Immunopathology and Infectious Diseases

IL-23 Dampens the Allergic Response to *Cryptococcus neoformans* through IL-17–Independent and –Dependent Mechanisms

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The cytokines IL-23 and IL-17 have been implicated in resistance to cryptococcal disease, but it is not clear whether IL-23–mediated production of IL-17 promotes fungal containment following pulmonary challenge with *Cryptococcus neoformans*. We used mice lacking IL-23 (*IL-23p19*−/−) or IL-17RA (*IL-17RA*−/−), and wild type (WT) C57BL/6 mice to examine the IL-23/IL-17 axis after intranasal infection with the *C. neoformans* strain 52D. The absence of IL-23 or IL-17RA had no effect on pulmonary or brain fungal burden at 1 or 6 weeks after infection. However, survival of *IL-23p19*−/− mice was reduced compared to *IL-17RA*−/− mice. IL-17 production by CD4 T cells and natural killer T (NKT) cells was impaired in *IL-23p19*−/− lungs, but was not completely abolished. Both *IL-23p19*−/− and *IL-17RA*−/− mice exhibited impaired neutrophil recruitment, increased serum levels of IgE and IgG2b, and increased deposition of YM1/YM2 crystals in the lung, but only *IL-23p19*−/− mice developed persistent lung eosinophilia. Although survival of *IL-17RA*−/− and WT mice was similar after 17 weeks of infection, only surviving *IL-17RA*−/− mice exhibited cryptococcal dissemination to the blood. These data demonstrate that IL-23 dampens the allergic response to cryptococcal infection through IL-17–independent suppression of eosinophil recruitment and IL-17–dependent regulation of antibody production and crystal deposition. Furthermore, IL-23, and to a lesser extent IL-17, contribute to disease resistance. (Am J Pathol 2012, 180:1547–1559; DOI: 10.1016/j.ajpath.2011.12.038)

Cryptococcus neoformans is a fungal pathogen that causes significant morbidity and mortality, primarily in immunocompromised, but also in immunocompetent individuals. In Africa, cryptococcal meningitis is the cause of death of 20% to 30% of HIV-infected patients.[1] Infection often occurs early in life when the organism is inhaled from the environment.[2,3] It is believed that most individuals resolve acute pulmonary infection, but that yeasts persist latently in granulomata.[4–6] If the immune system subsequently becomes compromised, the organism can re-establish pulmonary infection and disseminate from the lungs to the central nervous system.[7,8] Depressed CD4+ T-cell–mediated immunity is associated with the development of cryptococcal disease in HIV-infected individuals,[9–11] but no biomarkers are available to predict which individuals will develop disseminated disease, regardless of CD4 counts, suggesting a complex immune response is required for fungal containment.

Mouse models of cryptococcal disease have established that a T_H1 polarized response is required for resolution of primary infection,[12–16] but innate[15,17,18] and humoral[19–21] factors also restrain fungal replication and facilitate the generation of adaptive T-cell–mediated immunity. Recent studies suggest a role for IL-17 in cryptococcal containment.[22–27] The IL-17 family consists of the cytokines IL-17a–f that are produced by innate cells such as lymphoid tissue inducer, γδ T, NKT, alveolar macrophages, and neutrophils,[27–31] as well as antigen-specific CD8 T cells[32] and T_H17 cells.[33,34] IL-17a, IL-17f, and IL-17a/f heterodimers signal through the IL-17RA/IL-17RC receptor complex to induce antimicrobial peptide, cytokine, and chemokine production,[35–37] whereas IL-17e (IL-25) signals through IL-17RA/IL-17RB to induce T_H2 inflammatory responses.[38] IL-17a and IL-17RA were shown to limit lung fungal burden in mice infected with a genetically engineered, IFN-γ–produc-

ing strain of *Cryptococcus neoformans* that elicits elevated IFN-γ and IL-17 production and fungal clearance,[22,27] corroborating *in vitro* studies demonstrating that IL-17 can inhibit yeast replication within macrophages.[39] In addition, mice lacking IL-13 or both IL-4 and IL-13, produce increased amounts of IL-17, which is associated with reduced burden in lungs.[23,24]

IL-17 production is enhanced by the cytokine IL-23.[34,40,41] IL-23 is a heterodimeric cytokine composed of a unique subunit, p19, and a shared subunit with IL-12, p40.[42] IL-23 is produced by dendritic cells on dectin-1 or dectin-2 recognition of fungal antigens,[43,44] and possibly Toll-like receptors since bone-marrow–derived dendritic cells produce IL-23 in response to *C. neoformans* in a MyD88-dependent manner.[45] In many infection models, IL-23 contributes to disease resistance in an IL-17–dependent manner.[31,46–50] In a chronic, systemic cryptococcal infection model, administration of recombinant IL-23 increased production of IL-17 by T_H17 cells and non-T cells and prolonged survival,[26] and lack of IL-23 impaired immunity.[25] However, the role of IL-23 in the lungs in the setting of respiratory acquisition of *C. neoformans*, the natural route of infection, has not been examined.

We sought to determine whether IL-23 promotes IL-17 production and subsequent control of chronic pulmonary infection caused by *C. neoformans*, strain 52D. Using *IL-23p19*^{−/−} or *IL-17RA*^{−/−} mice, we found that IL-23 and IL-17 did not significantly contribute to control of lung fungal burden or yeast dissemination to the brain during the first 6 weeks of infection, but the absence of IL-23 was associated with increased mortality. IL-23 and IL-17 both suppressed mediators of the allergic response to *C. neoformans*; specifically IgE production and the formation of YM1/YM2 crystals; however, IL-23 also inhibited eosinophil recruitment to the lung.

Materials and Methods

Mice

Male 6- to 8-week-old C57BL/6 WT control mice were purchased from the National Cancer Institute (Charles River Laboratories, Wilmington, MA). *IL-23p19*^{−/−} mice were obtained from Genentech (San Francisco, CA), and *IL-17RA*^{−/−} mice were supplied by Amgen (Seattle, WA). *IL-23p19*^{−/−} and *IL-17RA*^{−/−} mice were previously backcrossed 10 generations onto the C57BL/6 background and were bred under pathogen-free conditions in the Institute for Animal Studies at the Albert Einstein College of Medicine (AECOM). All mice were given unrestricted access to food and water. All mouse experiments were conducted with prior approval from the Animal Care and Use Committee of AECOM following established guidelines.

Cryptococcal Infection Model

A serotype D strain (52D) of *C. neoformans*, ATCC 24067 (American Type Culture Collection, Manassas, VA), was used for intranasal (i.n.) infection of mice. Strain 52D has been used extensively to evaluate the immune response to experimental pulmonary cryptococcosis.[25,51–54] The *C. neoformans* strain was kept in 15% glycerol aliquots at

−80°C until needed. Thawed aliquots of *C. neoformans* were grown in Difco Sabouraud Dextrose Broth (Becton Dickinson, Franklin Lakes, NJ) for 48 hours at 37°C with shaking, washed twice in PBS (Mediatech, Herndon, VA), and counted in a hemocytometer using Trypan Blue for viability. For the i.n. infection, mice were anesthetized with isoflurane (Halocarbon, River Edge, NJ) and placed in a vertical position. A volume of 20 μL containing 5×10^5 colony-forming units (CFU) of *C. neoformans* was administered via the nares. For survival studies, infected mice were observed at least once daily.

Measurement of Tissue and Blood Fungal Burden

Blood was collected from three to five animals per group by retro-orbital puncture under deep isoflurane anesthesia. After blood collection, mice were euthanized by cervical dislocation. Lungs and brains were removed and homogenized in 1 mL of HBSS (Lonza, Walkersville, MD). CFU were determined by making 10-fold serial dilutions of each tissue or twofold dilutions of blood and plating 20 μL of the sample and dilutions on Sabouraud Dextrose agar plates (BBL, Sparks, MD). Each sample was processed in duplicate. Plates were incubated at room temperature for 72 hours, after which colonies were visually counted.

Measurement of Cytokine Levels

Lung homogenates that were used to determine fungal burden were centrifuged at $3000 \times g$ for 30 minutes at 4°C and the supernatants collected. The supernatants were centrifuged at $13,000 \times g$ at 4°C for an additional 10 minutes to remove any remaining debris. Samples were stored at −80°C before use. All cytokine concentrations were determined using a DuoSet ELISA Development Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

Isolation of Lung Cells

In independent experiments, three to five mice per group were anesthetized with isoflurane, sacrificed by cervical dislocation, and lungs removed. Single-cell lung suspensions were obtained from using a gentleMACS Dissociator (Miltenyi Biotec, Auburn, CA) following the manufacturer's protocol for dissociation of mouse lung. Briefly, lungs were excised, washed in PBS, and added to gentleMACS Dissociator C tubes containing 5 mL of HEPES buffer, 2 mg/mL Collagenase D, and 40 U/mL DNase I (Roche, Indianapolis, IN). Lungs were briefly dissociated using the gentleMACS, incubated for 30 minutes at 37°C for tissue digestion, and then further dissociated with the gentleMACS to obtain a single-cell suspension. Red blood cells were lysed by addition of 0.17 mol/L NH4Cl (Sigma-Aldrich, St. Louis, MO), and the lung homogenate was passed through 70-μm filters (BD Biosciences, San Jose, CA) to remove debris.

Flow Cytometry

The phenotypes of isolated lung cells were determined by flow cytometry. Before staining for cell-surface markers, cells were incubated with CD16/32 in 1% bovine serum albumin–PBS for 10 minutes at 4°C to limit nonspecific binding. Cells were then stained for 15 minutes at 4°C with combinations of the following antibodies: CD45-Pacific Blue or Alexa 700, Ly6G-APC-Cy7, CD11b-Percp-Cy5.5, or APC-Cy7, CD11c-Pe-Cy7, MHCII-Pe, Ly6C-FITC, F4/80-Alexa 647, CD19-Pe-Cy7, B220-Percp-Cy5.5, IgD-Alexa 647, IgM-FITC, CD5-PE, CD49b-APC, CD4-APC-Cy7, CD8-Pacific Blue, CD3-Alexa 647. Antibodies were purchased from BD Biosciences (Franklin Lakes, NJ) with the exceptions of F4/80-Alexa 647 and CD11b-Percp-Cy5.5 (eBioscience, San Diego, CA), and CD45-Pacific Blue (Biolegend, San Diego, CA). Appropriate fluorescence minus one and isotype controls were also included. Data were collected on an LSRII (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR).

Calculation of the Number of CD45$^+$ Lung Leukocytes

Isolated mammalian lung cells and extracellular yeast were counted using a Scepter handheld automated cell counter (Millipore, Billerica, MA). Cells ranging from 6 to 24 μm in size were included in the cell count, with >90% of the cells being between 6 and 14 μm. To arrive at the number of CD45$^+$ lung leukocytes, the relative percentage of live lung cells as determined by forward and side scatter and CD45$^+$ double gating was multiplied by the automated Scepter cell count, therefore excluding yeast and CD45$^-$ mammalian leukocytes.

Intracellular Staining for IL-17a

A Lympholyte M (Cedarlane, Ontario, Canada) gradient was used for enrichment of CD45$^+$ leukocytes from lung cell suspensions. Isolated leukocytes were plated in 96-well plates at a concentration of 1×10^6 lung cells/200 μL of RPMI-10% bovine serum albumin. The lung cells were stimulated for 5 hours at 37°C with 50 ng/mL PMA, 1 μg/mL ionomycin (Sigma-Aldrich), and 1 μL of GolgiPlug (BD Biosciences). Leukocytes were washed and stained with LIVE/DEAD Fixable Violet Dead Cell stain (Invitrogen, Carlsbad, CA) for live-cell gating followed by staining with surface antibodies as described above. Leukocytes were then permeabilized in Cytofix/Cytoperm, and stained in Permwash buffer with anti–IL-17a-PE (BD Biosciences) at a 1:100 dilution for 30 minutes at 4°C.

Determination of Serum Antibody Concentrations

Concentrations of serum antibodies were determined by enzyme-linked immunosorbent assay as described previously.[55] EIA/RIA 96-well plates (Costar, Corning, NY) were coated with 10 μg/mL of goat anti-mouse IgM or IgG (SouthernBiotech, Birmingham, AL) or 2 μg/mL rat anti-mouse IgE (BD Biosciences) for 1 hour at 37°C. Plates were then blocked overnight with 1% bovine serum albumin–PBS (Sigma-Aldrich, St. Louis, MO) and washed with PBS-Tween 20 using an Aquamax 2000 plate washer (Molecular Devices, Bethesda, MD) before use. Serum was added at a dilution of 1:5 for IgE, 1:15 for total IgM, and 1:1000 for total IgG and IgG isotypes, and serially diluted 1:3 with 1% bovine serum albumin–PBS. Mouse IgM and IgG (SouthernBiotech) and IgE (BD Biosciences) standards were applied at a starting concentration of 10 μg/mL for IgM and IgG and 1 μg/mL for IgE. Samples and standards were incubated for 1 hour at 37°C, plates were washed, and then antibody detected with a 1:2500 dilution of goat anti-mouse IgM, IgG, IgG1, IgG2b, IgG2c, or IgG3 or 1:1000 dilution of goat anti-mouse IgE for 1 hour at 37°C. Plates were subsequently developed with 1 mg/mL p-nitrophenyl phosphate (Sigma-Aldrich) dissolved in bicarbonate buffer (pH 9.8). A titration curve was generated by curve-fit analysis using GraphPad Prism 5 software (San Diego, CA). The titer for IgG subtypes was defined as the point at which the titration curve crossed an optical density of 0.1 after subtraction of the background.

Determination of GXM-Specific IgM and IgG

Plates were coated with 10 μg/mL *C. neoformans* 24067 GXM for 3 hours at room temperature and processed as described.[55] Briefly, serum samples were added at starting dilution of 1:5 and serially diluted. GXM-specific antibody was detected by addition of goat anti-mouse IgM or IgG, plates were developed, and the titer determined as described above.

Histology

At 7, 42, and 123 days after infection, groups of three to four mice were anesthetized with isoflurane, sacrificed by cervical dislocation, and lungs and brains removed. Tissues were fixed in 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA). Following 48 to 72 hours of fixation, samples were sent to the Histopathology Core of AECOM for routine processing into paraffin blocks. Five-micrometer lung and brain tissue sections were routinely stained with H&E and examined under a Zeiss AxioScope II microscope (Carl Zeiss, Thornwood, NY) by a board-certified veterinary pathologist (R.S.S.).

Statistical Analysis

Mouse survival data were evaluated by comparing Kaplan-Meier survival curves with a log-rank (Mantel-Cox) test. For experiments in which only WT and *IL-23p19*$^{-/-}$ mice were compared, a Student's *t*-test was applied. For multiple comparisons between groups of WT, *IL-23p19*$^{-/-}$, and *IL-17RA*$^{-/-}$ mice, data that exhibited equal variance and equal normality were analyzed for significance by one-way analysis of variance followed by Holm-Sidak post-test. For data that did not exhibit equal variance and normality, a Kruskal-Wallis rank-based one-
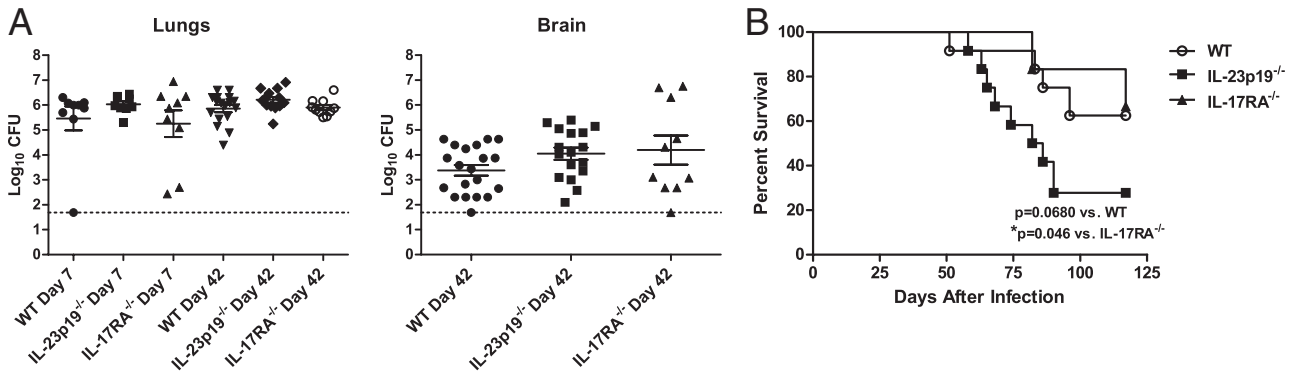
Figure 1. IL-23 and IL-17RA do not significantly contribute to reduction in *C. neoformans* CFU, but survival of *IL-23p19$^{-/-}$* mice is decreased. Fungal burden in lungs and brains (as labeled) was determined at the indicated time points after infection (**A**), ($n = 9$ to 20). Error bars represent the SEM from two to four independent experiments. Survival of *IL-23p19$^{-/-}$* and C57BL/6 WT controls (**B**). Results are from two independent experiments, $n = 6$ (WT and *IL-23p19$^{-/-}$*), $n = 6$ (WT, *IL-23p19$^{-/-}$*, and *IL-17RA$^{-/-}$*).

way analysis of variance was performed followed by Dunn's post-test. A *P* value of <0.05 was considered significant. All statistical tests were performed using the advisory statistical software, SigmaStat 3.0 (SyStat, San Jose, CA).

Results

Tissue Fungal Burden in IL-23p19$^{-/-}$ and IL-17RA$^{-/-}$ Mice

Previous studies have demonstrated impaired fungal clearance and reduced survival of *IL-23p19$^{-/-}$* mice infected intraperitoneally or intravenously with *C. neoformans*, which was associated with reduced production of IL-17.[25] We sought to expand on these studies by determining whether IL-23 or IL-17 also contributes to control of pulmonary burden and/or yeast dissemination to the brain in an intranasal infection model of chronic cryptococcosis.[56] *IL-23p19$^{-/-}$*, *IL-17RA$^{-/-}$*, and C57BL/6 WT mice were infected intranasally with 5×10^5 CFU of *C. neoformans* strain 52D, and the fungal burden in lungs, brain, and blood evaluated at days 7 and 42 after infection. Fungal burden in lungs of *IL-23p19$^{-/-}$* or *IL-17RA$^{-/-}$* mice were similar ($P > 0.05$) to WT at both time points examined (Figure 1A). No yeast was cultured (<50 CFU/mL) from the brain at day 7 or in blood at day 7 or day 42 from any WT, *IL-23p19$^{-/-}$*, or *IL-17RA$^{-/-}$* mice ($n = 9$ to 15). At day 42 after infection, the mean brain fungal burden was statistically comparable ($P > 0.05$) in all groups of mice (Figure 1A).

We evaluated the survival of *IL-23p19$^{-/-}$*, *IL-17RA$^{-/-}$*, and WT mice. Although C57BL/6 mice can initially contain infection caused by strain 52D, the T_H2-dominated allergic response to the fungus promotes yeast dissemination and death.[12,51] Survival 13 weeks post-infection was only 23% in *IL-23p19$^{-/-}$* mice, whereas 83% of *IL-17RA$^{-/-}$* and 75% of WT mice were alive at week 13 (Figure 1B). The decreased survival of *IL-23p19$^{-/-}$* mice was significant ($P < 0.05$) compared to *IL-17RA$^{-/-}$* mice, and there was a trend toward significance ($P < 0.1$) compared to WT (Figure 1B).

Measurement of Lung Cytokines

IL-17 is produced in response to IL-23,[26,50,57] as well as other stimuli.[30,34,41,58,59] We asked whether IL-23 was required for IL-17 production during *C. neoformans* infection. IL-17 levels were increased in both WT ($P < 0.001$) and *IL-23p19$^{-/-}$* ($P < 0.01$) lungs at day 7 after infection compared to naive WT and *IL-23p19$^{-/-}$* mice, respectively, but IL-17 was lower ($P < 0.01$) in *IL-23p19$^{-/-}$* and *IL-17RA$^{-/-}$* lungs at day 7 compared to WT (Figure 2). At day 42, there was no difference ($P > 0.05$) in IL-17 levels between WT and *IL-23p19$^{-/-}$* lungs (Figure 2). We also examined other cytokines that can be regulated by IL-23/IL-17 and/or influence cryptococcal containment,[13,16,28,35,51,56,60] but we did not find differences ($P > 0.05$) in levels of IL-4, IL-5, IL-13, IL-12, IFN-γ, IL-10, TGF-β, or IL-6 (Figure 2).

To identify leukocytes that were producing IL-17 in WT and *IL-23p19$^{-/-}$* mice, we performed intracellular staining for IL-17a followed by flow cytometry. We found that >95% of the IL-17a–producing leukocytes in the lungs of WT and *IL-23p19$^{-/-}$* mice were CD3$^+$ (Figure 3, A and B), whereas no CD11b$^+$ cells were IL-17a$^+$ (Figure 3A). Further analysis revealed that the IL-17a$^+$ cells were primarily composed of CD3$^+$CD4$^+$ T cells and CD3$^+$CD49b$^+$ NKT cells (Figure 3, A and B). The percentage of CD45$^+$ leukocytes producing IL-17a was similar ($P > 0.05$) between WT and *IL-23p19$^{-/-}$* mice (Figure 3C), as well as the absolute number (mean $\times 10^4 \pm$ SEM, $n = 5$) of IL-17a$^+$ leukocytes (WT: 3.00 ± 0.22, *IL-23p19$^{-/-}$*: 3.28 ± 0.22), but the median fluorescent intensity of the IL-17a$^+$ cells was decreased ($P < 0.01$) in *IL-23p19$^{-/-}$* compared to WT (Figure 3D).

Cellular Inflammation in Lungs

IL-23–mediated IL-17 production enhances neutrophil and monocyte recruitment to sites of inflammation,[33,37,61,62] therefore, we assessed lung leukocyte phenotype in *IL-23p19$^{-/-}$*, *IL-17RA$^{-/-}$*, and WT mice before and after 7 and 42 days of infection by flow cytometry. The number of CD45$^+$ lung leukocytes was similar
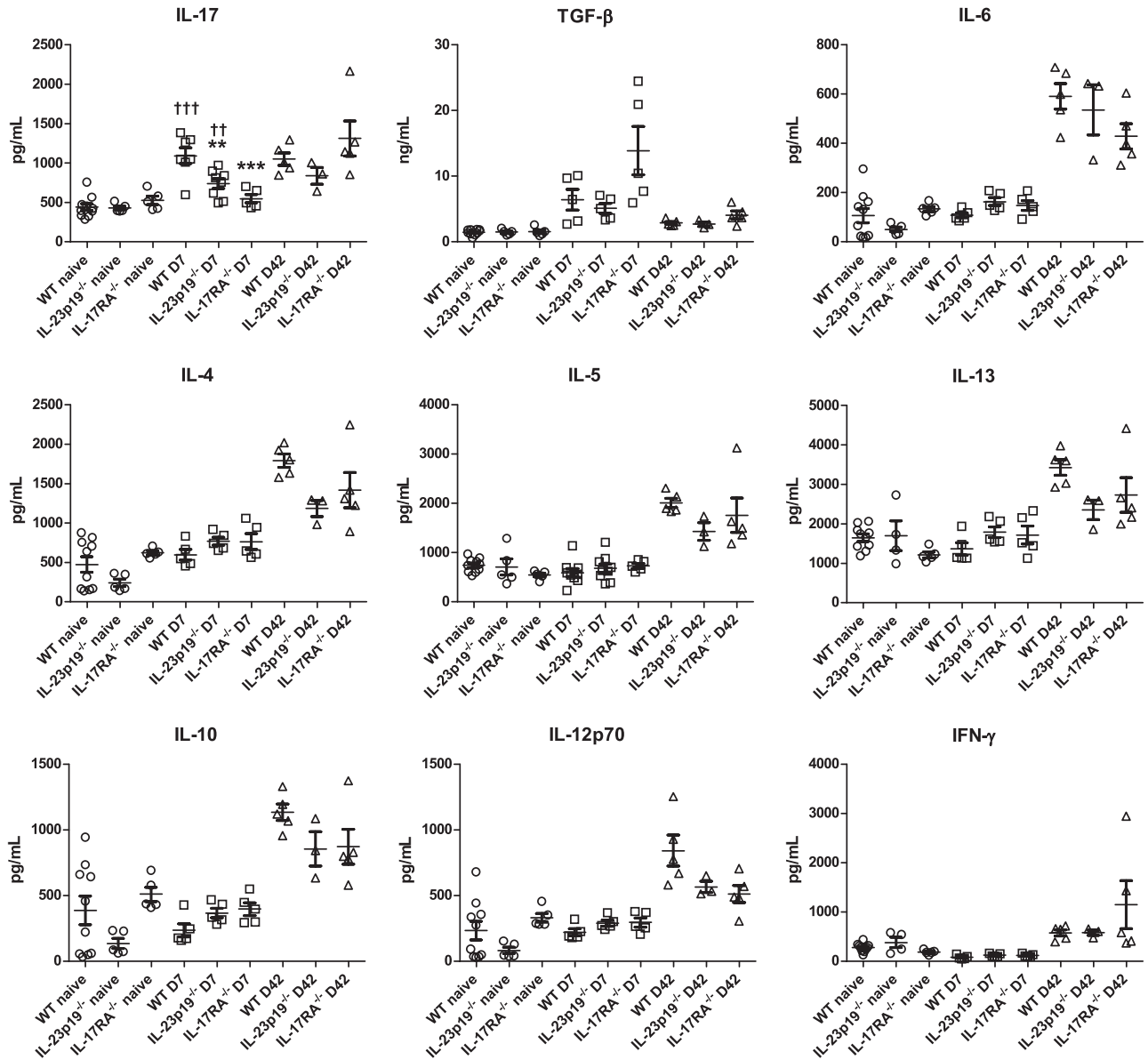
Figure 2. The absence of IL-23 or IL-17RA results in decreased IL-17 levels in lungs early after infection but has no influence on production of other cytokines. Lung homogenates were used to quantitate cytokine production in naive mice or 7 days (D7) and 42 days (D42) after infection ($n = 3$ to 8). $**P < 0.001$, $***P < 0.001$ versus WT D7; $\dagger\dagger P < 0.01$ versus *IL-23p19$^{-/-}$* naive; $\dagger\dagger\dagger P < 0.001$ versus WT naive mice. Error bars represent the SEM of one to two experiments.

($P > 0.05$) between all strains at all time points examined (Table 1). However, there were differences in the proportions of leukocyte populations. Naive *IL-17RA$^{-/-}$* and *IL-23p19$^{-/-}$* mice exhibited an increase ($P < 0.001$, $P < 0.01$, respectively) in CD3$^+$CD8$^+$ T cells in lungs compared to WT (Figure 4). There were also more ($P < 0.001$) CD3$^+$CD49b$^+$ NKT cells in naive *IL-17RA$^{-/-}$* lungs compared to WT (Figure 4). At day 7 after infection, SiglecF$^+$CD11c$^-$SSC^{hi} eosinophils were increased ($P < 0.001$) in lungs of *IL-23p19$^{-/-}$* mice compared to WT, whereas *IL-17RA$^{-/-}$* lungs contained more ($P < 0.001$) CD3$^+$CD4$^+$ T cells and NKT cells (Figure 4). At day 42, eosinophils remained increased ($P < 0.001$) in *IL-23p19$^{-/-}$* lungs, and CD11b$^+$CD11c$^-$F4/80$^+$ macrophages were decreased ($P < 0.001$) compared to WT. Ly6G^{hi}CD11b$^+$ neutrophils were diminished in both *IL-*

23p19$^{-/-}$ ($P < 0.05$) and *IL-17RA$^{-/-}$* lungs ($P < 0.01$) compared to WT at day 42 (Figure 4). There was no difference ($P > 0.05$) in numbers of CD11c^{hi}CD11b^{lo} alveolar macrophages, CD11c$^+$CD11b$^+$ dendritic cells, CD11c$^-$CD11b$^+$Ly6C^{hi} monocytes, CD49b$^+$CD3$^-$ NK cells, or CD19$^+$B220^{hi} B cells between the strains at any time point (data not shown).

Antibody Response

Lung eosinophil influx is enhanced by IgE,[63] and both eosinophil recruitment and IgE production are associated with cryptococcal disease susceptibility.[13,64] We found that serum IgE was significantly increased ($P < 0.05$) in naive *IL-17RA$^{-/-}$* mice (Figure 5A) and at day 7 in *IL-23p19$^{-/-}$* and *IL-17RA$^{-/-}$* mice compared to WT, but at
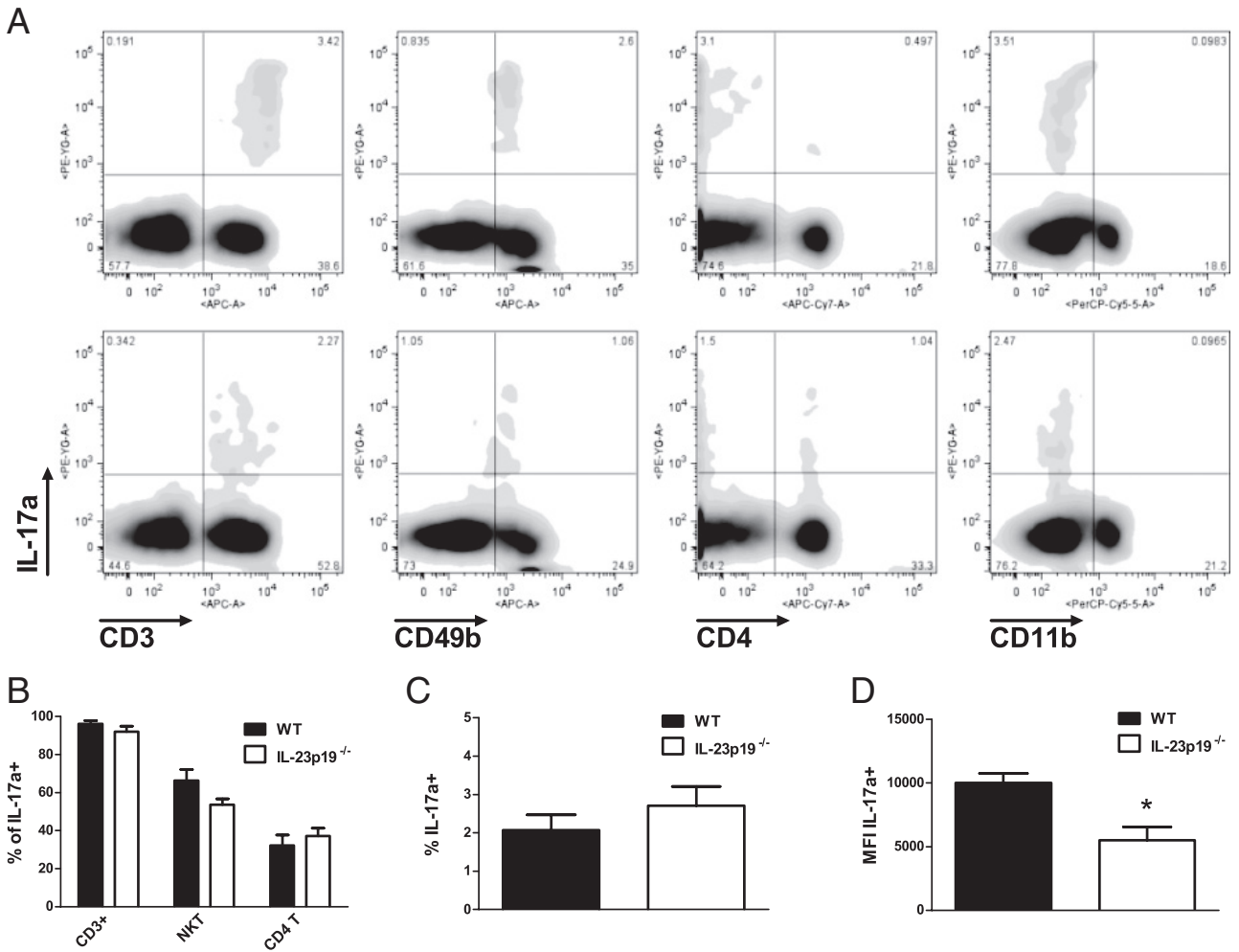
Figure 3. CD4T and NKT cells in lungs of *IL-23p19$^{-/-}$* mice produce decreased amounts of IL-17 compared to WT mice. At day 7 post-infection, lung leukocytes were isolated from WT (top panels) and *IL-23p19$^{-/-}$* (bottom panels) mice, and intracellular production of IL-17a was assessed by flow cytometry (**A**). Graphs represent the percentage of CD45^{+}IL-17a^{+} cells that were CD3^{+}, CD3^{+}CD4^{+} T cells, or CD3^{+}CD49b^{+} NKT cells (**B**); percentage of CD45^{+} lung leukocytes producing IL-17a (**C**); and median fluorescent intensity (MFI) of CD3^{+}IL-17a^{+} cells (**D**), (n = 5). *P < 0.05 versus WT.

day 42, there was no difference (P > 0.05) between the strains (P > 0.05) (Figure 5).

In contrast to IgE, secretion of IgM and antigen-specific IgG can contribute to resistance to cryptococcosis.[19–21,65] Total serum IgM concentration did not differ (P > 0.05) between *IL-23p19$^{-/-}$*, *IL-17RA$^{-/-}$*, and WT mice, but total serum IgG was significantly elevated in naive *IL-17RA$^{-/-}$* and in both *IL-23p19$^{-/-}$* and *IL-17RA$^{-/-}$* mice compared to WT at day 7 and day 42 after infection (Figure 5A). We determined the titer of specific IgG isotypes and found that serum titers of IgG2b were increased (P < 0.001) in *IL-23p19$^{-/-}$* and *IL-17RA$^{-/-}$* mice at day 7 compared to WT, whereas differences in

IgG1, IgG2c, and IgG3 were not significant (P > 0.05), (Figure 5B). Despite the increase in total serum IgG in both *IL-23p19$^{-/-}$* and *IL-17RA$^{-/-}$* mice at day 7, titers of anti-cryptococcal GXM were only significantly elevated (P < 0.01) in *IL-17RA$^{-/-}$* mice (Figure 5C).

Histopathology of Lungs and Brains

Eosinophil recruitment to the lungs of C57BL/6 mice infected with *C. neoformans* results in deposition of Charcot-Leyden–like crystals, resulting in tissue damage.[51] These crystals are composed of the chitinases YM1 and YM2 that are produced by alternatively activated macrophages; therefore, it is hypothesized that eosinophils induce crystal formation by influencing macrophage activation.[56,66,67] Lung sections from *C. neoformans*–infected mice were examined by H&E staining to determine whether the increased eosinophilia in *IL-23p19$^{-/-}$* mice was associated with amplified lung pathology. On day 7 after infection, there was no significant difference in lung pathology between *IL-23p19$^{-/-}$*, *IL-17RA$^{-/-}$*, and WT

Table 1. Lung CD45^{+} Leukocytes

	Day 0	Day 7	Day 42
WT	1.53 ± 0.05*	3.09 ± 0.06	5.46 ± 0.12
IL-23p19$^{-/-}$	1.61 ± 0.40	3.04 ± 0.10	5.62 ± 0.20
IL-17RA$^{-/-}$	1.41 ± 0.35	3.93 ± 0.25	5.85 ± 0.51

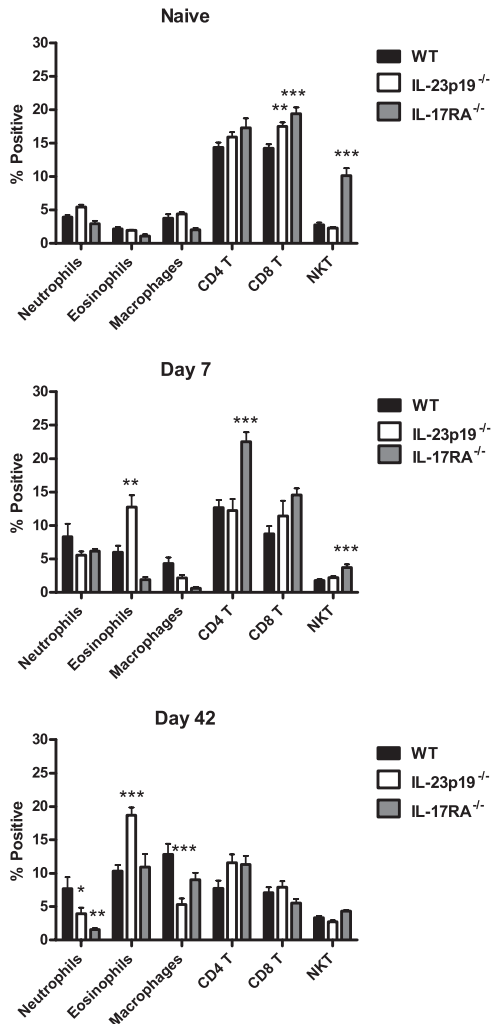*Mean number of CD45^{+} leukocytes ± SEM × 10^5, (n = 4 to 9).

Figure 4. Eosinophils are increased in lungs of infected *IL-23p19*⁻/⁻ mice, whereas CD4 T and NKT cells are increased in lungs of infected *IL-17RA*⁻/⁻ mice. Lung cells were analyzed by flow cytometry to determine the relative percentages of the indicated leukocyte populations after gating on CD45⁺ cells in WT, *IL-23p19*⁻/⁻, and *IL-17RA*⁻/⁻ lungs before and during infection (*n* = 4 to 9). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$versus WT. Data represent the mean ± SEM of one to two experiments.

mice (data not shown). However, at day 42, there were increased amounts of acidophilic YM1/YM2 crystals in lungs of both *IL-23p19*⁻/⁻ and *IL-17RA*⁻/⁻ mice (Figure 6, Table 2). The inflammation was primarily eosinophilic and neutrophilic except in the WT mice, which had notably more neutrophilic inflammation. In the WT mice, the neutrophils were generally associated with focal tissue destruction (Table 2). We also examined brain sections at day 42 after infection, but there were no notable differences in cryptococcal burden or inflammatory infiltrate (Table 2).

After 17 weeks of infection (day 123), four of five surviving WT mice displayed little granulomatous inflammation in lungs (Figure 7, A–D, Table 3), and one WT mouse exhibited minimal to mild acidophilic macrophage pneumonia with YM1/YM2 crystals (Table 3). Cryptococci were not apparent in lungs of any WT mice (Table 3). By contrast, all *IL-17RA*⁻/⁻ mice (*n* = 4), exhibited acidophilic macrophage pneumonia in lungs, ranging from

mild to severe, with cryptococci. Cryptococcal foci contained mild numbers of eosinophils and aggregates of fragmented neutrophils that were associated with necrosis, presumably resulting from tissue damage from pointed YM1/YM2 crystals (Figure 7, A–D, Table 3).

The brains of WT mice at week 17 had focal areas of chronic inflammation with no typical cryptococcal organisms (Table 3). Two of four *IL-17RA*⁻/⁻ mice had similar inflammatory infiltrates, with one containing aggregates of viable-appearing organisms. Cryptococci were cultured from the blood of *IL-17RA*⁻/⁻ mice at week 17, but were below the detection limit (<50 CFU/mL) in WT (Figure 7E).

Discussion

Our findings reveal IL-17–independent and -dependent roles of IL-23 in limiting the allergic response during chronic pulmonary cryptococcal disease. Although IL-23 was not required for induction of IL-17, it enhanced IL-17 production by NKT and CD4T cells during early cryptococcal infection. Both IL-23 and IL-17 suppressed serum IgE, IgG2b, and formation of pulmonary YM1/YM2 crystals, suggesting that IL-23–dependent production of IL-17 contributes to control of cryptococcal-induced allergy. However, an IL-17–independent function of IL-23 was also evident, since *IL-23p19*⁻/⁻ mice had increased numbers of eosinophils in the lung at all time points after cryptococcal infection. This was in sharp contrast to *IL-17RA*⁻/⁻ and WT mice, which did not exhibit these findings. Based on these data, pulmonary eosinophilia might promote mortality, because IL-23p19 null mice had higher mortality than IL-17 mutant mice, despite having a similar cryptococcal burden in lung and brains.

Cryptococcal infection in C57BL/6 mice results in a T_H2-dominated response that is accompanied by allergic markers that are similar to those observed in humans with allergic bronchopulmonary mycosis.[68,69] Although our data indicates that IL-23 dampens the allergic bronchopulmonary mycosis phenotype of *C. neoformans*–infected mice, IL-23 enhanced allergic lung inflammation and eosinophilia in experimental models of ovalbumin-induced asthma in C57BL/6 × 129 and BALB/c mice.[70,71] The contrast between the roles of IL-23 in cryptococcal versus ovalbumin-induced allergy suggests that cryptococcal antigens drive an allergic response that is unique from that of ovalbumin-induced allergy and in which IL-23 is immunoregulatory. In support of this hypothesis, recent work has demonstrated that the mechanisms of eosinophil recruitment induced by fungal chitin and ovalbumin are distinct.[72] Furthermore, an immunoregulatory role for IL-23 and IL-17a has been indicated in chronic asthma induced by *Aspergillus fumigatus* or house dust mite antigen. Lung inflammation is exacerbated in asthmatic TLR6 null mice as a result of decreased IL-23 production, and IL-17a inhibits goblet cell metaplasia in asthmatic C57BL/6 mice.[73] Taken together, these studies indicate that the role of IL-23 and IL-17 signaling in the lung is
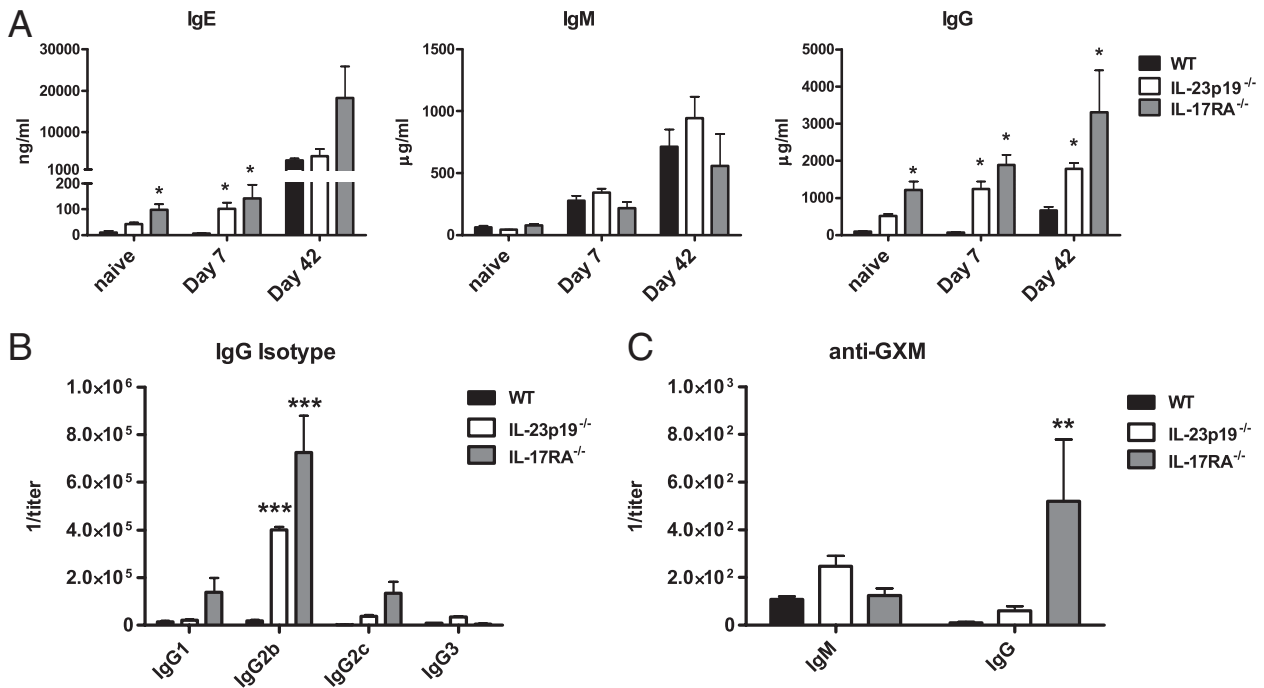
Figure 5. IgG and IgE is elevated in serum of *IL-23p19*^{−/−} and *IL-17RA*^{−/−} mice. Concentration of serum IgE, IgM, and IgG at the indicated time points (**A**), titers of serum IgG isotypes 7 days after infection (**B**), and titers of serum cryptococcal GXM-specific IgM and IgG 7 days after infection (**C**), (*n* = 5 to 10). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus WT. Data represent the mean ± SEM from one to two experiments.

complex, and likely dependent on many immune factors that are modulated by exogenous antigens.

Eosinophils are held to be deleterious in the mouse model of cryptococcosis. Early studies demonstrated

Figure 6. *IL-23p19*^{−/−} and *IL-17RA*^{−/−} mice exhibit increased YM1/YM2 crystal formation in lungs during chronic infection compared to WT. Representative H&E-stained lung sections from WT (**A** and **B**), *IL-23p19*^{−/−} (**C** and **D**), and *IL-17RA*^{−/−} (**E** and **F**) 42 days after infection (*n* = 3 to 4). **Large arrowheads** designate YM1/YM2 crystals, and **small arrows** indicate eosinophils. Original magnification: ×10 (**A**, **C**, and **E**); ×40 (**B**, **D**, and **F**).

that IL-5–dependent eosinophil recruitment results in crystal formation in macrophages and lung damage.[51] More recent work, using mice that lack eosinophils, confirm these findings and demonstrate decreased fungal burden in the absence of eosinophils.[74] These findings contrast with those in rat models of cryptococcosis, in which primary infection can resolve and eosinophils contribute to a protective T_H1 response.[75,76] Our data support a destructive role for eosinophils, possibly by promoting tissue damage and subsequent mortality of *IL-23p19*^{−/−} mice. Eosinophilic granule proteins are toxic to bystander cells, resulting in lung damage,[77] and contribute to airway constriction and impaired respiration.[78] Additional deleterious functions of eosinophils were demonstrated in an *Aspergillus*-induced allergic asthma model, in which mice lacking eosinophils or CCR3, an eosinophil chemokine receptor, displayed reduced mucous production and decreased transcription of genes involved in coagulation.[79]

Similar to our data, survival of *IL-23p19*^{−/−} mice infected intraperitoneally or intravenously with *C. neoformans* strain 1841D is decreased.[25] In the foregoing systemic infection model, the absence of IL-23 resulted in increased hepatic fungal burden and decreased infiltration of inflammatory cells into the brain, but not increased brain fungal burden.[25] We could not attribute the increased mortality of *IL-23p19*^{−/−} mice infected intranasally with *C. neoformans*-52D to increased brain or lung fungal burden or differences in brain inflammation. However, we did find that their earlier time to death was associated with a higher number of lung eosinophils. One explanation for the increased mortality of *IL-23p19*^{−/−} mice in our model is that in combination, lung eosinophils
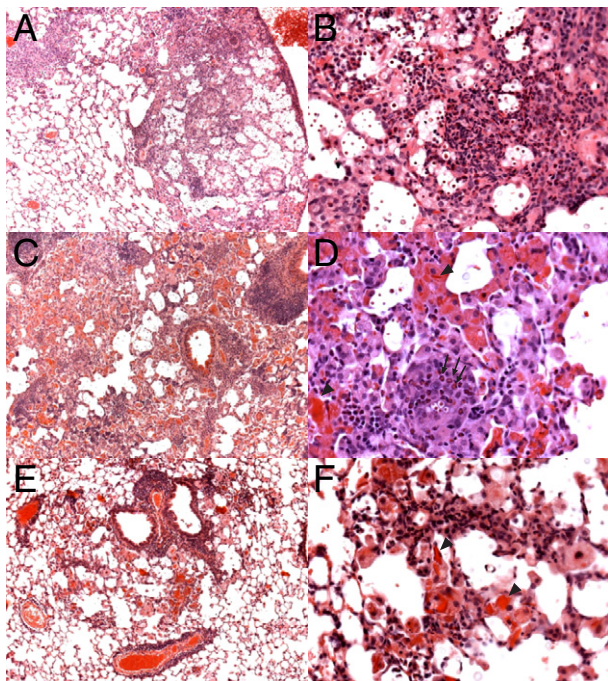
Table 2.   Histopathology of Lungs and Brains 42 Days after Infection

	WT				IL-23p19−/−			IL-17RA−/−			
Brain											
Mouse number	1	2	3	4	1	2	3	1	2	3	4
Cryptococcal nodules*	3	0	1–2	1–2	1	1	2	1	1	1	2
With pyogranulomatous inflammation*	2	0	0	0	0	0	0	0	0	0	2
Infiltrate, perivascular, chronic*	1	0	0	1	0	0	1	1	1	0	0
Lung											
Mouse number	1	2	3	4	1	2	3	1	2	3	4
Granulomatous pneumonia† (# lobes/5)											
Minimal	0	0	0	0	0	0	0	0	0	1	1
Mild	0	0	0	1	0	0	0	2	2	4	4
Moderate	1	3	1	3	3	1	1	2	2	0	0
Marked	1	2	4	1	2	2	3	1	1	0	0
Severe	3	0	0	0	0	2	1	0	0	0	0
Acidophilic crystalline material‡	1	2	1	2	3	4	4	4	4	4	2
Neutrophils in inflammation‡	3	3	3	2	1	1	1	1	1	1	1

*Severity modifiers: 0 = no lesion, 1 = minimal lesion, 2 = mild lesion, 3 = moderate lesion, 4 = marked lesion, 5 = severe lesion.
†Granulomatous pneumonia refers to dense pyogranulomatous and eosinophilic inflammation.
‡Pneumonia severity modifiers: 1 = 1% to 10%, 2 = 10% to 25%, 3 = 25% to 45%, 4 = 45% to 75%, 5 = >75%.

and YM1/YM2 crystals could impair respiration, whereas crystals without increased eosinophils do not impact pulmonary physiology in *IL-17RA*−/− mice. Although alveolar accumulation of needle-shaped YM1/YM2 crystals can induce mechanical damage, lung consolidation, insuffi-

Figure 7. Surviving *IL-17RA*−/− mice display acidophilic macrophage pneumonia and fungal dissemination to the blood after 17 weeks of infection. Representative H&E-stained lung sections from WT (**A** and **B**) and *IL-17RA*−/− mice (**C** and **D**), (n = 4 to 5). Fungal burden (Log₁₀ CFU/mL) in blood of WT and *IL-17RA*−/− mice (**E**), (n = 4 to 5). Original magnification: ×10 (**A** and **C**); ×40 (**B** and **D**). KO, knockout.

cient respiration, and fatality without eosinophilia,[66,80,81] comparable crystal formation in the lungs of *IL-23p19*−/− and *IL-17RA*−/− mice suggests that additional factors, such as lung-damaging eosinophils, could have contributed to respiratory demise and mortality in infected-*IL-23p19*−/− mice.

Eosinophil recruitment to lungs can be mediated by CD4 T-cell production of T_H2 cytokines.[23,51,82] Although the increase in lung eosinophils in *IL-23p19*−/− mice was not accompanied by an increase in lung levels of IL-5, IL-4, or IL-13, it is possible that non–T-cell sources masked a decrease in CD4 T-cell production of one or more of these cytokines in whole-lung homogenates. At present, it is not known whether the cellular source and/or the location of T_H2 cytokine production are important factors in eosinophil emigration into the lung during cryptococcal infection. Given that ligation of Ox40, a T-cell–activating receptor, on CD4 T cells limits eosinophil recruitment in an IFN-γ–dependent manner during cryptococcal infection, signaling via OX40 could be decreased in *IL-23p19*−/− mice.[83] Alternatively, eosinophil generation and release from bone marrow or migration from the circulation into the lung could be mediated by other factors. However, we found that transcription of eotaxin, an eosinophil chemoattractant that can promote eosinophil recruitment,[84] was not increased in lungs of *IL-23p19*−/− mice at day 7 or 21 after infection (data not shown). Furthermore, we note that the increase in eosinophils in lungs of *IL-23p19*−/− mice could reflect a defect in eosinophil clearance, rather than an increase in eosinophil recruitment. Though not specific to eosinophils, IL-23 was recently shown to inhibit cellular infiltration into the lungs in an *A. fumigatus* allergy model,[73] but the mechanism was not examined. Therefore, more work is needed to determine how IL-23 directly or indirectly inhibits eosinophil recruitment in fungal infections.

IgE secretion during cryptococcal infection is associated with reduced lung function.[64] It appears that IL-23 and IL-17RA restrain IgE production, since IgE was ele-
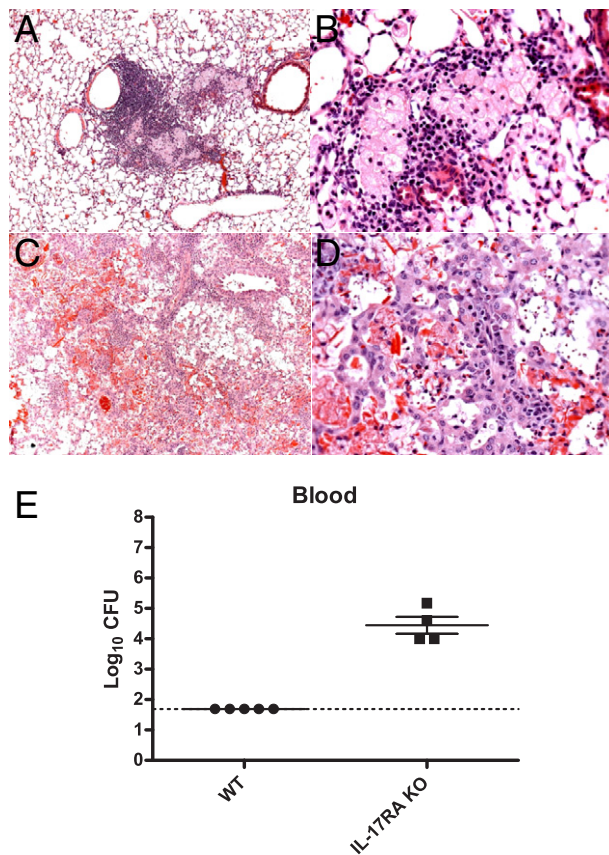
Table 3. Histopathology of Lungs and Brains of Surviving WT and *IL-17RA*$^{-/-}$ Mice 123 Days after Infection

	WT					IL-17RA$^{-/-}$			
Brain									
Mouse number	1	2	3	4	5	1	2	3	4
Cryptococcal nodules*	0	0	0	0	0	0	0	0	3
Granulomatous inflammation*	0	0	0	2	0	0	2	0	2
Infiltrate, perivascular, chronic*	0	0	0	0	0	0	1	0	2
Lung									
Mouse number	1	2	3	4	5	1	2	3	4
Granulomatous pneumonia† (# lobes/5)									
No lesions	4	0	5	0	3	4	5	4	3
Minimal	1	5	0	3	2	1	0	1	0
Mild	0	0	0	2	0	0	0	0	2
Acidophilic Macrophage pneumonia‡									
No lesions	0	1	0	0	0	2	2	4	2
Minimal	0	2	0	0	0	0	0	1	1
Mild	0	2	0	0	0	0	0	0	0
Moderate	0	0	0	0	0	0	3	0	2
Marked	0	0	0	0	0	1	0	0	0
Severe	0	0	0	0	0	2	0	0	0

*Severity modifiers: 0 = no lesion, 1 = minimal lesion, 2 = mild lesion, 3 = moderate lesion, 4 = marked lesion, 5 = severe lesion.
†Granulomatous pneumonia refers to dense pyogranulomatous and eosinophilic inflammation.
‡Acidophilic macrophage pneumonia refers to dense inflammation composed of macrophages with acidophilic YM1/YM2 crystals.

vated in *IL-23p19*$^{-/-}$ and *IL-17RA*$^{-/-}$ mice during infection. Increased IgE levels also occurred in naive *IL-17RA*$^{-/-}$ mice. To our knowledge, elevated IgE has not been reported in naive *IL-17RA*$^{-/-}$ mice. To confirm that increased IgE was not a response to parasites,[85] the cecum and colon were evaluated histologically to confirm the lack of intestinal parasites, and sentinel mice were routinely verified to be parasite free. Interestingly, a link between IL-23/IL-17 and IgE regulation is also seen in humans. Individuals with hyper-IgE syndrome exhibit a genetic mutation in STAT3 that results in impaired IL-23 and IL-17 production; however, the cause of the elevated IgE is not known. These individuals also have increased susceptibility to infections by the fungi *Candida* and *Aspergillus,* as well as to various bacteria.[86–89]

In addition to elevated IgE, the absence of IL-23 and IL-17RA resulted in increased serum IgG, primarily IgG2b. Elevated total serum IgG in naive *IL-17RA*$^{-/-}$ mice and IgG2b after *Porphyromonas gingivalis* has also been reported.[90] It is not clear from our studies whether the increase in IgG in *IL-23p19*$^{-/-}$ mice or *IL-17RA*$^{-/-}$ has any effect on immunity. Previous studies have demonstrated that GXM-specific IgG2b can have protective functions in mice infected with a lethal dose of *C. neoformans*,[91,92] but IgG2b has also been shown to be a weaker opsonin than IgG2a and IgG1,[93] and is associated with decreased survival time when administered to mice before cryptococcal infection in comparison to other antibody isotypes.[91,94,95] Another possibility is that the elevated IgG to IgM ratio in *IL-23p19*$^{-/-}$ and *IL-17RA*$^{-/-}$ mice impacts binding of IgM, which is diluted by the excess IgG. Secreted IgM promotes phagocytosis of *C. neoformans*, inflammation, and granuloma formation that is associated with disease resistance.[20]

Although the absence of IL-17RA did not impact mortality up to 17 weeks, our data suggest that IL-17 contributes to disease resistance by limiting dissemination of yeast to the blood late in infection. The presence of cryptococci in blood of *IL-17RA*$^{-/-}$ mice correlated with increased acidophilic YM1/YM2 crystal deposition in lungs, which progressed between weeks 6 and 17 after infection. A role for IL-17 late in infection is also demonstrated in a vaccination model in which *C. neoformans*-H99γ, a γ-interferon–producing H99 strain, elicits protection from subsequent infection of BALB/c mice with H99.[27,96] IL-17 is not required for survival, but vaccinated *IL-17RA*$^{-/-}$ mice exhibited dissemination to the brain after rechallenge, indicating protective functions of IL-17.[27] However, our findings contrast with this vaccination model, in which IL-17RA also contributed transiently to control of pulmonary burden.[27] We found that lung cryptococcal burden was similar between WT and *IL-17RA*$^{-/-}$ mice at day 7 and day 42 after infection, suggesting that there are compensatory pathways that can sufficiently control fungal burden during early infection of C57BL/6 mice with cryptococcal strain 52D. *IL-17RA*$^{-/-}$ lungs did contain elevated numbers of CD8T and NKT cells before infection and CD4T and NKT cells at day 7 after infection, and these cellular effectors are known to contribute to cryptococcal disease resistance.[9,97,98]

Although our studies did not address a potential deleterious role of the IL-23/IL-17 axis, it is important to note that IL-17 is also associated with cryptococcal disease susceptibility. Increased production of IL-17 by alveolar macrophages is associated with accelerated mortality in mice challenged with a highly virulent mucoid strain of *C. neoformans*[99] or in mice lacking secretory IgM.[20] Also, increasing IL-17 levels are associated with mortality in HIV patients with immune reconstitution syndrome following cryptococcal meningitis.[100] Future studies are needed to understand whether yeast virulence factors, the timing of IL-17 production, the cellular sources of IL-17, or additional host factors influence the outcome of IL-17 signaling.

The findings presented herein provide evidence that IL-23 and IL-17 may contribute to disease resistance by

limiting the allergic response to cryptococcal infection. Since IL-23 has opposing effects in non-fungal allergy models,[70,71] it will be important to further examine IL-23 signaling in specific IL-23–responsive leukocyte populations and determine how cryptococcal antigen and host factors influence IL-23 activity.

Acknowledgments

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