histological appearance, these data demonstrate that the influx of the cellular inflammatory response was delayed by approximately 2 to 3 weeks relative to the onset of infection and peak fungal burden. Furthermore, the influx of BIL on day 21 postinfection was synchronized with the onset of fungal clearance and mortality (Fig. 1A and D).

We also quantified and determined the phenotypes of $CD45^{int}$ microglia throughout infection. As brain resident macrophages, microglia are among the first cells to encounter *C. neoformans* during CNS infection and are capable of antimicrobial effector responses (22, 45, 50–53). Thus, we initially anticipated that these cells would become activated in response to *C. neoformans* within the infected CNS early during infection prior to the accumulation of BIL. The number of $CD45^{int}$ microglia remained relatively stable throughout the course of infection (Fig. 2E). However, these cells were not immediately activated by rapid expansion of *C. neoformans* in the infected brains. Resting microglia from naive animals expressed low levels of CD11c and major histocompatibility complex class II (MHCII) markers of activation, and expression of these activation markers in infected animals occurred gradually, continuing through day 21 postinfection, at which point nearly all microglia had acquired a $CD11c^{hi}$ $MHCII^{hi}$ phenotype (Fig. 2F). Together, these data support the idea that the CNS inflammatory response involving both resident and infiltrating cells does not develop immediately during cryptococcal infection but is delayed by approximately 3 weeks and aligns with the onset of neurological injury and mortality.

Production of inflammatory cytokines in the *C. neoformans*-infected brain is significantly delayed. We also asked whether inflammatory cytokines were produced by early resident cells in response to CNS infection and found that the early response

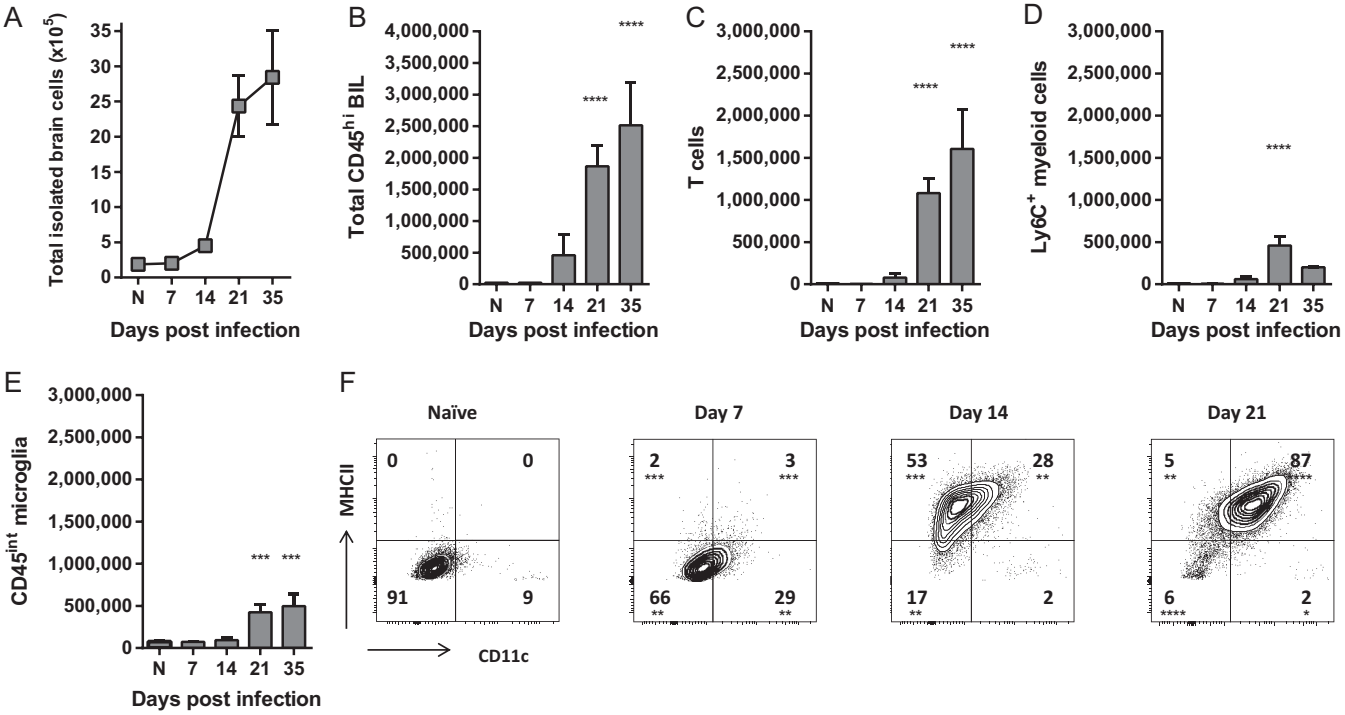

FIG 2 Recruitment of brain-infiltrating leukocytes and activation of microglia is delayed relative to early fungal growth in the CNS. (A) The total number of cells isolated from the leukocyte-enriched fraction of brains, containing predominantly leukocytes and microglia, was quantified at various time points postinfection. (B to D) The number of $CD45^{hi}$ brain-infiltrating leukocytes (BIL) (B), including T cells (C) and $CD11b^{+}$ LyC^{+} myeloid cells (D) was determined by flow cytometry. (E and F) Numbers of $CD45^{int}$ microglia (E) and expression of surface activation markers CD11c and MHCII (F) were also quantified. Note that there was not significant accumulation of BIL or complete activation of microglia until late on day 21 postinfection, delayed relative to peak fungal growth but coinciding with the onset of mortality (Fig. 1). Data shown are the mean ± SEM from a representative experiment of two to four independent experiments with three to eight animals per time point. Values that are statistically significantly different from the means for naive (N) animals are indicated by asterisks as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

to *C. neoformans* infection in the CNS was relatively quiescent. The levels of tumor necrosis factor alpha (TNF-α) remained at basal levels through day 14 and only showed statistically significant increases on day 21 postinfection and beyond (Fig. 3A). Chemokines CCL5 (CC chemokine ligand 5) (RANTES [*r*egulated on *a*ctivation, *n*ormal *T* cell *e*xpressed and *s*ecreted]) and CCL2 (macrophage chemoattractant 1 [MCP-1]) also did not increase until day 21 (Fig. 3B and C), whereas CCL3 (macrophage inflammatory protein 1α [MIP-1α]), CCL4 (MIP-1β), CCL20 (MIP-3α), CXCL5 (CXC chemokine ligand 5) (lipopolysaccharide-induced CSC chemokine [LIX]), and CXCL13 (B lymphocyte chemoattractant [BLC]) were even further delayed (Fig. 3D to H). Interestingly, we did not find significantly elevated levels of interleukin 12p70 (IL-12p70), IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), or CCL17 (thymus and activation-regulated chemokine [TARC]) at any time point throughout the course of infection (data not shown). These data further support that molecular signals consistent with antigen recognition are not immediately correlated with increasing fungal burdens early during infection but are delayed and synchronized with the leukocyte recruitment (Fig. 2A to D) beginning on day 21 postinfection.

Brain-infiltrating leukocytes consist primarily of antigen-experienced $CD4^{+}$ and $CD8^{+}$ T cells in mice with cryptococcal meningoencephalitis. To determine the dynamics and immune phenotypes of BIL entering the CNS on day 21 and beyond, we extensively analyzed the $CD45^{hi}$ recruited population by flow cytometry. $CD4^{+}$ T cells composed the majority of the $CD45^{hi}$ cellular infiltrate, overall accounting for 40 to 50% of the total recruited cells from days 21 and 35 postinfection (Fig. 4A and B). $CD8^{+}$ T cells composed a smaller fraction of approximately 15% of recruited cells (Fig. 4C). Nearly all (>90%) of both $CD4^{+}$ and $CD8^{+}$ T cells isolated from the *C. neoformans*-infected CNS were antigen-experienced ($CD44^{hi}$ $CD62L^{lo}$ [CD62L stands for CD62

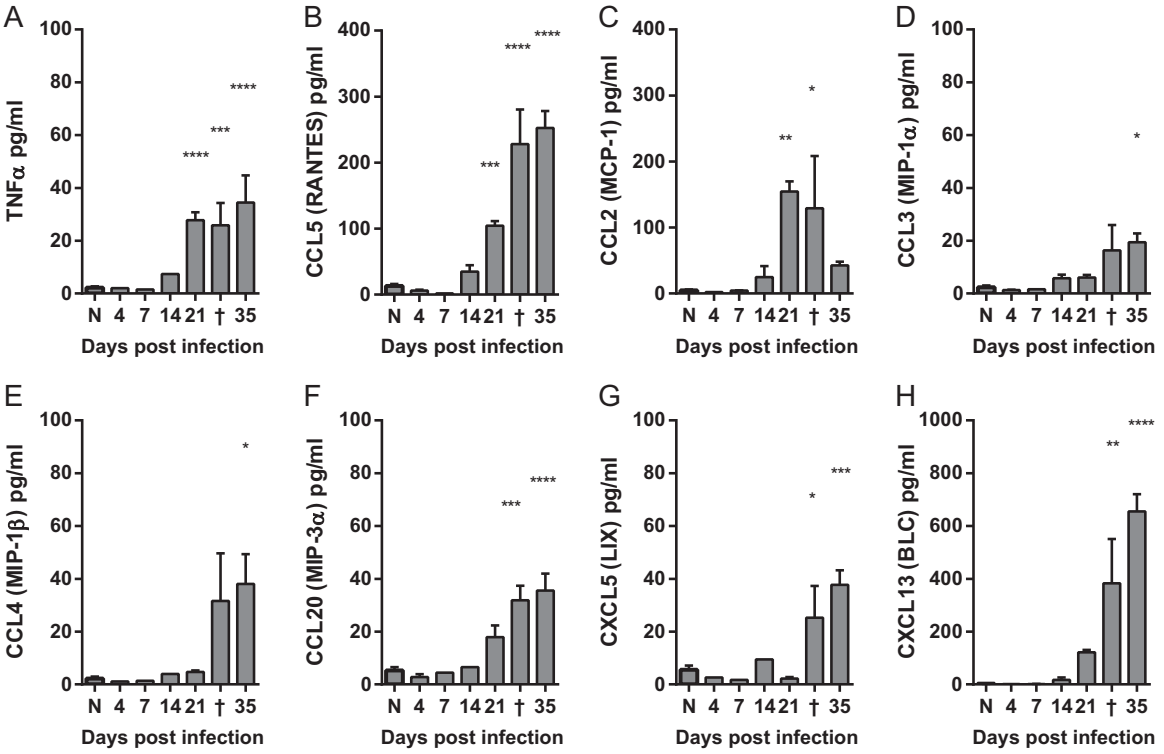

FIG 3 Production of inflammatory cytokines in the brains of mice with cryptococcal meningoencephalitis is delayed but coincides with leukocyte recruitment. (A to H) Levels of inflammatory cytokines were measured in the supernatants of whole-brain homogenates. Each brain was homogenized in 5 ml of medium. Naive mice (N) and animals that succumbed to infection and were euthanized between days 31 to 33 postinfection (†) are indicated. Note that there was not significant induction of any cytokines measured until late on day 21 postinfection. Data shown are the means ± SEM from a representative of two to four independent experiments with three to eight mice per time point. Values that are statistically significantly different from the means for naive animals are indicated by asterisks as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

ligand]) (Fig. 4D and E). These frequencies were significantly greater than those found in the peripheral tissues, including the cervical lymph nodes, blood, and lungs for both CD4+ and CD8+ T cells, suggesting that these antigen-experienced cells have preferential access to the *C. neoformans*-infected CNS.

T cell inflammatory response in the CNS during cryptococcal meningoencephalitis is highly Th1 polarized, and nearly all CD4+ and CD8+ T cells produce IFN-γ. The subsets of patients with paradoxical inflammatory responses to cryptococcal infection (IRIS and PIIRS) display dominant Th1 T cell polarization with elevated levels of IFN-γ in the serum and CSF (32, 33, 36–40). Thus, we sought to determine whether murine brain-infiltrating T cells would mimic this phenotype by analyzing T cell cytokine production and expression of polarizing Th-associated transcription factors in the *C. neoformans*-infected CNS. Brain-infiltrating T cells demonstrated an overwhelming Th1 bias, as nearly all (>90%) CD4+ and CD8+ cells isolated from the brains of *C. neoformans*-infected mice on days 21 to 35 postinfection produced IFN-γ (Fig. 5A). This influx of IFN-γ-producing T cells correlated with significant elevation of secreted IFN-γ in whole-brain tissue samples (Fig. 5B), robust increases in IFN-γ transcript and induction of the Th1-associated transcription factor Tbet (Fig. 5D and E). Remarkably, there was no evidence of either a Th17 or Th2 response in the infected CNS, either at the level of protein or transcript (Fig. S2), indicating that virtually all of the immune response was Th1 driven. Cumulatively, these data corroborate the increased T cell responses and IFN-γ levels found in patients with IRIS and PIIRS and suggest that overexuberant Th1-biased CD4+ T cell responses may contribute to detrimental clinical outcomes during cryptococcal CNS infections.

Depletion of CD4+ T cells prevents mortality of mice with cryptococcal meningoencephalitis despite impaired fungal clearance. To conclusively determine

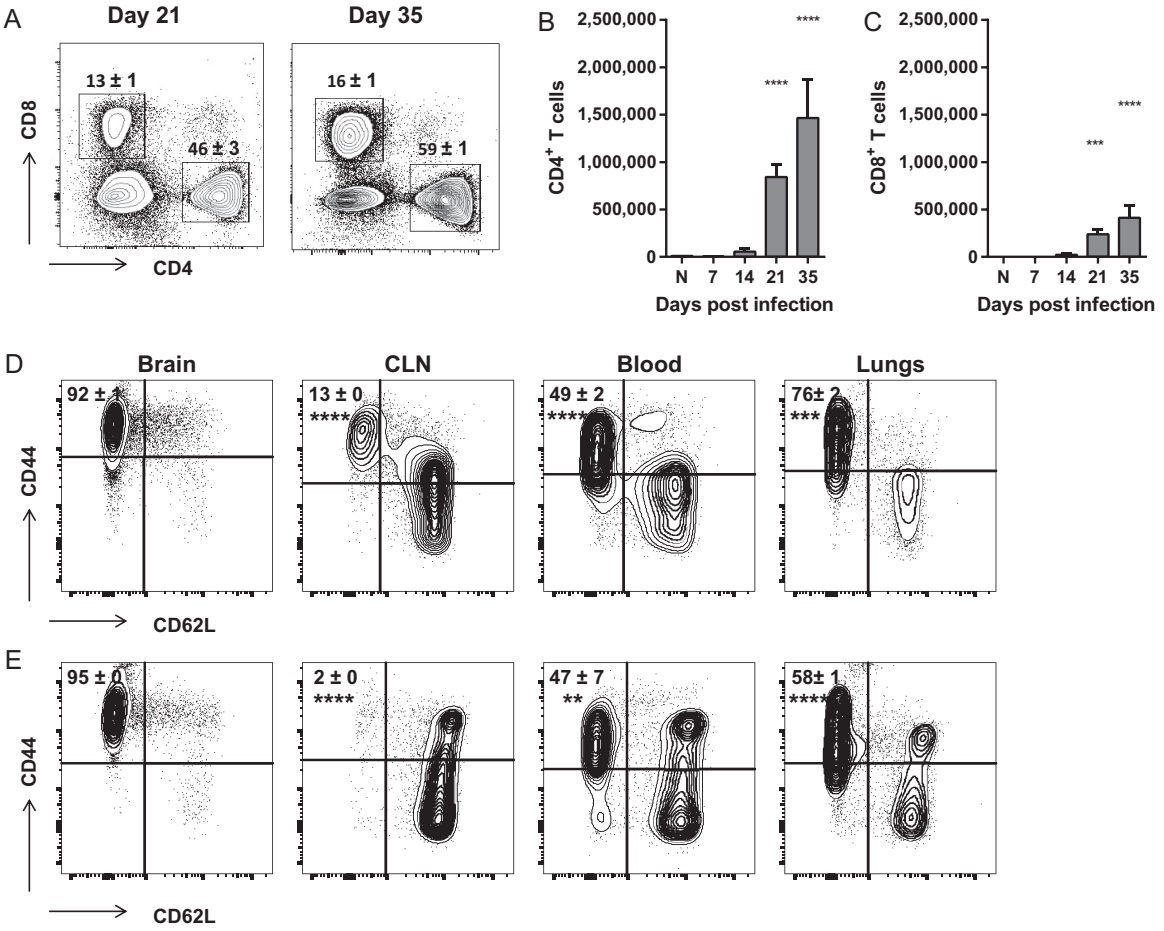

FIG 4 CD4$^+$ and CD8$^+$ T cells that accumulate in the brains of mice with cryptococcal meningoencephalitis are antigen-experienced cells. (A to C) The percentages (of total CD45^{hi} BIL) (A) and total numbers of CD4$^+$ and CD8$^+$ T cells (B and C) that accumulate in the brains of *C. neoformans*-infected mice. (D and E) The frequencies of antigen-experienced (CD44^{hi} CD62L^{lo}) CD4$^+$ (D) and CD8$^+$ (E) T cells isolated from the brains, cervical lymph nodes (CLN), blood, and lungs of infected mice on day 35 postinfection. Similar frequencies were observed on day 21. Data shown are the mean ± SEM from a representative experiment of two to four independent experiments with three to eight mice per time point. Representative flow cytometry plots are shown. Values that are statistically significantly different from the means for naive (N) animals (B and C) or brain-infiltrating cells (D and E) are indicated by asterisks as follows: **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

whether CD4$^+$ T cells contribute to CNS damage and mortality in our murine model of cryptococcal meningoencephalitis, we depleted CD4$^+$ T cells from mice beginning 2 days prior and throughout the course of infection. This depletion regimen successfully eliminated >99% of CD4$^+$ T cells from the brains of *C. neoformans*-infected mice (Fig. 6A) and in the periphery (data not shown). Unlike their isotype-treated counterparts, nearly 100% of CD4-depleted mice survived to day 35 post *C. neoformans* infection and did not succumb to immune pathology (Fig. 6B). Notably, the survival of CD4-depleted mice occurred despite their failure to clear fungal organisms. The brain fungal burdens of CD4-depleted mice peaked at levels 10-fold higher than isotype-treated animals (Fig. 6C) and remained elevated out to day 35 without apparent symptoms of CNS disease. CD4-depleted infected mice also demonstrated behavioral responses indistinguishable from naive animals (Fig. 6D), whereas isotype-treated animals had worse MCBS scores and succumbed to disease despite fungal clearance (Fig. 6C and D). These data demonstrate that while CD4$^+$ T cells are necessary to control fungal burden, they ultimately contribute to disease pathology and clinical deterioration. Further supporting this conclusion, animals treated with a partial-depletion regimen consisting of only two anti-CD4 treatments beginning the day of infection, which reduced CNS CD4$^+$ T cells by only approximately 30%, still exhibited delayed mortality and a trend toward increased survival (Fig. S3).

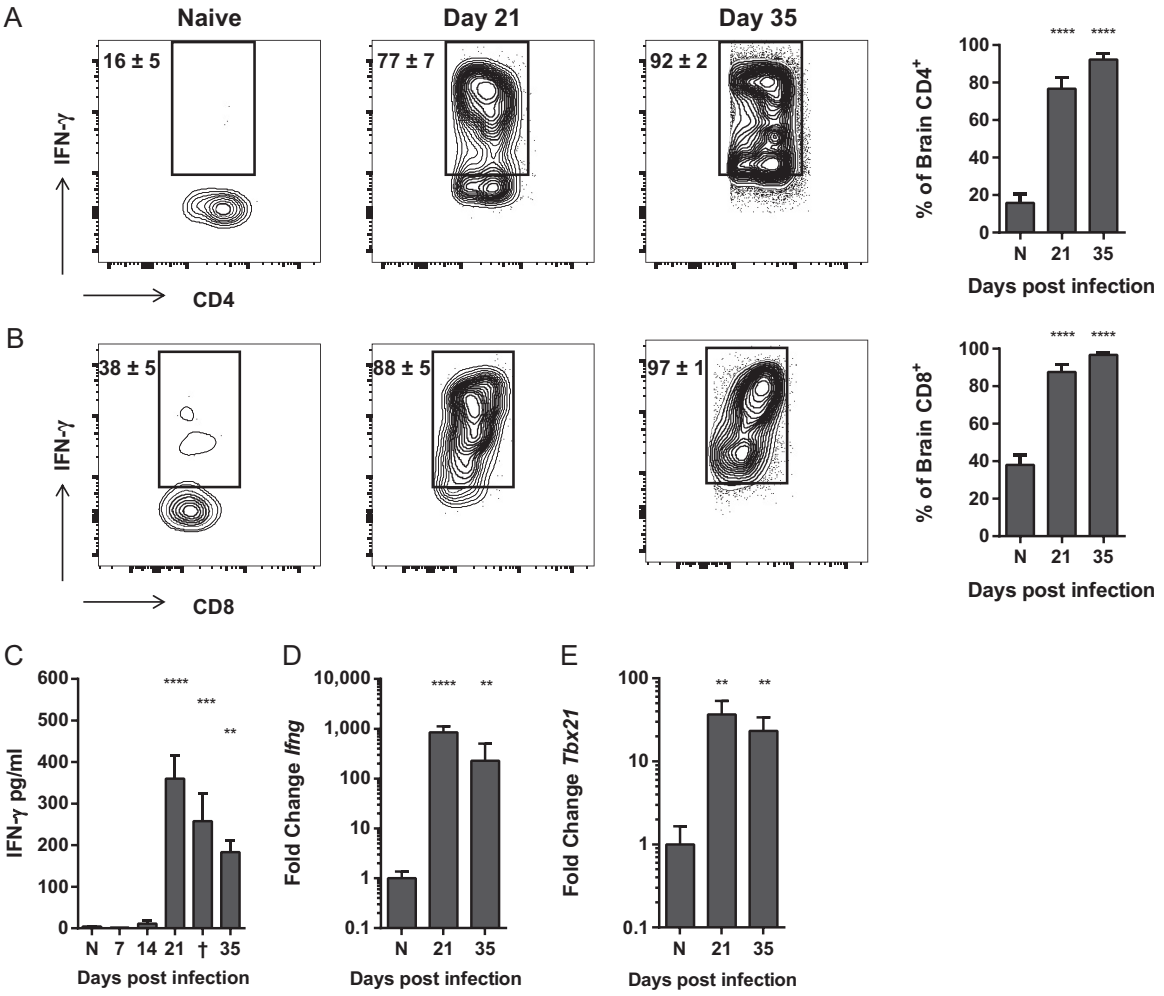

FIG 5 CD4+ and CD8+ T cells that accumulate in the brains of mice with cryptococcal meningoencephalitis are uniformly Th1 polarized and produce IFN-γ. (A and B) Frequency of IFN-γ-producing CD4+ (A) and CD8+ (B) T cells isolated from the brains of naive (N) and infected mice on day 21 and 35 postinfection. (C to E) Levels of IFN-γ protein and expression of IFN-γ (*Ifng*) (D) and Tbet (*Tbx21*) transcript (E) were measured in whole-brain homogenates. Note that fold expression data are on a log scale. Data shown are the means ± SEM from a representative of two to four independent experiments with three to eight mice per time point. Representative flow cytometry plots are shown. Values that are statistically significantly different from the means for naive (N) animals are indicated by asterisk as follows: **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Finally, depletion of CD4+ T cells during *C. neoformans* meningoencephalitis also broadly inhibited all other aspects of the CNS inflammatory response. Expression of IFN-γ was dramatically reduced in CD4-depleted mice (Fig. 6E) consistent with CD4+ T cells being an abundant source of this cytokine in the CNS during infection. Depletion of CD4+ T cells also led to a striking reduction in the accumulation of total CD45hi BIL (Fig. 6F), including CD8+ T cells (Fig. 6G) and CD11b+ Ly6C+ myeloid effector cells (Fig. 6H). Taken together, these data strongly support the idea that CD4+ T cells exert dual but opposing roles: promoting the accumulation of immune cells and driving fungal clearance but simultaneously orchestrating lethal pathology during cryptococcal meningoencephalitis.

DISCUSSION

In this study, we established an intravenous infection model using *C. neoformans* strain 52D in C57BL/6 mice that reproduces features of highly lethal cryptococcal meningoencephalitis occurring in human patients with severe CNS manifestations in the setting of microbiological control, including immune reconstitution inflammatory syndrome (IRIS) and post-infectious inflammatory response syndrome (PIIRS). Using this

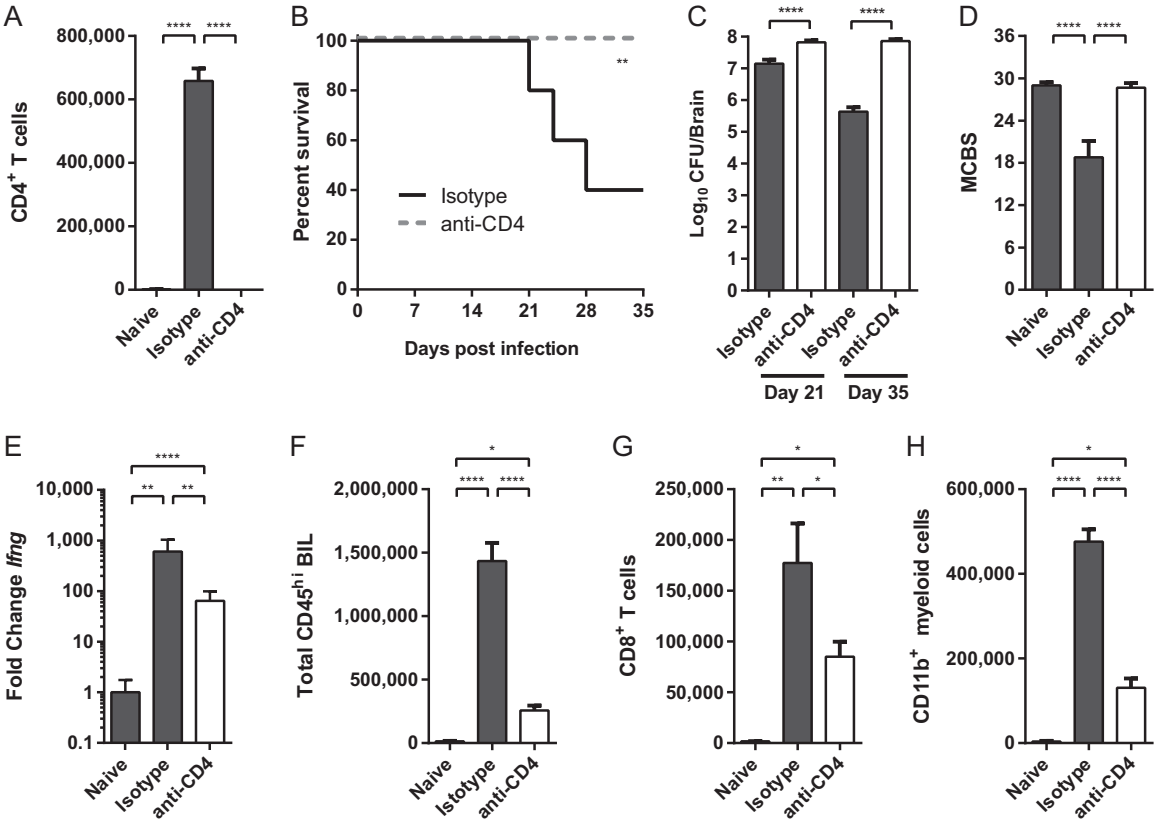

FIG 6 Depletion of CD4$^+$ T cells rescues survival despite failure to clear infection during cryptococcal meningoencephalitis. (A) Treatment with anti-CD4 depleting antibody (white bar) reduces the total number of brain-infiltrating CD4$^+$ T cells by >99% compared to infected isotype-treated controls (dark gray bar). (B) Survival of infected CD4-depleted (broken line) and isotype-treated mice through day 35 postinfection. (C and D) Brain fungal burdens were calculated on day 21 and 35 postinfection (C), and MCBS scores were calculated on day 21 (D). (E) Expression of IFN-γ transcript was quantified in whole-brain homogenates on day 35. Note that fold expression data are on a log scale. (F to H) The total numbers of CD45^{hi} BIL (F), CD8$^+$ T cells (G), and CD11b$^+$ Ly6C$^+$ myeloid cells (H) were quantified by flow cytometry on day 35 postinfection. Similar reductions were also observed on day 21. Data shown are the means ± SEM from a representative experiment of three independent experiments with 5 to 10 mice per group. Values that are statistically significantly different are indicated by brackets and asterisks as follows: *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

model, we showed that although *C. neoformans* rapidly enters and expands within the murine CNS within a few days postinfection, it does not induce an immediate inflammatory response at this site. Rather, the cellular inflammatory responses did not become pronounced until day 21 postinfection, which coincided with the onset of fungal clearance, development of neurological symptoms, and progressive mortality. The major immune cell subset that accumulated within the infected CNS were Th1-polarized IFN-γ-producing CD4$^+$ T cells. Importantly, the development of lethal CNS symptoms could be prevented by depletion of CD4$^+$ T cells, despite the persistent and increased growth of fungus within the CNS. Thus, despite being required for fungal clearance, Th1 cells are a central mediator of detrimental CNS pathology. These results support the paradigm that regulation of T cell responses within the CNS needs to be considered part of the therapeutic strategy to prevent CNS damage in certain patients with cryptococcal CNS infection.

The treatment of cryptococcal CNS infections presents many challenges due to the need to balance microbial control with a patient's own potentially paradoxical responses during therapy (26, 27, 29, 30, 32). In these patients, treatment strategies must strike a balance between activation of antifungal T cell responses and limiting CNS damage due to inflammation. Although indiscriminate use of steroids is not universally justified for all cases of cryptococcal meningitis (54), including initial treatment of most HIV-infected patients where the immune response is generally poor and microbiological control has not been achieved (55), steroid therapy has been used with some

success in refractory cases where elevated levels of IFN-γ, CNS T cell reactivity, and neuronal injury are predominate features of disease, such as in IRIS and PIIRS (6, 11, 27, 28, 32–34, 41, 42). However, a more complete understanding of immune pathogenesis is desperately needed to develop more precise and effective therapies. Much of the current understanding regarding immune defense against cryptococcal infection results from studies using models of pulmonary infection aimed at deciphering mechanisms preventing fungal escape from the lungs (12–18), which were presumed to be beneficial in the CNS (20–24). However, to date, little has been shown about either the pathogenesis or the cellular and molecular mechanisms mediating anticryptococcal defenses within the CNS itself. The data we present here using our CNS infection model demonstrates dramatic and significant distinctions between immune responses within the CNS compared to the lungs, which has important implications for clinical treatment of CNS infections.

First, we observed that there is a surprisingly long quiescent period between entry of *C. neoformans* into the CNS before evidence of cryptococcal antigen recognition and subsequent development of the immune response. We did not measure significant chemokine production or inflammatory cell recruitment to the infected CNS until 21 days postinfection with high inoculum (10^6 CFU i.v. seeding 10^4 CFU in the CNS within 24 h) (Fig. 2 and 3), unlike in the lungs where the robust cytokine responses and leukocyte accumulation occur by 7 days postinfection, even after a relatively small dose (10^4 CFU) of *C. neoformans* is instilled (13, 56, 57). This delay occurred despite the fact that the fungus rapidly expanded within CNS, at rates comparable to that reported in the lungs; expansion of 10^4 organisms at 24 h to 10^7 organisms within 7 days. This is likely due to the poor response of CNS resident cells in recognizing and responding to *C. neoformans*. The fact that we did not observe full activation of microglia until 21 days postinfection is supported by previous studies showing that *C. neoformans* can be phagocytosed and induce cytokine and chemokine production by microglia, including CCL3, CCL4, and CCL5, but these effects are severely hindered in the absence of opsonizing antibody or additional stimulatory factors (45, 47, 50, 51, 53). Thus, it appears that substantial time is required to overcome the poor activation and impaired phagocytosis of *C. neoformans* by microglia and to provide significant antigenic stimulation to prompt local reactivation and proliferation of peripherally licensed T cells surveilling the CNS. Likewise, we did not measure any significant breakdown in blood-brain barrier function through at least day 21 postinfection (by Evans blue dye exclusion [data not shown]) that would have permitted unrestricted immune cell entry. Furthermore, even at its peak, the relative abundance of accumulated leukocytes was at least 1 order of magnitude lower in the CNS than in the lungs despite relatively similar fungal loads achieved in both tissues (13, 56, 57).

The second, but perhaps most important, distinction was the unique compartmentalization of polarized T cell responses in the CNS compared to the lungs. Notably, we observed a total dominance of Th1 polarization in the CNSs of *C. neoformans* 52D-infected mice; nearly all $CD4^+$ and $CD8^+$ T cells were found to be antigen experienced, in contrast to the periphery, and virtually all produced IFN-γ. This is in stark contrast to years of data gained from studies of pulmonary and systemic responses where the balance of Th1/Th2/Th17/Treg (regulatory T cell) responses ultimately shape disease outcome. Data generated by multiple labs, using multiple strains of mice and of *C. neoformans*, uniformly support the idea that Th1 responses are protective, promoting fungal clearance and recovery, while Th2 responses allow for fungal persistence and drive immune pathology in the infected lungs. In particular, C57BL/6 mice develop a mixed Th2/Th1/Th17 response to pulmonary infection with *C. neoformans* 52D, resulting in immunological stalemate and persistent infection, but outcomes can be improved by provoking a greater Th1 bias (12, 58, 59) or worsened when stronger Th2 bias is induced (44, 60, 61). Our data significantly differ from this balanced Th polarization paradigm, in that we did not observe any hallmarks of Th2 or Th17 response within the infected CNS of C57BL/6 mice at any time point.

Rather, the seminal finding of our study is that antifungal Th1-polarized IFN-γ-

producing CD4$^+$ T cells have immense pathogenic potential within the CNS. The timing of IFN-γ-producing T cell accumulation in *C. neoformans*-infected CNS just prior to the onset of fatalities and declining neurological function, despite fungal clearance, strongly suggests that these cells contribute to mortality in certain patients with severe or refractory CNS disease despite successful microbiological control. This notion is further supported by our results using CD4$^+$ T cell depletion in *C. neoformans*-infected mice. CD4-depleted mice uniformly survived to day 35 after CNS infection despite fungal burdens maintained for weeks at levels 10- to 1,000-fold higher than controls, which develop neurological sequelae and succumb to infection despite fungal clearance. These data conclusively show that Th1-polarized CD4$^+$ T cells are required to directly or indirectly orchestrate a catastrophic inflammatory cascade that can lead to the CNS damage independent of fungal burden during cryptococcal CNS infection. On the other hand, the high susceptibility and mortality of HIV-associated cryptococcal meningitis, along with increased fungal burdens in CD4-depleted mice, support the idea that restoration of CD4$^+$ T cells and treatment with antifungal drugs are still required for pathogen clearance and long-term patient survival. Thus, the goal of the present study is to begin to dissect microbiologically from immunologically induced pathological effects in order to minimize overall CNS damage (62). While our data emphasize that inhibition of T cell responses in the CNS may be protective against severe inflammatory damage in select cases of CNS cryptococcosis, such as IRIS and PIIRS, long-term inhibition may pose other risks due to microbiological factors (i.e., unrestricted growth of the fungus). Thus, future T cell-targeted therapies for patients with severe cryptococcal CNS disease will need to identify critical windows of T cell functions and carefully weigh their pathological versus beneficial effects.

These data showing the pathological potential of IFN-γ-producing T cells in the CNS during *C. neoformans* meningoencephalitis are supported by notable damaging roles of CD4$^+$ and CD8$^+$ T cells in the CNS during infections, including cerebral malaria (63–65), viral encephalitis (66), toxoplasmosis (67–69), tuberculosis meningitis (27), and immunological disorders such as multiple sclerosis (70). However, a similar paradigm exalting paradoxical roles for T cells has only recently begun to emerge in the study of cryptococcal infections. Clinical review of complex and refractory human infections increasingly implicate T cells in immune-driven CNS pathology in both HIV+ and non-HIV patients. In HIV+ patients, IRIS frequently arises following restoration of T cell-mediated immunity, and in non-HIV individuals, PIIRS is correlated with elevated CSF CD4$^+$ T cell counts. Both are associated with elevated IFN-γ and often occur despite successful antifungal therapy and clearance supporting the idea that, as in our murine model, immune pathology is Th1 cell driven and is not solely determined by fungal burden (32, 33, 36–40). How the overexuberant Th1 responses develop in these patients is unknown, but they are possibly linked to myeloid cell dysfunction leading to a persistent overly trigger-sensitive (low-threshold) and hyperactivated state (35, 39, 71).

Finally, our findings that CD4 depletion conferred a survival benefit during severe CNS cryptococcosis in mice correlate with cases of severe paradoxical or refractory cryptococcal CNS disease in humans, where the use of steroids has been shown to aid or even be essential for the resolution of clinical symptoms (6, 11, 27, 32–34, 41). Together, these studies clearly demonstrate that therapies to control or fine-tune the antifungal immune response within CNS can be invaluable to preventing detrimental brain injury. However, a recent report found that the use of steroids, which have broad immunosuppressive properties but many detrimental side effects, is not universally justified in broadly treating HIV+ patients with cryptococcal meningitis (54). Even in subsets of patients initially benefitting from steroid therapy, the risks associated with increased cumulative dosing limit their long-term use. Next-generation therapies must overcome these limitations in order to develop more precisely targeted and safe treatments for this devastating CNS infection. However, our findings crucially reenforce that therapies targeting the inflammatory processes have definitive use in cases such as IRIS and PIIRS and suggest that approaches targeting more-specific immune path-

ways can be refined in order to tailor the most appropriate therapies for treating CNS injury in these patients. While optimal treatment strategies for CNS cryptococcosis still need to be elaborated, our study provides a robust tool to systematically investigate these processes and shows that the ultimate goal of these approaches will be to balance fungal control with immunomodulation to minimize inflammatory damage within the CNS.

MATERIALS AND METHODS

Mice. C57BL/6 mice (Jackson Laboratory) were housed under specific-pathogen-free (SPF) conditions in microisolator cages at the Ann Arbor Veterans Affairs Medical Center and were provided with food and water *ad libitum*. Animals were 8 to 12 weeks old at the time of infection. Mice were euthanized using CO_2 inhalation followed by exsanguination. All experiments were approved by the Veterans Affairs Institutional Animal Care and Use Committee under protocol 1408-004 and were performed in accordance with NIH guidelines and the *Guide for the Care and Use of Laboratory Animals*. In mortality studies, animals were euthanized when they lost 20% body weight, had persistent cranial swelling, and/or developed neurological symptoms.

***C. neoformans* infection.** *Cryptococcus neoformans* strain 52D was grown for 4 days in Sabouraud dextrose broth (Difco). Fungal cells were washed in phosphate-buffered saline (PBS), counted on a hemocytometer with trypan blue, and adjusted to a concentration of 5×10^6/ml just prior to infection. Mice were infected with 10^6 organisms (in 200 μl PBS) via retro-orbital intravenous injection under inhaled isoflurane anesthesia. Serial dilutions of the *C. neoformans* suspension were plated on Sabouraud dextrose agar to confirm the number of viable fungi in the inoculum.

Inoculation with a substantially reduced infectious dose of *C. neoformans* 52D, such as the 10^4 CFU typically used to establish pulmonary infection of the lungs (14, 72), failed to induce substantial fungal growth and immune cell accumulation within the CNS on day 21 postinfection (see Fig. S1 in the supplemental material) and therefore was not suitable as a model of meningoencephalitis. Thus, the inoculum dose of 10^6 CFU was used for all experiments.

Murine coma and behavioral scale. A modified murine coma and behavioral scale (MCBS) to assess the overall physical and neurological condition of infected mice was adapted from the MCBS of Carroll et al. (49) using expanded scoring criteria to reflect the more subtle degree of symptoms observed in mice with cryptococcal infection. Additional smell, vision, and touch reflexes were also incorporated. Briefly, mice were scored on a scale of 0 to 3 for exploration, balance, gait, body posture, coat condition, grip strength, reflexes (body, neck, pinna and footpad reflexes), and response to visual and olfactory stimuli. Scores are reported with a maximum of 36 possible points, with lower scores reflecting more-pronounced symptoms. Detailed scoring criteria are in Table S1.

Cell isolation. Mice were euthanized with CO_2 and then perfused with PBS. The brains were aseptically removed, transferred to GentleMACs C tubes containing 5 ml of sterile complete RPMI 1640 (with 5% fetal bovine serum [FBS], 25 mM HEPES, GlutaMAX, penicillin-streptomycin, nonessential amino acids, sodium pyruvate, and beta-mercaptoethanol) supplemented with collagenase (50 μg/ml; Roche) and DNase (100 U/ml; Worthington). The tissue was minced and then processed on a GentleMACs homogenizer (Miltenyi). Small samples of the homogenate were collected immediately after processing for whole-brain fungal burden and RNA and cytokine measurements. The remaining homogenate was washed with RPMI 1640 and filtered through a 70-μm cell strainer. A discontinuous 30%/70% Percoll (GE Healthcare) gradient was used to remove cell debris, myelin, and neurons, and then microglia and brain-infiltrating leukocytes (BIL) were recovered from the interface. Isolated cells were washed twice to remove residual Percoll before use in assays. Total cell numbers were determined by counting live cells on a hemocytometer with trypan blue.

Flow cytometry. Isolated cells were stained with fixable live/dead dye (Life Technologies), blocked with anti-CD16/32, and stained with antibodies for CD45, CD3, CD4, CD8, CD11b, CD11c, Ly6C, Ly6G, major histocompatibility complex class II (MHCII), and gamma interferon (IFN-γ). All antibodies were purchased from BioLegend. For IFN-γ production, the cells were stimulated for 6 h with phorbol myristate acetate (PMA) and ionomycin in the presence of brefeldin A and monensin for the final 4 h. The cells were stained for extracellular markers and then fixed with fixation/permeabilization buffer (eBioscience), and intracellular staining was performed in permeabilization buffer. Fluorescence minus one (FMO) controls were used for all experiments. Data were collected on an LSRII cytometer (BD) and analyzed using FlowJo (Treestar).

Fungal burdens. To enumerate fungal burdens, samples of whole-brain homogenate were serially diluted in distilled water, and 10-μl aliquots were deposited on Sabouraud dextrose agar. The number of CFU per whole brain was calculated from the average of replicate counts.

CD4+ T cell depletion. Mice were depleted of CD4+ T cells via intraperitoneal injection of monoclonal anti-CD4 antibody (GK1.5; 300 μg) on days -2, 0, and 5 postinfection and then weekly thereafter. Control animals received corresponding anti-keyhole limpet hemocyanin (anti-KLH) isotype antibody (LTF-2; 300 μg). All depleting antibodies were purchased in *in vivo* grade purity from BioXCell, West Lebanon, NH. This regimen resulted in >99% depletion of CD4+ T cells in the brains, blood, and spleens of infected mice.

Cytokine and gene expression. Cytokine protein levels were measured from the supernatants of whole-brain homogenate after centrifugation ($600 \times g$, 5 min) using LegendPlex cytometric bead assays (BioLegend) according to the manufacturer's instructions. For gene expression, RNA was isolated from the whole-brain cell pellets using TRIzol (Life Technologies) and converted to cDNA using Quantitech

reverse-transcription kit (Qiagen). Gene expression was calculated relative to *Gapdh* using SYBR-based amplification (Radiant Green master mix; Alkali Science) on a LightCycler 96 thermocycler (Roche) and the $2^{-\Delta\Delta}$Ct method.

Statistical analysis. Statistical analysis was performed using GraphPad Prism v6 software with Student's *t* test or analysis of variance (ANOVA) plus Tukey's posthoc test for multiple comparisons. Asterisks on figures (in graphs or in the corners of flow cytometry plots) indicate statistical significance as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.01415-17.

FIG S1, EPS file, 0.5 MB.

FIG S2, EPS file, 1.1 MB.

FIG S3, EPS file, 0.8 MB.

TABLE S1, DOCX file, 0.02 MB.

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