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Early or Late IL-10 Blockade Enhances Th1 and Th17 Effector Responses and Promotes Fungal Clearance in Mice with Cryptococcal Lung Infection


The potent immunoregulatory properties of IL-10 can counteract protective immune responses and, thereby, promote persistent infections, as evidenced by studies of cryptococcal lung infection in IL-10–deficient mice. To further investigate how IL-10 impairs fungal clearance, the current study used an established murine model of C57BL/6J mice infected with Cryptococcus neoformans strain 52D. Our results demonstrate that fungal persistence is associated with an early and sustained expression of IL-10 by lung leukocytes. To examine whether IL-10–mediated immune modulation occurs during the early or late phase of infection, assessments of fungal burden and immunophenotyping were performed on mice treated with anti–IL-10R–blocking Ab at 3, 6, and 9 d postinfection (dpi) (early phase) or at 15, 18, and 21 dpi (late phase). We found that both early and late IL-10 blockade significantly improved fungal clearance within the lung compared with isotype control treatment when assessed 35 dpi. Immunophenotyping identified that IL-10 blockade enhanced several critical effector mechanisms, including increased accumulation of CD4+ T cells and B cells, but not CD8+ T cells; specific increases in the total numbers of Th1 and Th17 cells; and increased accumulation and activation of CD11b+ dendritic cells and exudate macrophages. Importantly, IL-10 blockade effectively abrogated dissemination of C. neoformans to the brain. Collectively, this study identifies early and late cellular and molecular mechanisms through which IL-10 impairs fungal clearance and highlights the therapeutic potential of IL-10 blockade in the treatment of fungal lung infections. The Journal of Immunology, 2014, 193: 000–000.

Cryptococcus neoformans is an encapsulated fungus acquired by the inhalational route. Depending on the virulence of the organism and the host’s immune status, lung infection results in one of three primary outcomes: clearance, persistence, or progressive infection (1). Failed clearance may result in lethal dissemination to the CNS (1). Infections with C. neoformans are the leading cause of fatal mycosis in HIV+ individuals [1 million new cases and 680,000 deaths/y (2)] and the second most common fungal infection in patients with organ transplants (1). In addition to the exceedingly high mortality (up to 70%) observed in infected HIV+ patients treated with antifungal therapy (2), up to 15% of these patients relapse, indicating that the infection can persist despite therapy and the development of partial immunity (3). Thus, novel approaches that can augment traditional antifungal therapies are needed. Cytokine networks, critically important in the pathogenesis of this disease (4, 5), represent potential new targets for immune-based therapies.

IL-10, a potent regulatory cytokine, exerts pleotropic effects on numerous subsets of immune cells (6, 7). The effects of IL-10 are mediated through IL-10R, a heterodimer consisting of α and β subunits (7, 8). These effects may be prominent during the innate (afferent) and/or adaptive (effferent) phase of immune responses. Among cells of the innate immune system, macrophages, in particular, are susceptible to the anti-inflammatory effects caused by IL-10 (9). Within adaptive immunity, IL-10 regulates many T and B cell responses, although many of the effects are indirect, being mediated via a direct effect of IL-10 on APCs, including dendritic cells (DCs) (6, 7).

Limited evidence implicates IL-10 in the pathogenesis of progressive or persistent cryptococcal infection in humans. In both HIV+ patients and transplant patients infected with C. neoformans, high IL-10 levels in the peripheral blood correlated with fungemia and disseminated disease (10, 11). In vitro studies demonstrated that human monocytes and DCs exposed to cryptococcal Ag express high amounts of IL-10 and fewer MHC class II (MHCII) molecules (12–14). The addition of exogenous IL-10 to cocultures of cryptococcal-laden human monocytes and T cells decreased T cell proliferation and IL-2 production (15).

Murine studies extended these clinical observations and yielded additional important insights into the role of IL-10 in the...
IL-10 BLOCKADE IMPROVES FUNGAL LUNG INFECTION

pathogenesis of cryptococcal lung infection. Most notably, Hernandez et al. (16) demonstrated that IL-10-deficient mice (C57BL/6J genetic background) were more resistant than wildtype mice to lung infection with a moderately virulent and encapsulated strain of C. neoformans (strain 52D; ATCC 24067). Although this study showed that IL-10 altered the overall balance between T1 and T2 adaptive immune responses (away from T1), numerous important questions remained unanswered. Were the effects of IL-10 mediated early or late during infection? Did IL-10 alter Th17 cell development [because we (17) recently showed an important protective role for IL-17 in this model]? Was there an effect of IL-10 on myeloid cells, including DCs and macrophages? Lastly, given the aforementioned limited efficacy of current anticytotoxic therapy in humans, could targeting of the IL-10-signaling axis be achieved in wild-type mice in a manner that could one day be translated into clinical trials?

In the current study, we show that persistent cryptococcal infection of C57BL/6J mice is associated with sustained expression of IL-10. By blocking IL-10 signaling (using Abs directed against IL-10R) during the early or late phase of cryptococcal lung infection, we demonstrate that both approaches enhance fungal clearance, likely through mechanisms involving increases in Th1 and Th17 cells and enhanced accumulation and activation of lung DCs and macrophages. Moreover, IL-10 blockade limited CNS dissemination. These novel findings identify the IL-10-signaling pathway as a potential therapeutic target in patients with fungal lung infections.

Materials and Methods

Mice

Wild-type (C57BL/6J) mice were obtained from Charles River Laboratory (Wilmington, MA) and housed under specific pathogen–free conditions in the Animal Care Facility at the Ann Arbor VA Health System. All studies were conducted according to a protocol approved by the VA Institutional Animal Care and Use Committee. Mice were 8–12 wk of age at the time of infection. Within this age range, we observed no age-related differences in the responses of these mice to C. neoformans infection. C. neoformans

C. neoformans strain 52D was obtained from the American Type Culture Collection (24067; Manassas, VA); this strain displayed smooth colony morphology when grown on Sabouraud dextrose agar. For intratracheal (i.t.) inoculation, C. neoformans was grown to a late logarithmic phase (48–72 h) at 37°C in Sabouraud dextrose broth (1% neopeptone and 2% dextrose; Difco, Detroit, MI) on a shaker. Cultured yeasts were washed in nonpyrogenic saline, counted in the presence of trypan blue using a hemocytometer, and diluted to 3.3 × 10^5 CFU/ml in sterile nonpyrogenic saline immediately prior to i.t. inoculation.

Surgical i.t. inoculation

Mice were anesthetized by i.p. injection of ketamine (100 mg/kg; Fort Dodge Laboratories, Shenandoah, IA) and xylazine (6.8 mg/kg; Lloyd Laboratories, Shenandoah, IA). Through a small midline neck incision, the strap muscles were divided and retracted laterally to expose the trachea. Intratracheal inoculation was performed under direct vision using a 30-gauge needle attached to a 1-ml syringe on a hypodermic (stepper; Tridak, Brookfield, CT). An inoculum of 10^4 CFU (30 μl) was injected into the trachea, and skin was closed using cyanoacrylate adhesive.

Intravenous inoculation

An inoculum of 10^6 CFU suspended in 100 μl PBS was injected into the lateral tail vein using a 30-gauge needle attached to a 1-ml syringe.

Tissue collection

Lungs were perfused in situ via the right heart using 5 ml PBS containing 0.5 mM EDTA until pulmonary vessels were grossly clear. Lungs were excised, minced, and enzymatically digested, and a single-cell suspension of lung leukocytes was obtained as previously described (18). After erythrocyte lysis, cells were washed, filtered over 70-μm mesh, and resuspended in complete medium. Dead cells were removed by centrifugation over a Percoll gradient. Total numbers of viable lung leukocytes were assessed in the presence of trypan blue using a hemocytometer. All cell preparations were washed twice in sterile PBS before use for culture or Ab staining. Brain and (in select experiments) spleen tissues were homogenized in 2 ml sterile water and used for CFU assays. In select experiments, blood obtained from the inferior vena cava was placed in heparinized tubes and subsequently used in CFU assays.

Measurement of cytokine production

Lung leukocytes obtained from uninfected mice (day 0) and mice infected for 7, 14, or 28 d were cultured at 5 × 10^5 cells/ml in 24-well plates in 2 ml complete RPMI 1640 media at 37°C and 5% CO₂ for 24 h without additional stimulation. At the end of culture, supernatants were harvested, and IL-10 concentration was assessed in triplicates by Luminex assay (Luminex, Austin, TX), per the manufacturer’s instructions. In selected experiments, supernatants from lung leukocyte cultures obtained in a similar manner were quantified for IFN-γ and IL-4 by ELISA using DuoSet kits (R&D Systems, Minneapolis, MN), following the manufacturer’s specifications. All plates were read on a VersaMax plate reader (Molecular Devices, Sunnyvale, CA).

Histology

Lungs were fixed by inflation with 1 ml 10% neutral buffered formalin, excised en bloc, and immersed in neutral buffered formalin. After paraffin embedding, 5-μm sections were cut and stained using H&E. Sections were analyzed with light microscopy, and microphotographs were taken using Digital Microphotography system DFX1200 with ACT-1 software (Nikon, Tokyo, Japan).

IL-10R–blocking Ab

Ultralow endotoxin, azide-free purified blocking Ab against the murine IL-10R (CD210; anti–IL-10R; clone 1B1.3a) or IgG1.k isotype control Ab (clone RTK2071) (BioLegend, San Diego, CA) was administered i.p. three times at a dose of 0.5 mg Ab in 200 μl sterile PBS (cumulative dose of 1.5 mg/mouse) to mice during the early or late phase of pulmonary C. neoformans infection. Early treatment consisted of i.p. injections at 3, 6, and 9 d postinfection (dpi); late treatment consisted of i.p. injections at 15, 18, and 21 dpi.

CFU assay

To quantify fungal CFU, aliquots (10 μl) of lung digests, brain and spleen homogenates, and blood samples were plated on Sabouraud dextrose agar plates in duplicate and in serial 10-fold dilutions and incubated at room temperature. Colonies were counted 2–3 d later, and the number of CFU organ was calculated. All recovered C. neoformans colonies retained their smooth morphology.

mAbs

The following mAbs (BioLegend, San Diego, CA) were used: N418 (anti-murine CD11c, hamster IgG1), 2.4G2 (Fc block; anti-murine CD16/CD32, rat IgG2b, 30-F11 (anti-murine CD45, rat IgG2b), 16-10A1 (anti-murine CD80, hamster IgG2), GL1 (anti-murine CD86, rat IgG2a), AF6-120.1 (Fc block; anti-murine I-Ab, mouse IgG2a), 145-2C11 (anti-murine CD3e, hamster IgG1, k), 6D5 (anti-murine CD19, rat IgG2a), and BMS (anti-murine F4/80, rat IgG2a). The mAbs AL-21 (anti-murine Ly-6C, rat IgM) and M1/70 (anti-murine CD11b, rat IgG2b) were purchased from BD Biosciences Pharmingen (San Diego, CA). mAbs were primarily conjugated with FITC, PE, PerCP-Cy5.5, allophycocyanin, allophycocyanin-Cy7, or Pacific blue. Isotype-matched control mAbs (BioLegend) were tested simultaneously in all experiments.

Cell staining and flow cytometric analysis

Cell staining, including blockade of FcRs, and sample analysis by flow cytometry were performed as described previously (18). A minimum of 100,000 events was acquired per sample on a FACSCanto flow cytometer using CellQuest software (both from BD Pharmlingen). Data were analyzed using FlowJo software (TreeStar, San Carlos, CA). Lung leukocyte subsets were identified using established gating strategies (17–20). Briefly, initial gates eliminated debris and identified CD45+ leukocytes. Within this CD45+ population, we identified CD4+ T cells (CD45+ FSC<median side scatter (SSC)<median CD4+ CD68−), CD8+ T cells (CD45+ FSC>median SSC>median CD4− CD68+), and B cells (CD45+ FSC<median SSC<median CD19+). To identify lung myeloid cells, lymphocytes were eliminated (by gating out CD3+ and CD19+ cells). Thereafter, additional gating identified the
following myeloid subsets: polymorphonuclear neutrophils (FSC$^{\text{low/mode}}$ Ly-6G$^{\text{high}}$), eosinophils (FSC$^{\text{moderate}}$ SSC$^{\text{moderate}}$ Ly-6G$^{\text{moderate}}$), Ly-6G$^{\text{high}}$ monocytes (CD45$^+$ FSC$^{\text{low}}$ Ly-6G$^{\text{low}}$ Ly-6Chigh), CD11b$^+$ DCs (CD45$^+$ FSC$^{\text{moderate}}$ Ly-6G$^{\text{high}}$), and alveolar macrophages (AMs; FSC$^{\text{moderate/high}}$ Ly-6G$^{\text{auto}}$ fluorescent CD11c$^+$ CD11b$^+$). Myeloid cell activation was assessed by measuring cell surface expression of MHCII (I-Ab), CD80, and CD86 relative to an isotype-matched control Ab.

For intracellular cytokine staining, $2 \times 10^6$ lung leukocytes/ml were stimulated in vitro for 6 h with PMA (50 ng/ml) and ionomycin (1 µg/ml) in the presence of monensin (1 µg/ml) (17). After stimulation, cells were washed and stained for CD4 to identify CD4$^+$ T cells. Subsequently, cells were washed and stained for intracellular IFN-$\gamma$ and IL-17A using the BD Cytotox/Cytoperm kit, according to the manufacturer’s instructions (BD Pharmingen).

To ensure consistency in data analysis, gate positions were held constant for all samples. To calculate the total number of cells in each population of interest in each sample, the corresponding percentage was multiplied by the total number of CD45$^+$ cells in that sample. The latter value was calculated for each sample as the product of the percentage of CD45$^+$ cells and the original hemocytometer count of total cells identified within that sample.

Statistical analysis
All data are expressed as mean ± SEM. Continuous ratio scale data were evaluated by an unpaired Student t test (for comparison between two samples) or by ANOVA (for multiple comparisons) with post hoc analysis by two-tailed Dunnett test, which compares treatment groups with a specific control group (21). Statistical calculations were performed on a Dell 270 computer using GraphPad Prism version 6.00 for Windows (GraphPad, San Diego, CA). Statistical difference was accepted at $p < 0.05$.

Results
Persistent lung infection with C. neoformans strain 52D in C57BL/6J mice is associated with sustained expression of IL-10
The effectiveness of host defenses in murine models of cryptococcal lung infection is related to the strain of mice studied and the virulence of the infecting organism. Our results demonstrated that infection of C57BL/6J mice with a moderately virulent encapsulated strain of C. neoformans, strain 52D (ATCC 24067), resulted in a rapid and sustained increase in lung CFU by 7 dpi that remained elevated ≥35 dpi (Fig. 1A), consistent with prior studies using this model system (16, 22, 23). Histologic evaluation of lung sections demonstrated that, at 7 and 14 dpi, many C. neoformans were located extracellularly within alveolar spaces (data not shown, Fig. 1B, middle panels, respectively). In contrast, at later time points (35 dpi; Fig. 1B, lower panels), the majority of viable C. neoformans was contained within granulomatous leukocyte infiltrates, many in intracellular forms within macrophages. Thus, these results demonstrate that i.t. inoculation of C57BL/6J mice with C. neoformans strain 52D results in persistent infection that is characterized by the localization of viable cryptococci within lung macrophages.

Prior studies by our colleagues and members of our research group (16) and other investigators (24–26) demonstrated that mice genetically deficient in IL-10 (C57BL/6J genetic background) are more resistant to cryptococcal lung infection. These studies provided important evidence that IL-10 critically modulates host defenses against this organism; however, the kinetics of IL-10 production in the lungs of wild-type C57BL/6J mice infected with strain 52D was not investigated. To address this point, we measured IL-10 production by lung leukocytes obtained from C57BL/6J mice at 0 (uninfected), 7, 14, and 28 dpi. IL-10 production by pulmonary leukocytes was significantly increased as early as 7 dpi and remained elevated relative to uninfected mice through 28 dpi (Fig. 1C). Thus, early and sustained production of IL-10 was strongly associated with persistent cryptococcal lung infection.

![FIGURE 1. Persistent infection of C57BL/6J mice with C. neoformans strain 52D is associated with early and sustained IL-10 expression. (A and B) C57BL/6J mice were infected i.t. with C. neoformans strain 52D. At day 0 (uninfected) and the indicated dpi, lungs were harvested for analysis. (A) Lung CFU analysis. (B) Representative lung sections from uninfected and infected mice (at 14 and 35 dpi; H&E, original magnification ×400, insets ×200). At 14 dpi, note the presence of numerous extracellular yeast within alveolar spaces (inset, arrows). At 35 dpi, note that most yeast are located intracellularly (inset, arrows) within large macrophages amid scattered foci of small mononuclear cells displaying lymphocyte morphology. (C) IL-10 production by lung leukocytes harvested at the designated time points postinfection (as described in Materials and Methods). Data in (A) and (C) are mean ± SEM of 5–21 mice assayed individually per time point in two separate experiments, *$p < 0.05$, versus day 0 (uninfected), ANOVA with Dunnett post hoc analysis.](http://www.jimmunol.org/Downloadedfrom)
Blocking either early or late IL-10 signaling improves fungal clearance in mice with cryptococcal lung infection

Our results demonstrated that IL-10 expression was elevated throughout lung infection. This finding suggested that IL-10 might be modulating both the early (primarily afferent and innate) phase of the infection (~0–14 dpi) and the late (primarily efferent and adaptive) phase when infection becomes established (~14–24 dpi) (17, 22, 23, 27–29). To assess the temporal impact of IL-10 production on disease pathogenesis in this model, we sought to transiently block IL-10 signaling in each of these two phases. Because of its demonstrated efficacy in other model systems (30–32), we chose to block IL-10 signaling by treating mice (by i.p. injection) with anti–IL-10R Ab. Treatment was administered to infected mice at 3, 6, and 9 dpi (early treatment) or at 15, 18, and 21 dpi (late treatment) (Fig. 2A). Additional cohorts of mice treated with isotype-control Ab during either the early or late phase of infection served as controls. All cohorts of mice were euthanized at 35 dpi because this advanced time point seemed the most appropriate for determining whether IL-10–signaling blockade would yield lasting effects on fungal clearance and the immunophenotype of the host response. Comparative analyses were performed between mice receiving anti–IL-10R Ab and isotype control during the same phase of infection.

Early treatment with anti–IL-10R Ab significantly reduced pulmonary fungal CFU at 35 dpi (Fig. 2B, left graph). This finding demonstrated that early IL-10 blockade yielded lasting effects on fungal clearance. Late treatment with anti–IL-10R Ab also significantly reduced pulmonary fungal CFU (Fig. 2B, right graph), demonstrating that late treatment with anti–IL-10R Ab could enhance fungal clearance even after lung infection was established. Note that differences in the elapsed time between IL-10 blockade and the time point selected for CFU analysis limits direct comparison between the early and late treatment groups.

We next sought to evaluate whether IL-10 blockade had a more immediate impact on fungal clearance. To this end, mice receiving either early or late treatment with anti–IL-10R Ab (or isotype-control Ab) were assessed for lung fungal burden by CFU assay 72 h following their last dose of Ab treatment (at 12 dpi for early treatment, 24 dpi for late treatment; Supplemental Fig. 1A). Early treatment resulted in a nonsignificant decline in pulmonary fungal CFU (Supplemental Fig. 1B, left graph), whereas late treatment resulted in a statistically significant, albeit small, reduction in fungal clearance (Supplemental Fig. 1B, right graph). Collectively, these results demonstrated that transient IL-10 blockade during either the early or late phase of infection improved clearance of cryptococcal lung infection. Reductions in lung fungal burden were observed most consistently when assessed at 35 dpi, >10 d posttreatment with anti–IL-10R Ab.

**Transient IL-10 blockade increases lung leukocyte accumulation in mice with cryptococcal lung infection**

To investigate the mechanism(s) whereby IL-10 blockade enhanced fungal clearance, we determined whether treatment with anti–IL-10R Ab altered the accumulation of lung leukocytes. Using specific Ab-mediated cell staining and flow cytometric analysis, we enumerated the total number of lung leukocytes (identified by expression of CD45) in each cohort of Ab-treated infected mice at 35 dpi. Early treatment with anti–IL-10R Ab resulted in a nonsignificant trend toward increased lung leukocyte accumulation (Fig. 3, left graph). Late treatment with anti–IL-10R Ab significantly increased lung leukocyte accumulation (Fig. 3, right graph).

In our study, no mice receiving either anti–IL-10R or isotype-control Ab died or appeared subjectively ill by 35 dpi, consistent with other studies using this model of persistent infection with a moderately virulent strain of *C. neoformans* (16, 33). However, in light of this observed increase in lung inflammation in mice subjected to IL-10 blockade, we monitored mice for weight loss as an objective measurement of morbidity. Mice were weighed every 2–3, up to 72 h following the last dose of Ab (Supplemental Fig. 1A). No significant differences in weight were observed in mice infected with either isotype-control Ab (white bars) or anti–IL-10R–blocking Ab (black bars) during the early phase or late phase of infection. Data represent mean ± SEM of 9 or 10 mice/experimental cohort assayed individually in two separate experiments. *p* < 0.05, versus treatment at same time point, unpaired Student *t* test.
receiving early treatment with anti–IL-10R Ab (mean weight, 19.4 ± 0.7 g for isotype-control treated mice versus 19.6 ± 0.6 g for anti–IL-10R–treated mice at 12 dpi), whereas a reduction in weight was observed in mice receiving late treatment with anti–IL-10R Ab (mean weight, 21.4 ± 0.8 g for isotype control–treated mice versus 17.0 ± 1.1 g for anti–IL-10R–treated mice at 24 dpi). Whether this weight loss persists in mice receiving late IL-10 blockade warrants follow-up in future studies.

**Transient IL-10 blockade increases the numbers of Th1 and Th17 cells in mice with cryptococcal lung infection**

Clearance of cryptococcal lung infection requires adaptive immunity (34, 35). Thus, we next investigated whether early or late anti–IL-10R Ab treatment altered the accumulation of lymphocytes in infected lungs. Data obtained using specific cell staining demonstrated that early and late IL-10 blockade increased the total numbers of CD4+ T cells (Fig. 4A), but it did not affect the accumulation of CD8+ T cells (Fig. 4B). Late (but not early) IL-10 blockade increased the total numbers of B cells (Fig. 4C).

Previous studies showed that Th1 cells accumulate in C57BL/6J mice persistently infected with *C. neoformans* strain 52D and that IFN-γ expression and signaling are critical for preventing progressive lung infection (22, 27, 28). More recently, we showed the importance of Th17 cells for cryptococcal clearance in this model (33). Therefore, we next determined whether early or late IL-10 blockade altered the frequency and total number of Th1 cells (identified as CD4+ T cells positive for intracellular IFN-γ staining) or Th17 cells (identified as CD4+ T cells positive for intracellular IL-17A staining). Early and late IL-10 blockade did not increase the frequency of Th1 cells but did increase their total numbers (Fig. 4D, upper panels). Early and late IL-10 blockade increased both the frequency and total numbers of Th17 cells (Fig. 4D, lower panels). Thus, transient early and late IL-10 blockade improved the accumulation of protective Th1 and Th17 cell populations in the lungs of infected mice.

To determine whether the total number of IFNγ+ CD4+ T cells in infected lungs correlated with the level of IFN-γ secretion by lung leukocytes, we measured the concentration of IFN-γ in the supernatants of lung leukocyte cultures obtained at 35 dpi using ELISA. We found a nonsignificant trend toward increased IFN-γ secretion in mice receiving early anti–IL-10R Ab treatment (Supplemental Fig. 2A, left graph) and a significant increase in IFN-γ release in mice receiving late IL-10 blockade (Supplemental Fig. 2A, right graph). IL-17 protein secretion by lung leukocytes was not assessed in the current study; however, using the same animal model, we recently showed a direct correlation between the incidence and the total number of IL-17+ CD4+ T cells and the level of IL-17 transcript expression and protein release (33). To determine whether transient IL-10 blockade affected Th2 responses, we also measured the concentration of IL-4 in the supernatants described above. No significant differences in the levels of IL-4 secretion were detected (Supplemental Fig. 2B).

**Transient IL-10 blockade increases the activation of lung DCs and macrophages in mice with cryptococcal lung infection**

The improvement in fungal clearance and enhancement in Th1/Th17 responses that we observed with IL-10 signaling blockade suggested a beneficial effect on myeloid effector cells. To investigate this, we used established gating strategies (17–20) for analysis of flow cytometric data to assess the effect of anti–IL-10R Ab treatment on the accumulation of specific lung myeloid cell...
subsets at 35 dpi (Fig. 5). Within granulocyte populations, early anti–IL-10R Ab treatment did not alter the number of neutrophils or eosinophils (Fig. 5A, 5B, left bars). Late anti–IL-10R Ab treatment resulted in a nonsignificant trend toward increased accumulation of neutrophils (Fig. 5A, right graph), perhaps reflecting the increase in Th17 cells. No difference in lung eosinophils was observed (Fig. 5B, right graph). Prior studies demonstrated an important role for recruited peripheral blood–derived Ly-6C<sup>high</sup> monocytes in mice that clear cryptococcal lung infection, because these cells can further differentiate into CD11b<sup>+</sup> DCs and ExMs (19, 20). In the current study, early anti–IL-10R Ab treatment did not alter the number of Ly-6C<sup>high</sup> monocytes or CD11b<sup>+</sup> DCs, but it did increase the numbers of ExMs (Fig. 5C–E, left graphs). Late anti–IL-10R Ab treatment did not alter the numbers of Ly-6C<sup>high</sup> monocytes, but it increased both CD11b<sup>+</sup> DCs and ExMs (Fig. 5C–E, right graphs). Neither treatment impacted the numbers of resident AMs (Fig. 5F).

We next sought to determine whether early or late treatment with anti–IL-10R Ab increased the expression of activation markers, including MHCII, CD80, and CD86, by lung DC and macrophage populations, because our prior studies demonstrated a strong association between the expression of these markers and improved clearance of C. neoformans from the lung (19, 20, 36, 37) (Fig. 6). Early treatment was associated with a trend toward increased expression of CD80 on CD11b<sup>+</sup> DCs (Fig. 6). In contrast, late IL-10 blockade was associated with significantly increased expression of I-Ab (MHCII) by ExMs, expression of CD80 by CD11b<sup>+</sup> DCs and ExMs, and expression of CD86 by AMs (Fig. 6). Collectively, these findings demonstrated that transient blockade of IL-10 signaling increases the accumulation and activation profile of lung DCs and macrophages.

**Late IL-10 blockade reduces CNS dissemination in mice with cryptococcal lung infection**

Our data demonstrated that transient IL-10 blockade enhanced protective immune responses within the lungs, yet the lethality of cryptococcal infections in mice and humans is determined primarily by whether the organism disseminates to the CNS. Our final objective was to explore whether early or late IL-10 blockade altered dissemination of C. neoformans from the lung to the brains of infected mice. To this end, cohorts of treated mice were assessed for fungal burden in the brain (by CFU assay) either 72 h following the last dose of Ab administration (refer to the treatment strategy depicted in Supplemental Fig. 2A) or at 35 dpi (refer to the treatment strategy depicted in Fig. 2A). At 72 h after early treatment (12 dpi), four of five mice treated with isotype-control Ab had fungal growth in brain tissue, whereas no mice (0/5) receiving early treatment with anti–IL-10R Ab had evidence of fungal growth in the brain (Fig. 7A, left graph). At 72 h after late treatment (24 dpi), fungal growth in the brain was noted in four of five mice treated with isotype-control Ab and in two of five mice treated with anti–IL-10R Ab (Fig. 7A, right graph).

At 35 dpi, three of four mice receiving early treatment with isotype-control Ab and four of five mice treated with anti–IL-10R Ab had fungal growth in brain tissue (Fig. 7B, left graph). In contrast, late IL-10 blockade significantly decreased the burden of C. neoformans within the CNS, because 9 of 10 mice receiving isotype-control Ab had evidence of fungal growth in brain tissue, whereas only 1 of 10 mice treated with anti–IL-10R Ab showed any fungal growth in the brain (Fig. 7B, right graph). Collectively, these results show that both early and late IL-10 blockade favorably reduced fungal growth in the brain in mice with primary cryptococcal lung infection.

Although the favorable reductions in brain fungal burden in mice treated with anti–IL-10R Ab could reflect diminished dissemination from the lung as a result of the reduced pulmonary fungal burden observed in these mice, we considered the possibility that IL-10 blockade might affect the blood–brain barrier or the immediate innate immune defenses within the CNS. To address this, cohorts of C57BL/6J mice were infected with C. neoformans i.v. 24 h after receiving one dose of either isotype-control Ab or anti–IL-10R Ab. CFU in the brain, lung, spleen, and blood were assessed 48 h postinfection. Results demonstrated no difference in fungal growth between treatment groups in the brain, lung, and spleen (Fig. 7C). No fungal growth was observed in the blood in either group (data not shown). These findings suggest that IL-10 blockade did not immediately alter the blood–brain barrier or innate CNS immune defenses.

**Discussion**

This study emphasizes that the microenvironment within persistently infected tissues is not static but rather results from an ongoing and dynamic balance between effector and regulatory...
mechanisms. Using a murine model of cryptococcal lung infection, we show that sustained elevations in IL-10 production play a critical role in maintaining persistent infection not only during the early, developing phase but even when the infection has been established. Specifically, using anti–IL-10R Ab to treat mice, we show that temporary blockade of IL-10 signaling during either the early or late phase of infection improved fungal clearance in the lung. Both early and late treatments enhanced generation of protective Th1 and Th17 responses and increased accumulation and activation of lung DCs and ExMs. Notably, treatment with anti-IL-10R Ab markedly reduced fungal dissemination to the CNS. Collectively, this study significantly advances our understanding of how and when IL-10 modulates immune microenvironments to promote cryptococcal persistence. Importantly, our findings suggest that IL-10 blockade may represent a novel therapeutic strategy for treating acute or established fungal lung infections.

Treatment of lung infections caused by invasive fungi (C. neoformans, Aspergillus fumigatus, Histoplasma capsulatum, Blastomyces dermatitidis, and Coccidioides immitis) requires lengthy, sometimes lifelong, courses of antibiotics that have limited efficacy.
and are toxic and expensive (1, 38). Persistent fungal infections result in parenchymal lung damage (39–43) and may progress once patients become further immunocompromised because of worsening of HIV/AIDS or immunosuppressive therapies (44). These realities motivate studies seeking to better understand the pathogenesis of persistent fungal lung infections with the hope that new treatments might be developed. In this study, we investigated the central role of IL-10 in fungal lung disease. Several features of our experimental design enhance the significance of our findings. First, infection was studied in the lung, the primary site through which humans acquire the organism (1). Second, the murine model used is well characterized (4, 16, 23, 28, 29, 45–48), which enabled studies evaluating the effects of IL-10 blockade on effector mechanisms known to be associated with fungal clearance. Third, we transiently blocked IL-10 during either the early or late phase of infection, which provided novel insights into the temporal significance of IL-10 production. This aspect was not investigated in prior studies using mice genetically deficient in IL-10 (16, 24–26). Moreover, to our knowledge, this study indicates for the first time that transient IL-10 blockade using mAbs has translational potential for treating persistent cryptococcal lung infections in humans. Fourth, we studied fungal dissemination to the CNS, an important surrogate of potentially lethal infection in both mice and humans (1).

Our data show that IL-10 production is increased in lung leukocytes as early as 7 dpi, at the time when afferent innate host responses are developing. Thus, it was tempting to speculate that IL-10-mediated suppression of early innate immune responses might account for the initial rapid intrapulmonary growth of *C. neoformans* that is consistently observed up to 14 dpi in C57BL/6J mice infected with strain 52D (5, 28, 49). However, a study by Hernandez et al. (16) showed no impairment in early fungal clearance in transgenic IL-10–deficient mice; only at later time points was improvement in fungal clearance observed. Taken together, these two findings motivated our strategy to block IL-10 during the early phase of infection.

Our results show that early blockade of IL-10 signaling variably reduced fungal clearance when assessed 72 h posttreatment; this initial improvement became much more consistent when assessed ~3 wk after the cessation of IL-10 blockade, suggesting that early treatment during the afferent and innate phase of infection favorably impacted the efferent and adaptive phase of infection. We investigated this hypothesis by focusing on immune effector mechanisms previously shown to be critical for fungal clearance in this model. Although dominated by Th2 responses (45), prior studies showed that both IFN-γ and IL-17A are essential for preventing progressive fungal lung infection and dissemination to the CNS (17, 22, 27). Our results showing that early anti–IL-10 Ab treatment increased the numbers of Th1 and Th17 cells not only underscore the importance of these cells to fungal clearance but, for the first time, to our knowledge, they show that IL-10 is actively impairing the induction of these cellular subsets, even during the very early phases of infection. Early anti–IL-10 Ab treatment also increased the accumulation of ExMs; these nonresident macrophages derived from recruited Ly-6C<sup>chil</sup> monocytes are more fungicidal than are resident AMs (20). Their fungicidal activity would be further enhanced under conditions of IL-10 blockade by the increased numbers of Th1 cells producing IFN-γ in the lung microenvironment.

We observed that IL-10 production is sustained during the development of the efferent and adaptive response in this model (measured at 14 dpi) and even later on into the persistent phase of infection (measured at 28 dpi). By delaying anti–IL-10 Ab treatment until the latter stage of infection, we were able to test, for the first time, whether this persistent presence of IL-10 within the infected microenvironment was continuing to actively suppress effector immune responses. Indeed, this was the case, as evidenced by our finding that late anti–IL-10 Ab treatment improved fungal clearance in the lung, with the most pronounced effect observed at 35 dpi. Similar to what we observed with early anti–IL-10 Ab treatment, late treatment increased the numbers of Th1 cells and Th17 cells in the lung and promoted increased accumulation of activated ExMs expressing higher amounts of MHCII and CD80. In contrast to early treatment, late IL-10 blockade also increased numbers of CD11b<sup>+</sup> DCs and B cells. These DCs expressed higher amounts of CD80 (than did those in the lungs of isotype control–treated mice) and, thus, may promote effector Th1 responses against *C. neoformans* (18, 19, 36). B cells might also improve cryptococcal clearance (50), perhaps through Ab-mediated improvements in the phagocytic activity of lung macrophages, as described recently (51, 52).
Our results show that both early and late treatment with anti–IL-10 Ab decreased the fungal burden of Cryptococcus within the CNS. These findings may have important clinical implications because CNS dissemination and meningococcalpalsitis are the leading cause of mortality in human cryptococcal disease (1). Interestingly, mice in the early treatment group showed no evidence of fungal growth in the brain when assessed 72 h after the last dose of anti–IL-10R Ab (at 12 dpi). However, CNS growth was detected when assessed at 35 dpi, suggesting that delayed growth within the brain may be possible in the absence of ongoing IL-10 blockade during the established phase of infection. In contrast, late treatment with anti–IL-10 Ab during this established phase almost completely abrogated CNS growth when assessed at 35 dpi. Future studies will need to address whether these mice might relapse and to determine whether extending treatment, perhaps at weekly intervals, with or without concurrent antifungal antibiotics, would further augment or sustain the reductions in pulmonary and CNS fungal burden achieved using IL-10 blockade.

In our experiment of i.v. acute systemic cryptococcal infection, we observed no differences in brain fungal growth in mice receiving anti–IL-10R Ab versus mice receiving isotype control when assessed 48 h postinfection. This finding suggests that IL-10 blockade does not immediately alter the blood–brain barrier. We also observed no difference in fungal growth in the lung, spleen, or blood, suggesting that the very initial innate immune events in these organs were not profoundly altered by IL-10 blockade. Collectively, our data suggest that the reduction in lung fungal burden observed in mice treated with anti–IL-10R Ab may promote containment of the organism within the lung and, thereby, reduce CNS dissemination. In support of this hypothesis, we observed increased numbers of activated ExMs in the lungs of mice treated with anti–IL-10R Ab, consistent with our prior studies demonstrating that increases in the number and activation of lung ExMs correlate with improved fungal clearance and reductions in fungal dissemination (17, 20, 37). Alternatively, our studies have not completely ruled out that IL-10 blockade may promote more effective adaptive immunity within the CNS against C. neoformans. Comprehensive studies investigating this intriguing possibility and potential cellular sources of IL-10 in the lung would be of interest, but they exceed the scope of the current study.

Collectively, our findings support an evolving paradigm based on a dynamic balance between effector and regulatory networks as the critical determinant of whether host–pathogen interactions in the lung result in clearance or persistence. Our data show that reductions in fungal dissemination (17, 20, 37). Alternatively, our studies demonstrating that increases in the number and activation thereby, reduce CNS dissemination. In support of this hypothesis, lung fungal burden observed in mice treated with anti–IL-10R Ab blockade. Collectively, our data suggest that the reduction in pulmonary and CNS fungal burden achieved using IL-10 blockade.

Disclosures
The authors have no financial conflicts of interest.

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