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Interleukin-17A Enhances Host Defense against Cryptococcal Lung Infection through Effects Mediated by Leukocyte Recruitment, Activation, and Gamma Interferon Production

Benjamin J. Murdock,[a,b] Gary B. Huffnagle,[b,c] Michal A. Olszewski,[a,b] John J. Osterholzer[a,b]

Pulmonary Section, VA Ann Arbor Healthcare System, Ann Arbor, Michigan, USA[a]; Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan, USA[b]; Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan, USA[c]

Infection of C57BL/6 mice with the moderately virulent *Cryptococcus neoformans* strain 52D models the complex adaptive immune response observed in HIV-negative patients with persistent fungal lung infections. In this model, Th1 and Th2 responses evolve over time, yet the contribution of interleukin-17A (IL-17A) to antifungal host defense is unknown. In this study, we show that fungal lung infection promoted an increase in Th17 T cells that persisted to 8 weeks postinfection. Our comparison of fungal lung infection in wild-type mice and IL-17A-deficient mice (IL-17A$^{-/-}$ mice; C57BL/6 genetic background) demonstrated that late fungal clearance was impaired in the absence of IL-17A. This finding was associated with reduced intracellular containment of the organism within lung macrophages and deficits in the accumulation of total lung leukocytes, including specific reductions in CD11c^{+} CD11b^{+} myeloid cells (dendritic cells and exudate macrophages), B cells, and CD8^{+} T cells, and a nonsignificant trend in the reduction of lung neutrophils. Although IL-17A did not alter the total number of CD4 T cells, decreases in the total number of CD4 T cells and CD8 T cells expressing gamma interferon (IFN-γ) were observed in IL-17A$^{-/-}$ mice. Lastly, expression of major histocompatibility complex class II (MHC-II) and the costimulatory molecules CD80 and CD86 on CD11c^{+} CD11b^{+} myeloid cells was diminished in IL-17A$^{-/-}$ mice. Collectively, these data indicate that IL-17A enhances host defenses against a moderately virulent strain of *C. neoformans* through effects on leukocyte recruitment, IFN-γ production by CD4 and CD8 T cells, and the activation of lung myeloid cells.

Cryptococcus neoformans is a globally distributed pathogenic fungus acquired by the inhalational route (1–3). When host defenses are impaired, *C. neoformans* becomes a devastating opportunistic pathogen. It is the leading cause of fatal mycosis in HIV-positive individuals (1 million new cases and 680,000 deaths per year [4]) and the second most common fungal infection in patients with organ transplants (5). Yet for most infections in non-HIV patients, either the organism is fully cleared (6) or it may persist at nonlethal levels (7), often resulting in destructive parenchymal lung disease or immune-mediated airway damage and bronchiectasis (5, 8). Thus, host defenses in immunocompetent humans are essential for clearance or containment of *C. neoformans*.

Host defense against *Cryptococcus neoformans* requires the successful interplay of both the innate and adaptive immune responses (9, 10). The effectiveness of the resultant adaptive immune response in clearing *C. neoformans* has largely been attributed to the balance between Th1 and Th2 responses (11). Th1 cytokine expression (characterized by gamma interferon [IFN-γ] production) enhances fungal resistance (12–17), while a Th2 response (characterized by interleukin-4 [IL-4], IL-5, IL-10, and IL-13 production) impairs clearance and promotes immune-mediated lung damage in mouse models of cryptococcosis (18–23). In the interim since many of these studies were performed, our understanding of adaptive immune regulation has expanded and now includes Th17 cells, a population of CD4 T cells that produce the proinflammatory cytokine IL-17A. IL-17A was originally implicated in mediating tissue damage in the context of autoimmune disease (24). IL-17A has since been studied in numerous infectious disease models, in which its role remains uncertain. IL-17A exacerbates some bacterial infections but protects against others (25, 26).

Our knowledge of the role of IL-17A in clearing fungal infections is similarly evolving. Using a model of repetitive exposure to *Aspergillus fumigatus* in IL-17A-deficient mice, we demonstrated that IL-17A impairs clearance of inhaled conidia (27), whereas another study using antibody-mediated neutralization of IL-17A suggested that IL-17A enhances clearance in response to acute *Aspergillus* infection (28). IL-17A provides some protection against mucocutaneous candidiasis and *Pneumocystis carinii* (29–31). Several studies have investigated the role of IL-17A in the context of vaccination and (or) primary infection with the endemic fungi *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Coccidioides immitis* (32–35). One study comparing wild-type and IL-17A-deficient mice demonstrated that vaccine efficacy for all three pathogens was reduced in the absence of IL-17A whereas impaired clearance in unvaccinated mice was observed only for

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Address correspondence to John J. Osterholzer, oster@umich.edu.
M.A.O. and J.J.O. contributed equally to this article as co-senior authors.
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mice infected with *B. dermatitidis* (35). Neutralization of IL-17A by blocking antibody blunted the clearance of *H. capsulatum* from the lungs but did not alter progressive infection or survival (32).

Our group and several others have investigated the role of IL-17A in response to a highly virulent strain of *Cryptococcus neoformans*, strain H99, in BALB/c mice (1, 36–39). In this model, progressive cryptococcal lung infection is observed associated with a high rate of lethal central nervous system (CNS) dissemination recapitulating the clinical course of cryptococcosis that develops in patients immunocompromised due to HIV/AIDS or other forms of immunosuppression. These murine studies using H99 (in wild-type [WT] BALB/c mice) have shown that little IL-17A is produced in the lung in either the early or the late phase of this aggressive infection.

In contrast to the progressive infection observed in BALB/c mice infected with strain H99, infection of C57BL/6 mice with the moderately virulent and encapsulated strain 52D results in a less aggressive infection characterized by the evolution of a complex adaptive immune response that contains the organism in the lung and limits dissemination to the CNS (40–44). This model is more representative of cryptococcal infections in immunocompetent human hosts, which either clear the infection or contain the organism within the lung; the latter outcome may result in lung damage similar to that observed in other chronic fungal infections (45–50). In this model, both Th1 and Th2 responses develop and evolve over time. In particular, signaling by activated antigen-presenting cells and the generation of IFN-γ are critically important for preventing progressive lung infection and lethal dissemination to the CNS (11, 13, 22, 51, 52). Whether IL-17A exerts a protective or detrimental effect in this model is unclear. One study using C57BL/6 mice deficient mice in the IL-17 receptor A (IL-17RA) showed no impairment in pulmonary clearance of strain 52D at 1 or 6 weeks postinfection and no difference in survival compared to WT C57BL/6 mice (44). Since IL-17RA is a receptor for more than one IL-17 family member, our current study utilized mice deficient in IL-17A to investigate the specific contribution of IL-17A to the evolution of the immune response to strain 52D. Our data demonstrate that IL-17A enhances intracellular containment and cryptococcal clearance within the lung during the latter stages of infection. Our findings suggest that IL-17A may exert these beneficial effects by directly or indirectly altering leukocyte recruitment, increasing IFN-γ production by CD4 and CD8 T cells, and enhancing myeloid cell activation.

MATERIALS AND METHODS

Mice. Wild-type (C57BL/6J) mice obtained from the Jackson Laboratories (Bar Harbor, ME) were housed under pathogen-free conditions in enclosed filter-topped cages. IL-17A knockout (IL-17A$^{-/-}$) mice that were originally generated on a C57BL/6 genetic background were obtained from a breeding colony at the University of Michigan. The IL-17A knockout breeders were kindly provided by Yoichiro Iwakura (Tokyo University) and have been described previously (53). Clean food and water were given to mice *ad libitum*. The mice were handled and maintained using microisolator techniques, with daily veterinarian monitoring. All studies involving mice were approved by the University Committee on Use and Care of Animals at the University of Michigan.

C. neoformans. *C. neoformans* strain 52D was obtained from the American Type Culture Collection (ATCC 24067); this strain displays smooth colony morphology when grown on Sabouraud dextrose agar. For the infection, yeast cells that were recovered from 10% glycerol stocks were grown to stationary phase (at least 72 h) at 36°C in Sabouraud dextrose broth (SDB) (1% neopeptone, 2% dextrose; Difco, Detroit, MI) on a shaker. The cultures were then washed in nonpyrogenic saline (Travenol, Deerfield, IL), cells were counted on a hemocytometer, and the cultures were diluted to 3.3×10^5 yeast cells/ml in sterile nonpyrogenic saline.

Intratracheal inoculations. Mice were anesthetized by intraperitoneal injection of pentobarbital (0.074 mg/g of body weight) and restrained on a surgical board. A small incision was made through the skin over the trachea, and the underlying tissue was separated. A bent 30-gauge needle (Becton, Dickinson, Rutherford, NJ) was attached to a tuberculin syringe (BD & Co, Franklin Lakes, NJ) filled with the diluted *C. neoformans* culture. The needle was inserted into the trachea, and 30 μl of inoculum was dispensed into the lungs (10^4 yeast cells). The skin was closed with cyanoacrylate adhesive. The mice recovered with minimal visible trauma.

CFU assays. For determination of microbial burden in the lungs, small aliquots of dispersed lungs or brains were collected after the digest procedure. Series of 10-fold dilutions of the lung samples were plated on Sabouraud dextrose agar plates in duplicates of 10-ml aliquots and incubated at room temperature. *C. neoformans* colonies were counted 2 days later, and the number of CFU was calculated on a per-organ basis.

Leukocyte isolation from lungs. The lungs from each mouse were excised, washed in phosphate-buffered saline (PBS), minced with scissors, and enzyme digested at 37°C for 30 to 35 min in 15 ml/lung of digestion buffer (RPMI, 5% fetal calf serum [FCS], antibiotics, 1 mg/ml collagenase [Boehringer Mannheim Biochemical, Chicago, IL], and 30 μg/ml DNase [Sigma]). 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% $KHCO_3$, 0.0372% Na_2-EDTA, pH 7.4) for 3 min 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 30 min at 1,500 × *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 medium and enumerated on a hemocytometer following dilution in trypan blue.

Lung histology. Lungs were fixed by inflation with 10% neutral buffered formalin (Sigma). After paraffin embedding, 5-μm sections were cut and stained with hematoxylin and eosin (H&E) for histological analysis (McClinchey Histology Lab, Stockbridge, MI).

Extracellular cryptococci. Histological sections obtained from WT and IL-17A$^{-/-}$ mice infected for 4 weeks were stained with H&E and examined by light microscopy at a magnification of ×400. Ten random, high-powered fields were examined in blinded fashion at sites of leukocyte accumulation for both mouse strains. The total number of extracellular *C. neoformans* cells was then determined for each field by counting.

Flow cytometry. Cells were washed and resuspended at a concentration of 10^6 cells/25 μl FA buffer (Difco) + 0.1% NaN_3, Fc receptors were blocked by the addition of unlabeled anti-CD16/32 (Fc block; BD Pharmingen, San Diego, CA). After Fc receptor blocking, 0.5×10^6 to 1×10^6 cells were stained in a final volume of 50 μl in 96-well round-bottom plates (Corning Incorporated, Corning, NY) for 30 min at 4°C. Cells were washed twice with FA buffer, resuspended in 120 μl of 4% formalin (Sigma), and transferred to 12- by 75-mm^2 polystyrene tubes (Becton, Dickinson, Franklin Lakes, NJ). A minimum of 100,000 events were acquired on a FACSCanto flow cytometer (BD PharMingen) using CellQuest software (BD Pharmingen). Acquired data were analyzed with FlowJo software (Tree Star, Stanford, CA). Fluorochrome-conjugated antibodies directed against the following antigens were obtained from the following vendors: CD45 (BioLegend, San Diego, CA), CD3, CD4, CD8, CD11b, CD11c, CD19, Gr1, siglec F, IFN-γ, Ly6C, Ly6G, IA-IE, CD80, CD86, and isotype controls (BD Pharmingen).

Lung leukocyte subset identification by flow cytometric analysis. Cells from whole-lung digest were analyzed as previously published (54, 55) and briefly described as follows. First, lung leukocytes were identified

by CD45 expression. The following leukocyte subsets were then identified within this gate: (i) neutrophils were identified using a CD11c versus Gr1 plot as cells expressing little CD11c but large amounts of Gr1; (ii) mature eosinophils were identified as cells expressing moderate amounts of CD11c and Gr1 and further expressing large amounts of siglec F; (iii) $CD11c^+$ $CD11b^+$ myeloid cells were identified as expressing high levels of CD11c, low to moderate levels of Gr-1, and moderate to large amounts of CD11b; this population includes $CD11b^+$ dendritic cells (DC) and $CD11b^+$ exudate macrophages (56, 57); (iv) resident $CD11b^-$ lung macrophages were identified as CD3-CD19-Ly6G-negative cells, which were large (FSC^{high}) and autofluorescent and expressed CD11c but not CD11b; (v) lymphocytes were identified within the population of small cells (FSC^{low} and SSC^{low}), further subdivided into CD4 T cells ($CD4^+$), CD8 T cells ($CD8^+$), or B cells ($CD19^+$) based on cell surface staining. Within the myeloid populations, cellular activation was assessed by measuring cell surface expression of MHC-II (IA-IE), CD80, and CD86 relative to isotype control.

Intracellular IL-17A and IFN-γ staining. Prior to intracellular cytokine staining, cells were stimulated *in vitro* for 6 h with phorbol myristate acetate (PMA; 50 ng/ml) and ionomycin (1 μg/ml) in the presence of monensin (1 μl/ml of cells) as per the manufacturer's instructions (BD Pharmingen) to promote the intracellular accumulation of cytokines. After stimulation, cells were washed twice prior to surface molecule staining. Subsequently, intracellular IL-17A or IFN-γ was stained using the BD Cytofix/Cytoperm kit according to the manufacturer's instructions (BD Pharmingen).

qPCR for IL-17A. For quantitative PCR (qPCR) assays, total RNA was prepared using the RNeasy Plus Minikit (Qiagen, Valencia, CA, USA) and first-strand cDNA was synthesized using SuperScriptIII (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. IL-17 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) 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 s followed by 60°C for 30 s and 72°C for 30 s) were performed on a cDNA template. The data were expressed as percent GAPDH mRNA levels.

IL-17A protein expression by Luminex. 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 h. Supernatants were separated from cells by centrifugation, collected, and frozen until tested. IL-17 protein expression was quantified by Luminex assay (Luminex, Austin, TX) following the manufacturer's specifications.

Statistical analyses. Results were obtained from at least two separate experiments, each containing 2 to 4 mice per group per experiment. Since these independent experiments produced consistent results, quantitative data were pooled (to obtain a minimum n of ≥4) and cumulative analysis was performed across the multiple experiments. All values are reported as means ± standard errors of the means. For figures comparing multiple time points with day 0 (uninfected mice) within the same mouse strain, statistical significance was determined using analysis of variance (ANOVA) with Dunnett's *post hoc* test. Differences between WT and IL-$17A^{-/-}$ mice at each time point were evaluated using an unpaired Student t test. P values of less than 0.05 were considered statistically significant.

RESULTS

Th17 cells increase in response to lung infection with *C. neoformans* strain 52D in WT C57BL/6 mice. Our prior studies demonstrated an expansion of both Th1 and Th2 cells in the lungs of C57BL/6 mice infected with *C. neoformans* strain 52D (11). The first objective of this study was to determine whether this mixed adaptive immune response includes an expansion in Th17 cells. To address this, C57BL/6 mice were infected by the intratracheal route with 1×10^4 organisms. The lungs of uninfected mice or mice infected for 1, 2, 4, and 8 weeks were enzymatically dispersed, and single-cell suspensions were subjected to antibody staining and flow cytometric analysis (as described in Materials and Methods). Compared to cells from uninfected mice, the percentage of lung CD4 T cells expressing intracellular IL-17A significantly increased (approximately 3-fold) as early as 1 week postinfection (Fig. 1A). This increase in IL-17A-positive CD4 T cells was consistent with a concurrent increase in IL-17A expression by total lung leukocytes, established by qPCR and Luminex assays in preliminary experiments (performed at 1 week postinfection; see Fig. S1 in the supplemental material). The percentage of CD4 T cells expressing IL-17A increased 5-fold by week 2, translating into a significant increase in the total numbers of IL-17A-expressing $CD4^+$ T cells, which persisted to 8 weeks postinfection (Fig. 1B). The percentage of CD4-negative lung leukocytes expressing IL-17A was consistently less than 2% and did not increase in response to infection (data not shown), suggesting that CD4 T cells are the predominant source of IL-17A in this model. Together, these data demonstrate that CD4 T cells expressing intracellular IL-17A, i.e., Th17 cells, increase in response to cryptococcal lung infection with strain 52D in C57BL/6 mice. Their presence adds to the complexity of the mixed adaptive immune response observed in the lungs of these mice.

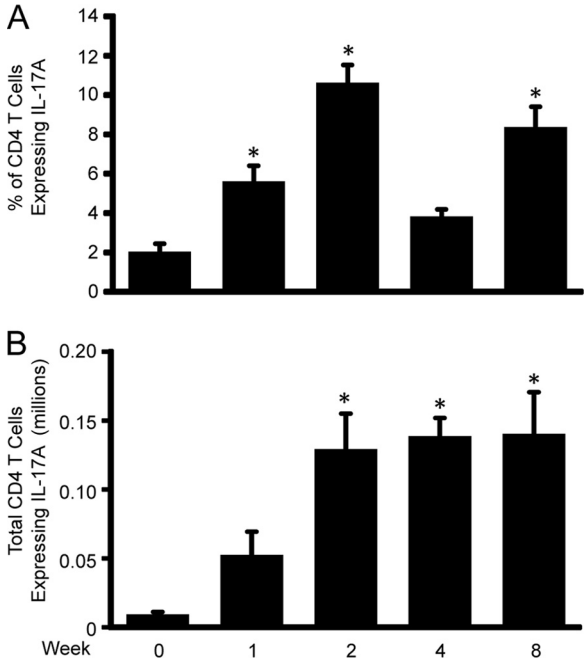

FIG 1 Th17 cells increase in C57BL/6 mice that develop persistent cryptococcal lung infection. C57BL/6 mice were infected by the intratracheal route with *C. neoformans* strain 52D. At weeks 0 (uninfected), 1, 2, 4, and 8, lungs were removed and enzymatically digested, and lung cells were antibody stained and analyzed by flow cytometric analysis to identify CD4 T cells expressing intracellular IL-17A as described in Materials and Methods. (A) Percentage of CD4 T cells expressing IL-17A. (B) Total number of CD4 T cells expressing IL-17A. Data are means ± standard errors obtained from 4 to 6 mice per time point in two separate experiments. *, $P < 0.05$ by ANOVA with Dunnett's *post hoc* analysis versus day 0 (uninfected) mice.

IL-17A promotes containment of chronic lung infection but does not alter survival at 8 weeks. We next sought to determine the role of IL-17A in fungal clearance of strain 52D. CFU analysis was performed using aliquots of enzymatically dispersed lungs obtained from WT mice (C57BL/6 mice) and IL-17A-deficient mice (IL-$17A^{-/-}$ mice; C56BL/6 genetic background) infected for

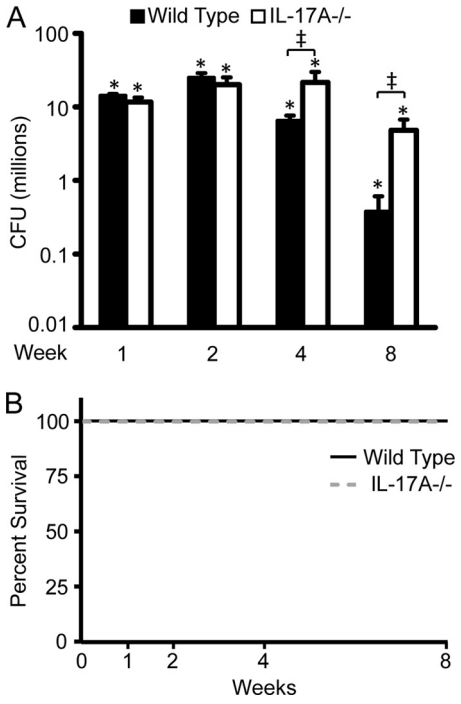

FIG 2 Fungal clearance from the lung is impaired in IL-17A-deficient mice. (A, B) Wild-type C57BL/6 mice and IL-17A-deficient mice (IL-17A$^{-/-}$ mice; C57BL/6 genetic background) were infected by the intratracheal route with *C. neoformans* strain 52D. (A) At 1, 2, 4, and 8 weeks postinfection, lungs were digested, and a CFU assay was performed to determine fungal burden. Black bars, WT mice; white bars, IL-17A$^{-/-}$ mice. The *y* intercept represents the initial inoculum of 10^4 organisms. (B) Survival of mice at each time point. Solid black line, WT mice; dashed gray line, IL-17A$^{-/-}$ mice. Data are means ± standard errors obtained from 4 to 6 mice per time point in two separate experiments. *, $P < 0.05$ by ANOVA with Dunnett's *post hoc* analysis versus day 0 (uninfected) mice of the same strain; ‡, $P < 0.05$ by unpaired Student *t* test between C57BL/6 mice and IL-17A$^{-/-}$ mice at the same time point.

1, 2, 4, and 8 weeks (Fig. 2). In both strains of mice, lung CFU were significantly increased by 1 week postinfection and remained persistently elevated out to 8 weeks postinfection relative to the initial inoculum of 10^4 organisms at day 0 (Fig. 2A). Our comparison of lung CFU between WT and IL-17A$^{-/-}$ mice revealed no differences at 1 or 2 weeks postinfection. However, by week 4, we observed that lung CFU in the IL-17A$^{-/-}$ mice was 3-fold higher than that observed for WT mice. By week 8, this disparity in fungal clearance had increased further, with the lung CFU in IL-17A$^{-/-}$ mice being 10-fold greater than that observed in WT mice.

We also examined whether the absence of IL-17A altered the rate of fungal dissemination to the central nervous system. Brain tissues were removed from infected WT and IL-17A$^{-/-}$ mice at 1, 2, 4, and 8 weeks postinfection, and CFU analysis was performed. Consistent with other studies using this model, CNS dissemination in WT mice was rare (2 of 20 mice evaluated; data not shown). In the infected IL-17A$^{-/-}$ mice, a modest nonsignificant trend toward increased CNS dissemination was observed (5 of 20 mice evaluated; $P = 0.21$ by chi-square analysis in comparison with WT mice). No deaths occurred in the subsets of WT and IL-17A$^{-/-}$ mice evaluated at 8 weeks postinfection (Fig. 2B). Collectively, our data demonstrate that IL-17A contributes to the clearance of chronic fungal infection within the lung microenvironment but is not essential for 8-week survival in this infection model.

IL-17A enhances lung inflammation and intracellular containment of *C. neoformans* strain 52D as assessed by light microscopy. To determine the role of IL-17A on the microanatomic response to lung infection with strain 52D, we examined (by light microscopy) H&E-stained lung sections of uninfected WT and IL-17A$^{-/-}$ mice (Fig. 3, left panels) and at 1 and 4 weeks postinfection (Fig. 3, middle and right panels, respectively). Lung sections from uninfected WT and IL-17A$^{-/-}$ mice appeared similar (Fig. 3A). We observed a substantial influx of lung leukocytes, many of them granulocytes, at 1 week postinfection in WT mice (Fig. 3A and B; week 1; wild type). In contrast, fewer lung leukocytes were observed in the lung sections obtained from IL-17A$^{-/-}$ mice at this time point. (Fig. 3A and B; week 1; IL-17A$^{-/-}$). In both strains of mice, extracellular cryptococci were abundant within alveolar spaces (Fig. 3B; week 1; wild type and IL-17A$^{-/-}$), suggesting that the well-described (58, 59) ineffectiveness of the early innate immune response at clearing strain 52D was not altered by the absence of IL-17A.

At week 4 postinfection, characteristic features of fungal containment were observed in WT mice, including the presence of loose granulomas containing large macrophages and numerous lymphocytes within the alveolar regions of the lung (Fig. 3A; week 4; wild type). At higher power, large foamy macrophages and multinucleated giant cells were observed, many of them containing intracellular cryptococci; extracellular cryptococci were rarely identified (Fig. 3B; week 4; wild type). In comparison, although a comparable number of lung leukocytes were observed in IL-17A$^{-/-}$ mice at 4 weeks postinfection (Fig. 3A; week 4; IL-17A$^{-/-}$), the pattern of inflammation differed qualitatively from that identified in WT mice. Specifically, macrophages appeared smaller, and fewer multinucleated giant cells were visible. Furthermore, in contrast to the intracellular location of cryptococci observed in WT mice, the number of extracellular cryptococci present in the lungs of IL-17A$^{-/-}$ mice was significantly increased (Fig. 3B; week 4; IL-17A$^{-/-}$; and Fig. 3C). Collectively, these data obtained by light microscopy suggest a role for IL-17A in lung leukocyte recruitment at early time points and an effect of IL-17A on intracellular fungal containment at later time points, which contributes to long-term infection control.

IL-17A promotes early lung leukocyte recruitment in response to infection with *C. neoformans* strain 52D. To substantiate and extend our observations regarding the effects of IL-17A on characteristics of the inflammatory response, we next investigated the role of IL-17A on leukocyte recruitment in this model system. Flow cytometric analysis was performed on lung cells isolated from WT and IL-17A$^{-/-}$ mice that were either uninfected or at 1, 2, 4, and 8 weeks postinfection with strain 52D (as described in Materials and Methods). In both strains of mice, total numbers of CD45$^+$ lung leukocytes increased beginning week 1 postinfection (relative to uninfected mice of the same strain [Fig. 4A]). Consistent with our findings using light microscopy, we observed a significant decrease in total CD45$^+$ lung leukocytes in IL-17A$^{-/-}$ mice (relative to WT mice) 1 week after intratracheal inoculation (Fig. 4A), which no longer reached statistical significance at weeks 2, 4, and 8.

We next identified and enumerated subsets of innate leukocytes in infected WT and IL-17A$^{-/-}$ mice. In both strains of mice, fungal lung infection promoted accumulations (relative to uninfected mice of the same strain) in the total number of neutrophils, eosinophils, CD11c$^+$ CD11b$^-$ myeloid cells (predominantly res-

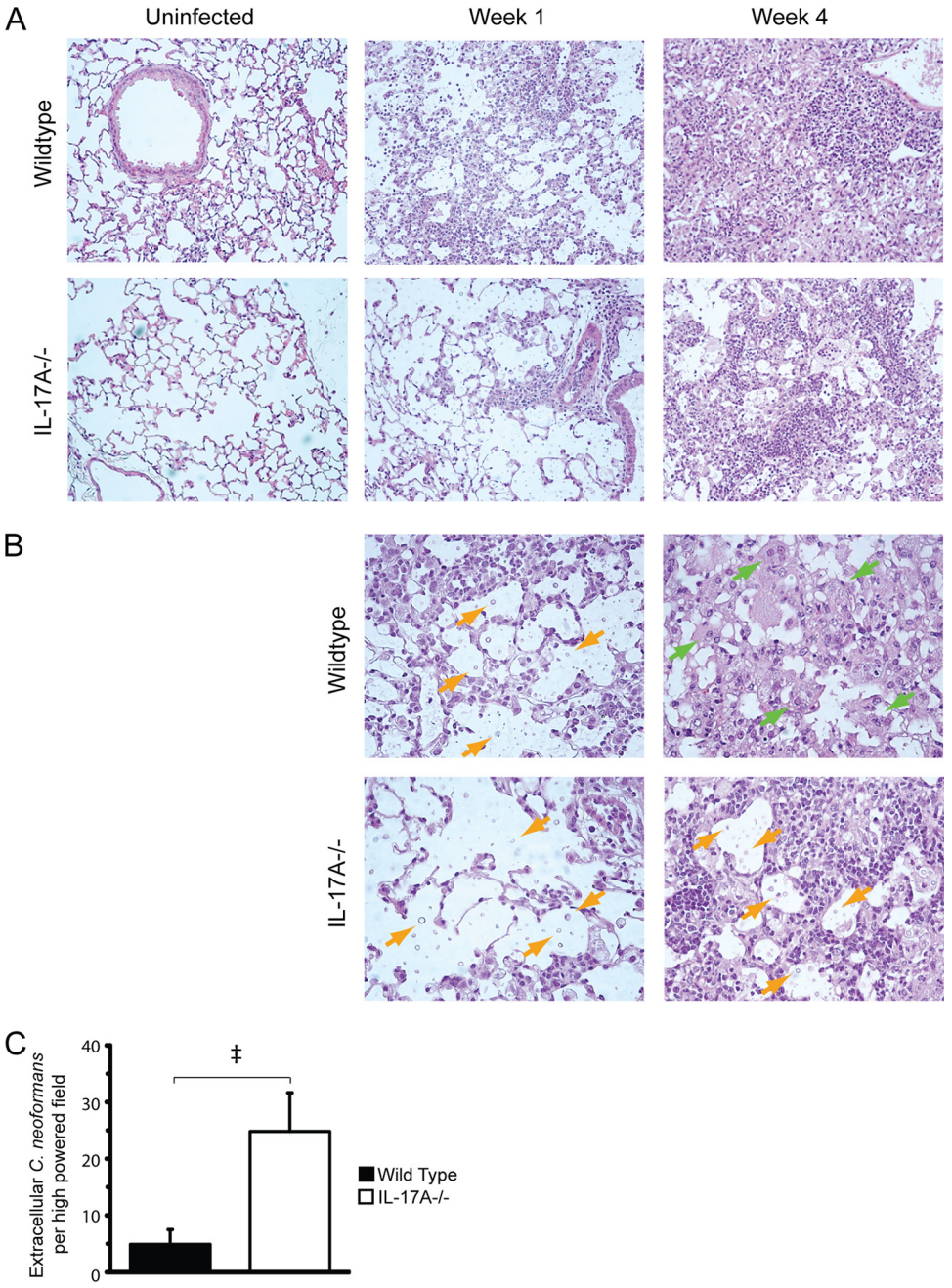

FIG 3 Intracellular containment of *C. neoformans* is impaired in IL-17A-deficient mice with persistent cryptococcal lung infection. (A to C) Lung sections obtained from uninfected and infected WT and IL-17A$^{-/-}$ mice were stained with H&E and examined by light microscopy at a magnification of ×200 (A) or ×400 (B). At 1 week postinfection (middle panels), fewer lung leukocytes are visible in the lungs of IL-17A$^{-/-}$ mice whereas numerous extracellular cryptococci can be seen in sections taken from both strains (orange arrows). At week 4 postinfection (right panels), lung sections in WT mice demonstrate loose granulomatous inflammation within alveolar spaces consisting of numerous large foamy macrophages and multinucleated giant cells (green arrows); most cryptococci are located intracellularly. In contrast, lung sections from IL-17A$^{-/-}$ mice contain fewer large macrophages or giant cells and extracellular cryptococci are common (orange arrows). (C) Total number of extracellular *C. neoformans* cells per high-powered field observed within lung sections obtained from WT mice (black bars) and IL-17A$^{-/-}$ mice (white bars) at week 4 postinfection. Data are means ± standard errors; bars represent data from 10 high-powered fields per strain examined at a magnification of ×400 in a blinded fashion. ‡, $P < 0.05$ by unpaired Student *t* test.

ident alveolar macrophages [57]), and CD11c^{+} CD11b^{+} myeloid cells (dendritic cells and exudate macrophages (56, 57) (Fig. 4B). Direct comparisons between infected WT mice and IL-17A$^{-/-}$ mice demonstrated that the observed reduction in total CD45^{+} lung leukocytes at week 1 was not attributable to a significant reduction in any single innate leukocyte subset. Neutrophil recruitment was consistently diminished in IL-17A$^{-/-}$ mice, although these reductions did not reach statistical significance at any single specified time point. Similarly, a nonsignificant trend toward impaired eosinophil recruitment was observed in IL-

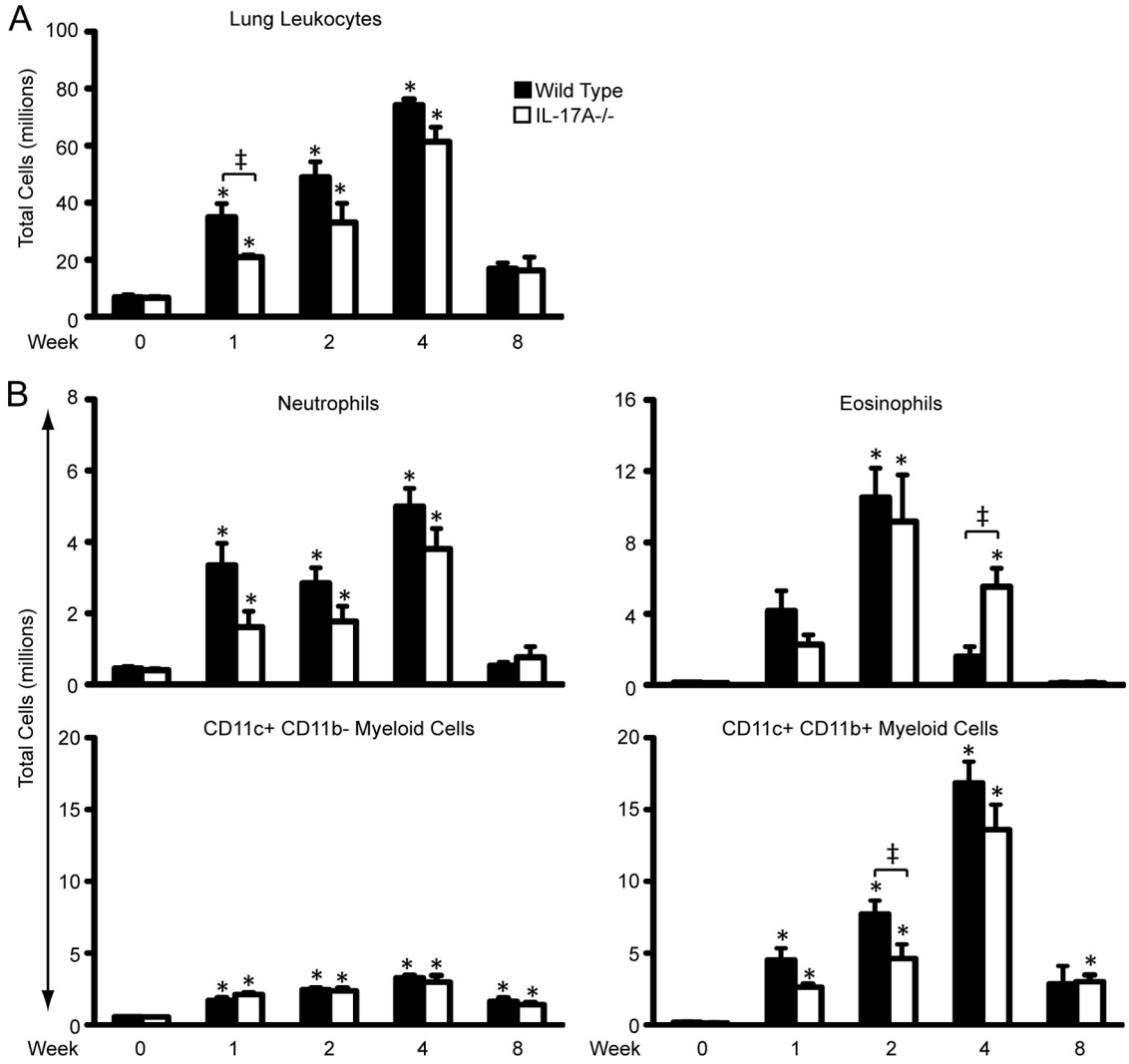

FIG 4 Accumulations of total lung leukocytes, including the $CD11c^+$ $CD11b^+$ myeloid cell subset, are modestly impaired in IL-17A-deficient mice with persistent cryptococcal lung infection. (A, B) WT C57BL/6 mice and IL-$17A^{-/-}$ mice were infected by the intratracheal route with *C. neoformans* strain 52D. At weeks 0 (uninfected), 1, 2, 4, and 8, lungs were removed and enzymatically digested, and lung cells were antibody stained and analyzed by flow cytometric analysis to identify total leukocytes and leukocyte subsets as described in Materials and Methods. (A) Total $CD45^+$ lung leukocytes. (B) Total numbers of neutrophils, eosinophils, $CD11c^+$ $CD11b^+$ myeloid cells (which include $CD11b^+$ dendritic cells and exudate macrophages), and $CD11b^-$ macrophages (which include resident alveolar macrophages). Black bars, WT mice; white bars, IL-$17A^{-/-}$ mice. Data are means ± standard errors obtained from 4 to 6 mice per time point in two separate experiments. ∗, $P < 0.05$ by ANOVA with Dunnett's *post hoc* analysis versus day 0 (uninfected) mice of the same strain; ‡, $P < 0.05$ by unpaired Student *t* test between C57BL/6 mice and IL-$17A^{-/-}$ mice at the same time point.

$17A^{-/-}$ mice at week 1, whereas at week 4, the total number of eosinophils was actually increased (relative to WT mice). Numbers of $CD11c^+$ $CD11b^-$ myeloid cells (resident alveolar macrophages) were comparable at all time points. In contrast, impaired accumulation of $CD11c^+$ $CD11b^+$ myeloid cells (dendritic cells and exudate macrophages) was observed in IL-$17A^{-/-}$ mice (relative to WT mice) at week 2 postinfection; nonsignificant reductions were observed at weeks 1 and 3. Collectively, these data indicate that IL-17A contributes to early leukocyte accumulation, likely through a combined effect on numerous leukocyte subsets, in response to cryptococcal lung infection.

IL-17A promotes B cell and early CD8 T cell accumulation but does not affect total CD4 T cell numbers in mice infected with *C. neoformans* strain 52D. Host defense against *C. neoformans* infection results from the interplay between innate and adaptive immune cells. To determine if IL-17A alters lymphocyte recruitment in this model, we used flow cytometric analysis to examine the influx of CD4 T cells, CD8 T cells, and B cells into the lungs of WT and IL-$17A^{-/-}$ mice either untreated or at 1, 2, 4, and 8 weeks postinfection with strain 52D. As expected from prior studies (51), the total number of all three lymphocyte subsets increased in infected WT mice (relative to uninfected WT mice [Fig. 5]). In IL-$17A^{-/-}$ mice, CD4 lymphocytes were increased by week 1 and remained elevated, whereas significant increases in CD8 and B lymphocytes were not observed until week 4 (relative to uninfected IL-$17A^{-/-}$ mice) (Fig. 5). Direct comparisons between infected WT mice and IL-$17A^{-/-}$ mice demonstrated that the total numbers of CD4 T cells were comparable in the two strains of mice at all time points (Fig. 5, top panel). In contrast, significant reductions in the numbers of CD8 T cells (at week 1) and B cells (at

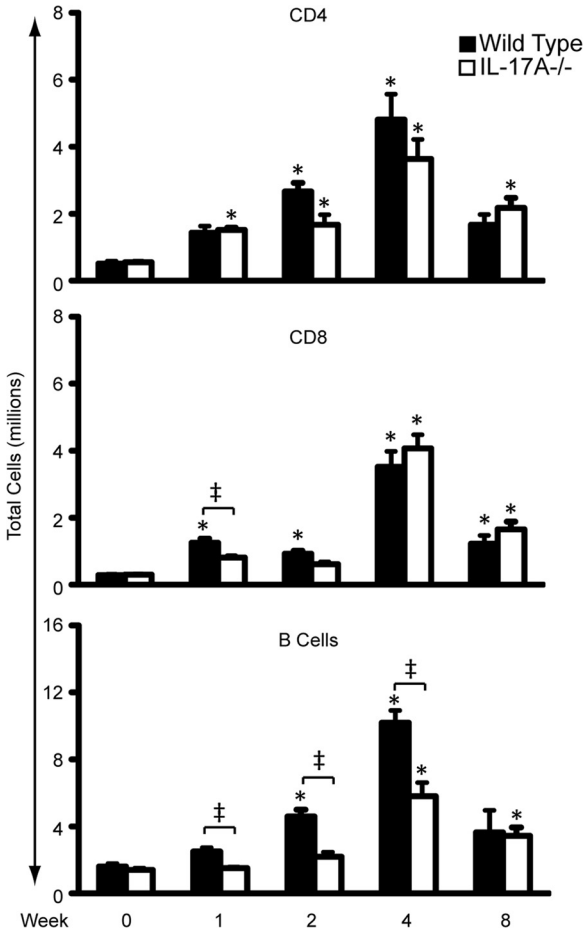

FIG 5 Accumulation of CD8 T cells and B cells but not CD4 T cells is impaired in IL-17A-deficient mice with persistent cryptococcal lung infection. WT C57BL/6 mice and IL-17A$^{-/-}$ mice were infected by the intratracheal route with *C. neoformans* strain 52D. At weeks 0 (uninfected), 1, 2, 4, and 8, lungs were removed and enzymatically digested, and lung cells were antibody stained and analyzed by flow cytometric analysis to identify the number of CD4 T cells (top panel), CD8 T cells (middle panel), and B cells (bottom panel) as described in Materials and Methods. Black bars, WT mice; white bars, IL-17A$^{-/-}$ mice. Data are means ± standard errors obtained from 4 to 6 mice per time point in two separate experiments. *, $P < 0.05$ by ANOVA with Dunnett's *post hoc* analysis versus day 0 (uninfected) mice of the same strain; ‡, $P < 0.05$ by unpaired Student *t* test between C57BL/6 mice and IL-17A$^{-/-}$ mice at the same time point.

weeks 1, 2, 4, and 8) were observed in IL-17A$^{-/-}$ mice relative to WT mice at the same time points (Fig. 5, middle and lower panels). These data indicate that IL-17A contributes to optimal CD8 T cell and B cell recruitment but does not significantly affect total CD4 T cell accumulation.

IL-17A is associated with increased IFN-γ production by CD4$^+$ T cells and CD8$^+$ T cells and the activation of antigen-presenting cells in mice infected with *C. neoformans* strain 52D. Our final objective was to investigate potential cellular and molecular mechanisms through which IL-17A enhanced fungal clearance and intracellular sequestration in this model system. We specifically questioned whether IL-17A altered the known relationships between fungal containment, IFN-γ production, and the activation status of lung dendritic cells and macrophages (56, 57, 60, 61). Using intracellular cytokine staining in conjunction with flow cytometric analysis, we first evaluated the expression of IFN-γ by CD4 and CD8 T cells at 1, 2, 4, and 8 weeks postinfection. Infection of WT C57BL/6 mice with *C. neoformans* strain 52D expands the percentage (Fig. 6A) and total numbers (Fig. 6B) of CD4 and CD8 T cells expressing IFN-γ (relative to uninfected WT mice); this Th1 component of the T-cell-mediated response is critical for containment of the infection in C57BL/6 mice and helps prevent progressive infection and lethal cryptococcal meningitis (11, 13, 22, 52, 62). In the infected IL-17A$^{-/-}$ mice, the percentage and total numbers of IFN-γ$^+$ CD4 and CD8 T cells also increased relative to uninfected IL-17A$^{-/-}$ mice (Fig. 6A and B), consistent with the relative containment of infection that we observed in these animals (Fig. 2). However, when direct comparisons between infected WT and IL-17A$^{-/-}$ mice were performed (at each time point), significant reductions in both the percentage and the total numbers of IFN-γ$^+$ CD4 T cells were observed in the IL-17A$^{-/-}$ mice relative to WT mice at week 1 postinfection as well as a nonsignificant trend noted at week 2. A significant early reduction in the total number of IFN-γ$^+$ CD8 T cells was also observed (at week 1 [Fig. 6B]).

We questioned whether the decrease in IFN-γ$^+$ CD4$^+$ T cells that we observed in the IL-17A$^{-/-}$ mice 1 week postinfection could be attributable to a loss of double positive, IFN-γ$^+$ IL-17$^+$ CD4 T cells, a unique but relatively rare T cell population that we had previously identified as expanding in the lungs of mice repetitively challenged with *A. fumigatus* (54). Although these cells could not be identified in IL-17A$^{-/-}$ mice (due to the absence of the IL-17A gene), we were able to identify a relatively small population of IFN-γ$^+$ IL-17$^+$ CD4 T cells in the lungs of uninfected C57BL/6 mice and observed that their percentage (Fig. 7A) and total number (Fig. 7B) had significantly increased at 8 weeks postinfection. However, the paucity of this population, especially at week 1 postinfection (approximately 2,000 cells), suggests that the observed decrease of approximately 200,000 IFN-γ$^+$ T cells observed in IL-17A-deficient mice relative to WT mice 1 week postinfection (Fig. 6B) cannot be accounted for by the loss of this population alone.

This observed decrease in IFN-γ production by lung T cells in concert with impaired fungal clearance suggested that the activation of lung dendritic cells and macrophages might be altered in IL-17A-deficient mice. We evaluated the expression of MHC-II and the costimulatory molecules CD80 and CD86, as their increased expression by lung dendritic cells is positively associated with Th1 polarization and increased expression on lung macrophages correlates with enhanced fungicidal activity (39, 61, 63). Expression of these markers was evaluated on both the CD11c$^+$ CD11b$^-$ myeloid cells (which include resident alveolar macrophages) and the CD11c$^+$ CD11b$^+$ myeloid cells (which include monocyte-derived dendritic cells and exudate macrophages) from uninfected WT and IL-17A$^{-/-}$ mice and at 1, 2, 4, and 8 weeks following infection with *C. neoformans* strain 52D. Within the population of CD11c$^+$ CD11b$^-$ myeloid cells, we observed an increase in MHC-II expression that did not differ between cells obtained from WT or IL-17A$^{-/-}$ mice, whereas a small reduction in the expression of CD86 (in IL-17A$^{-/-}$ mice) was identified at week 4 (Fig. 8A). In contrast, our comparative analysis on the population of CD11c$^+$ CD11b$^+$ myeloid cells revealed more-substantial findings. Specifically, whereas MHC-II expression increased over time in these cells in both strains of mice, expression was significantly reduced in the IL-17A$^{-/-}$ mice (relative to WT

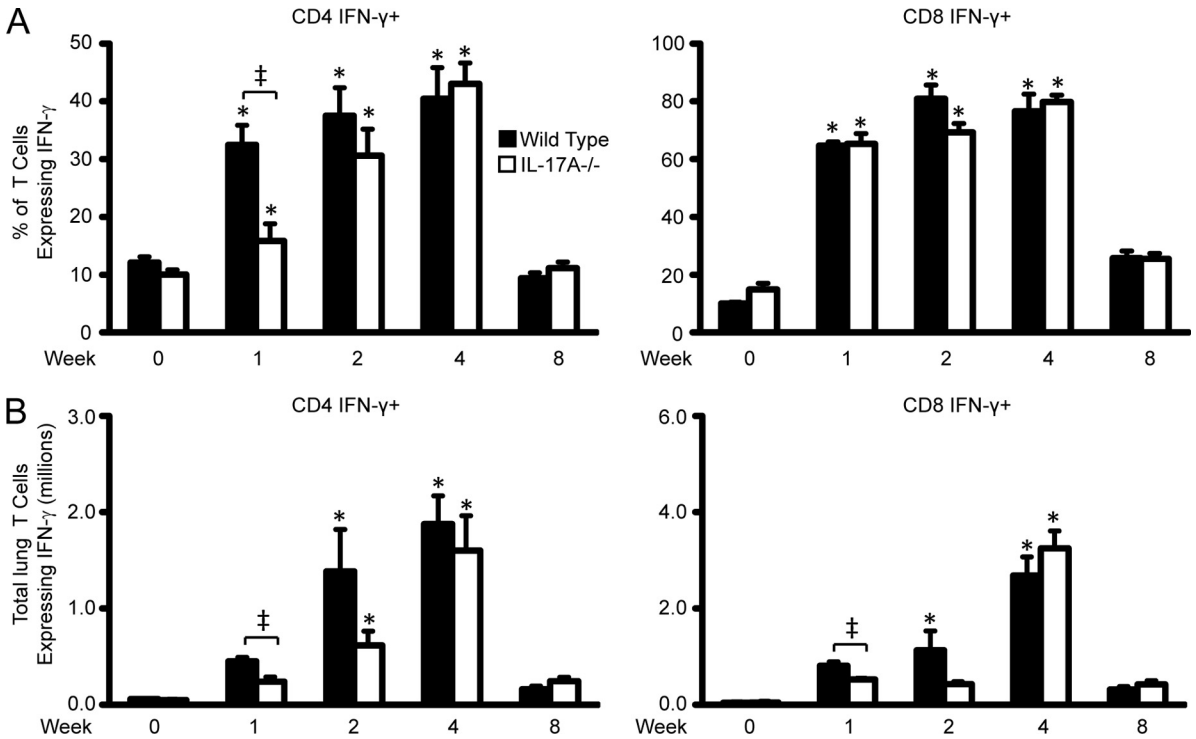

FIG 6 The percentages and total numbers of CD4 and CD8 T cells producing IFN-γ are impaired in the lungs of infected IL-17A-deficient mice with persistent cryptococcal lung infection. (A, B) WT C57BL/6 mice and IL-17A$^{-/-}$ mice were infected by the intratracheal route with *C. neoformans* strain 52D. At weeks 0 (uninfected), 1, 2, 4, and 8, lungs were removed and enzymatically digested, and lung cells were antibody stained and analyzed by flow cytometric analysis to identify the percentage (A) and total number (B) of CD4 T cells (left panels) and CD8 T cells (right panels) expressing intracellular IFN-γ as described in Materials and Methods. Black bars, WT mice; white bars, IL-17A$^{-/-}$ mice. Data are means ± standard errors obtained from 4 to 6 mice per time point in two separate experiments. *, $P < 0.05$ by ANOVA with Dunnett's *post hoc* analysis versus day 0 (uninfected) mice of the same strain; ‡, $P < 0.05$ by unpaired Student *t* test between C57BL/6 mice and IL-17A$^{-/-}$ mice at the same time point.

mice) at 2 and 4 weeks postinfection (Fig. 8B). Significant reductions were also observed for CD80 (weeks 2 and 4) and CD86 (week 4). Collectively, these findings suggest that IL-17A directly or indirectly enhances the production of IFN-γ by lung CD4 and CD8 T cells and the activation of lung dendritic cells and macrophages.

DISCUSSION

The current study investigated the role of IL-17A in pulmonary host defense against a moderately virulent strain of *C. neoformans* that causes a persistent infection in C57BL/6 mice. We demonstrate that the frequency and total numbers of lung Th17 cells expand during the course of infection in WT mice. Infection in IL-17A$^{-/-}$ mice (relative to WT mice) results in (i) increases in fungal burden and extracellular cryptococci in the lung; (ii) no change in 8-week survival; (iii) decreased accumulation of total lung leukocytes, including reductions in CD11c^{+} CD11b^{+} myeloid cells (dendritic cells and exudate macrophages), CD8 T cells, and B cells; (iv) specific decreases in the numbers of IFN-γ-producing CD4 and CD8 T cells; and (v) diminished expression of MHC-II, CD80, and CD86 on CD11c^{+} CD11b^{+} myeloid cells. Collectively, we identify Th1 polarization and macrophage and dendritic cell activation as two related cellular and molecular mechanisms through which IL-17A exerts a beneficial effect in a model of persistent cryptococcal lung infection.

This series of experiments provides some answers to an important question not previously investigated in prior studies of cryptococcal lung infection. Namely, what is the role of IL-17A in response to a moderately virulent and encapsulated strain of *C. neoformans* (strain 52D) in a model that recapitulates many features of fungal lung infection in immunocompetent (or mildly compromised) human hosts? Our results show that infection with strain 52D is associated with a substantial expansion of Th17 cells. This novel finding further illustrates the complexity of the evolving adaptive immune response in this model, which previously had been best characterized as a mixed Th1 and Th2 response. Our data provide evidence that a Th17 response is initiated within the first week of infection, at which time host defenses are dominated by a robust but relatively ineffective innate immune response. Thereafter, the Th17 response increases further, perhaps due to the influx of CD11c^{+} CD11b^{+} antigen-presenting cells, with an additional 3-fold increase in total numbers of Th17 cells observed at week 4 postinfection that persists to week 8. Although other cells can produce IL-17A, our data suggest that Th17 cells were likely the primary source of IL-17A in this model, as the percentage of non-CD4 T cells staining for intracellular IL-17A was low (~2%) and did not increase, in contrast to the substantial increase in CD4 T cells staining for IL-17A (which increased from 2% to 6%).

Our studies of fungal clearance revealed additional novel findings. First, we show that the absence of IL-17A did not affect fungal growth in the early, primarily innate phase of the infection (up to 2 weeks postinfection [Fig. 3]). Yet by week 4 postinfection, and

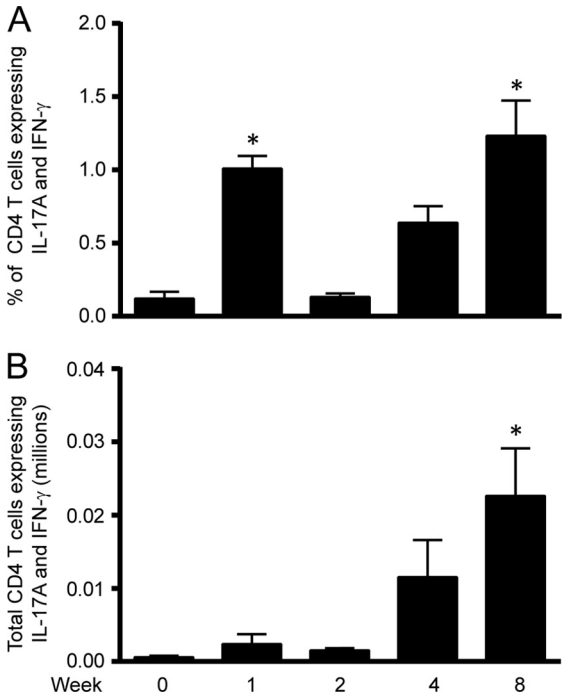

FIG 7 A small number of CD4 T cells expressing both IL-17A and IFN-γ accumulate in the lungs of C57BL/6 mice with persistent cryptococcal lung infection. C57BL/6 mice were infected by the intratracheal route with *C. neoformans* strain 52D. At weeks 0 (uninfected), 1, 2, 4, and 8, lungs were removed and enzymatically digested, and lung cells were antibody stained and analyzed by flow cytometric analysis to identify CD4 T cells expressing both intracellular IL-17A and IFN-γ. (A) Percentage of CD4 T cells expressing IL-17A and IFN-γ. (B) Total number of CD4 T cells expressing IL-17A and IFN-γ. Data are means ± standard errors obtained from 4 to 6 mice per time point in two separate experiments. *, $P < 0.05$ by ANOVA with Dunnett's *post hoc* analysis versus day 0 (uninfected) mice.

continuing to week 8, we observed that fungal clearance was impaired in the IL-17A-deficient mice, suggesting that IL-17A plays a role in protective adaptive immunity. This effect on late (but not early) fungal clearance parallels those reported by Deepe et al. in a study evaluating the effect of antibody-mediated neutralization of IL-17A in a model of chronic *H. capsulatum* infection (32). The manner in which IL-17A signaling is disrupted may be important, as in contrast to our study, a report by Szymczak et al. noted no impairment in fungal clearance in IL-17 receptor A-deficient mice (IL-17RA$^{-/-}$ mice; C57BL/6 genetic background) infected with *C. neoformans* strain 52D (44). Yet both our study and theirs provide evidence that IL-17A may help contain the organism within the lung. We observed a trend toward increased CNS dissemination in infected IL-17A$^{-/-}$ mice, whereas they showed evidence of increased organisms within the peripheral blood of persistently infected IL-17RA$^{-/-}$ mice. However, despite this evidence that IL-17A enhances antifungal defenses in the lung, neither the current study nor the previous two studies of chronic fungal lung infection were able to show that IL-17A signaling was essential for survival during the time periods studied. Thus, IL-17A contributes to pulmonary clearance of a moderately virulent strain of cryptococcus, yet other protective mechanisms remain active even in its absence.

Our analysis of the microanatomic pattern of the response to infection of WT and IL-17A$^{-/-}$ mice yielded important insights into the potential cellular and molecular mechanisms accounting for the beneficial effect of IL-17A observed in this model. We observed that the number of leukocytes accumulating within the lungs of IL-17A$^{-/-}$ mice was diminished relative to that seen in WT mice during the early phase of infection. These data are consistent with other reports showing that IL-17A can enhance the recruitment of multiple leukocyte subsets to sites of infection (27,

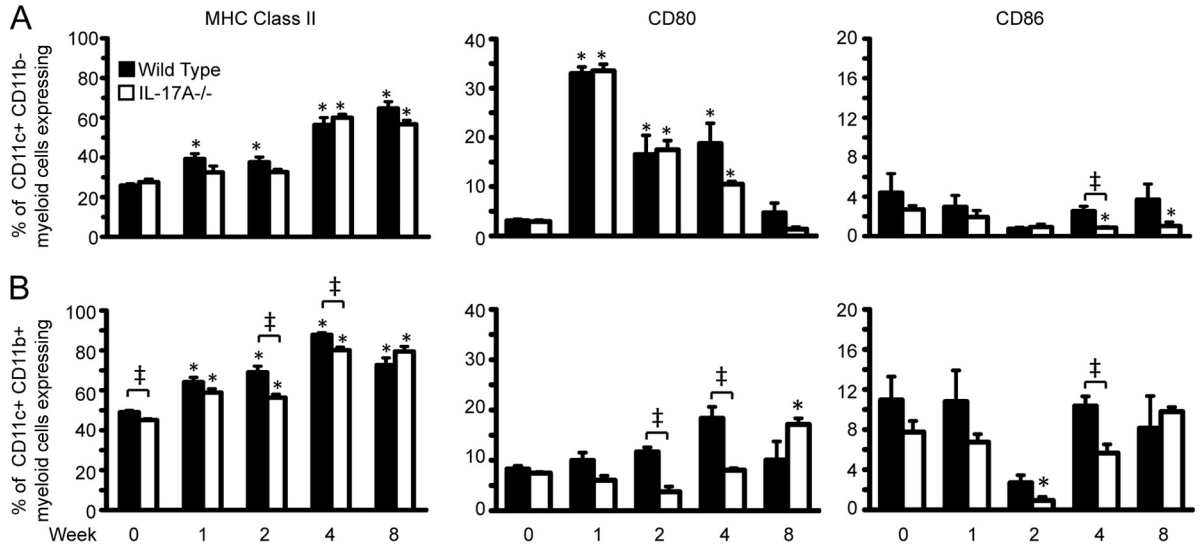

FIG 8 Expression of MHC-II and costimulatory molecules by lung macrophages and dendritic cells is impaired in the lungs of infected IL-17A-deficient mice with persistent cryptococcal lung infection. (A, B) WT C57BL/6 mice and IL-17A$^{-/-}$ mice were infected by the intratracheal route with *C. neoformans* strain 52D. At weeks 0 (uninfected), 1, 2, 4, and 8, lungs were removed and enzymatically digested, and lung cells were antibody stained and analyzed by flow cytometric analysis to identify the percent expression of MHC-II and the costimulatory molecules CD80 and CD86 by CD11c$^+$ CD11b$^-$ myeloid cells (resident alveolar macrophages) (A) and CD11c$^+$ CD11b$^+$ myeloid cells (dendritic cells and exudate macrophages) (B) as described in Materials and Methods. Black bars, WT mice; white bars, IL-17A$^{-/-}$ mice. Data are means ± standard errors obtained from 4 to 6 mice per time point in two separate experiments. *, $P < 0.05$ by ANOVA with Dunnett's *post hoc* analysis versus day 0 (uninfected) mice of the same strain; ‡, $P < 0.05$ by unpaired Student's *t* test between C57BL/6 mice and IL-17A$^{-/-}$ mice at the same time point.

64, 65). Our analysis using flow cytometric analysis confirmed this finding, and our subset analysis suggested that IL-17A mediated a nonsignificant increase in lung neutrophils and eosinophils (at week 1 postinfection), and a significant increase in $CD11c^+$ $CD11b^+$ myeloid cells (at week 2 postinfection) in response to infection. Furthermore, IL-17A influenced the recruitment of cells associated with the adaptive immune response, specifically, CD8 T cells and B cells. Thus, IL-17A contributes to lung leukocyte recruitment in response to cryptococcal lung infection with strain 52D. Whether IL-17A altered leukocyte recruitment through direct effects on cell migration or indirectly via effects mediated by chemokines and chemokine receptors (56, 57) remains uncertain but warrants additional investigation in future studies.

The presence of numerous extracellular cryptococci in the lungs of IL-$17A^{-/-}$ mice at 4 weeks postinfection was a second and striking finding resulting from our histopathologic analysis. This observation was very reminiscent of the numerous extracellular cryptococci that we identified in the lungs of mice deficient in either granulocyte-macrophage colony-stimulating factor (GM-CSF), a dendritic cell (DC) and exudate macrophage growth and differentiation factor, or in CD40, an important costimulatory molecule (51, 52). In those studies, the presence of numerous extracellular cryptococci was highly associated with reductions in IFN-γ and impairments in myeloid cell activation. This led us to question whether IFN-γ production was diminished in IL-17A-deficient mice. Our findings using intracellular staining for IFN-γ on both CD4 T cells and CD8 T cells confirmed this hypothesis. Prior studies in our lab have revealed a strong association between IFN-γ production by T cells and activation of lung myeloid cells. Here we advance our prior observations by demonstrating an association with IL-17A and the expression of MHC-II, CD80, and CD86 on lung $CD11c^+$ $CD11b^+$ myeloid cells (56, 63). Thus, the reduced MHC-II and costimulatory molecule expression on lung DC could explain the reduction in IFN-γ-producing T cells in IL-17A-deficient mice, consistent with our other studies linking impaired DC maturation with reductions in Th1 polarization (61, 62, 66). Since B cells can also function as antigen-presenting cells, it is possible that their diminished numbers in infected IL-$17A^{-/-}$ mice further contributed to our observed reduction in IFN-γ-producing T cells. Since IFN-γ is critical for the activation and fungicidal capabilities of lung macrophages (57), we believe that the reduced expression of MHC-II, CD80, and CD86 observed on $CD11c^+$ $CD11b^+$ cells could also reflect decreased local activation of exudate macrophages. Reductions in local concentrations of IFN-γ and IL-17A would also be expected to reduce intracellular cryptococcal proliferation (67). Collectively, these studies suggest that intracellular containment of strain 52D relies on the combined effects of IL-17A, IFN-γ, and activated myeloid cells.

Our observed findings complement studies investigating the host response to either the highly virulent strain of *C. neoformans*, H99, or the attenuated strains derived from H99 (H99γ and Δlac1), which are much less virulent (39, 60, 68). The original study by Wormley et al. demonstrated that infection with H99γ, a strain engineered to produce IFN-γ, enhanced lung IL-17A production and increased MHC-II expression by leukocytes (36). Likewise, infection with Δlac1 (laccase-deficient H99) resulted in increased pulmonary Th17 T cell recruitment, pulmonary IL-17A induction, and upregulation of MHC-II by macrophages (39). Lastly, Wozniak et al. demonstrated that antibody-mediated blockade of IL-17A impaired clearance of H99γ in BALB/c mice, although the rate of fungal dissemination to the CNS did not differ in the absence of IL-17 signaling (determined in their study using IL-17RA-deficient mice [68]). Thus, our findings and those obtained using strain H99γ suggest a strong interrelationship between IL-17A, IFN-γ, and the activation of lung dendritic cells and exudate macrophages in the containment or clearance of cryptococcal lung infection.

In summary, our findings further clarify the protective role of IL-17A in host defense against a moderately virulent strain of *C. neoformans* in C57BL/6 mice. In concert with IFN-γ, IL-17A enhances intracellular containment of the organism within activated lung macrophages, thereby reducing the opportunity for CNS dissemination. The insights gained from these studies are important, as therapeutic strategies designed to augment or suppress IL-17A production are being developed to treat a wide spectrum of infectious, autoimmune, and inflammatory diseases. Our findings suggest that therapies that suppress IL-17A may increase susceptibility to *C. neoformans* while enhancing IL-17A production might be beneficial in the treatment of cryptococcal lung infections.

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