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FUNGAL AND PARASITIC INFECTIONS

Interleukin-8 Receptor 2 (IL-8R2)-Deficient Mice Are More Resistant to Pulmonary Coccidioidomycosis than Control Mice

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ABSTRACT The pathology of human coccidioidomycosis is granulomatous inflammation with many neutrophils surrounding ruptured spherules, but the chemotactic pathways that draw neutrophils into the infected tissues are not known. We previously showed that formalin-killed spherules (FKS) stimulate mouse macrophages to secret macrophage inflammatory protein 2 (MIP-2), which suggested that CXC ELR+ chemokines might be involved in neutrophil recruitment *in vivo*. To test that hypothesis, we intranasally infected interleukin-8R2 (IL-8R2) (*Cxcr2*)-deficient mice on a BALB/c background with *Coccidioides immitis* RS. IL-8R2-deficient mice had fewer neutrophils in infected lungs than controls, but unexpectedly the IL-8R2-deficient mice had fewer organisms in their lungs than the control mice. Infected IL-8R2-deficient mouse lungs had higher expression of genes associated with lymphocyte activation, including the Th1 and Th17-related cytokines *Ifnγ* and *Il17a* and the transcription factors *Stat1* and *Rorc*. Additionally, bronchial alveolar lavage fluid from infected IL-8R2-deficient mice contained more IL-17A and interferon-γ (IFN-γ). We postulate that neutrophils in the lung directly or indirectly interfere with the development of a protective Th1/Th17 immune response to *C. immitis* at the site of infection.

KEYWORDS *Coccidioides*, neutrophil, CXC chemokines, IL-8R2, cytokine, IFN-γ, IL-17, RNA-seq, Nramp1, PMN

Coccidioidomycosis, one of the endemic mycoses in the United States, has been increasing in incidence in both Arizona and California in recent years (1, 2). It is caused by two closely related species, *Coccidioides immitis* and *Coccidioides posadasii*, which are thermally dimorphic fungi that cause the same clinical syndromes, pathology, and immune responses in humans and are distinguishable only by DNA analysis (3, 4). There is a report of differences in how mice respond to the two species, but only one isolate of each species was studied, so one cannot tell if those differences were characteristic of the species or of the individual isolates tested (5). Both species live in alkaline desert soil as molds that produce arthroconidia (spores). When their hyphae are disturbed, arthroconidia are aerosolized and can be inhaled by a mammalian host. Under the influence of temperature and partial CO_2 pressure (pCO_2), arthroconidia transform into spherules (6).

The outcome of the resulting pneumonia in humans is very variable. Most infections are mild or even asymptomatic (7). Nevertheless, coccidioidomycosis is one of the most common etiologies of symptomatic pneumonia in Arizona (8). In a small minority of cases, there is clinically evident spread from the lung, either during the episode of pneumonia or manifesting itself weeks to months after the pneumonia has resolved (9). Risk factors for dissemination include diseases and medications that adversely affect T cells (10, 11). Mendelian gene mutations in the interleukin-12 (IL-12)/interferon-γ

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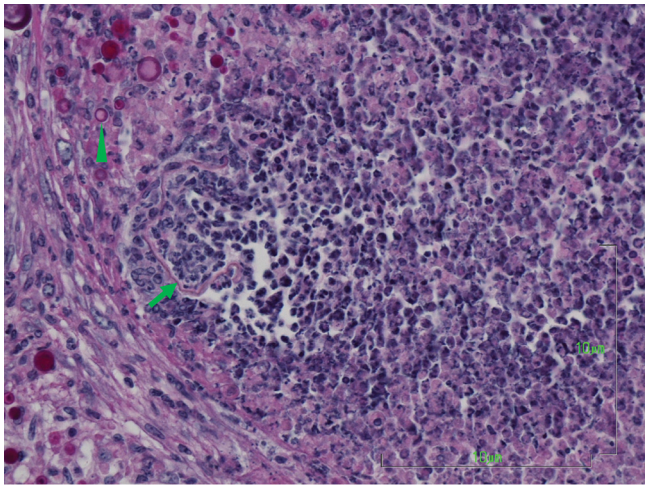

FIG 1 Periodic acid-Schiff (PAS) stain of a ruptured spherule in a mouse lung. Part of the pink-stained, fragmented spherule cell wall is indicated by the arrow. The triangle points to an endospore or a small spherule in the vicinity of the ruptured spherule. The small intact fungal cells are inside and/or surrounded by a few mononuclear cells, in contrast to the intense mixed cellular infiltrate around and inside the ruptured spherule. ×40 magnification.

(IFN-γ) axis also predispose to disseminated coccidioidomycosis, confirming the importance of IFN-γ in human immunity to these fungi and suggesting that Th1 immunity is critical in humans (12, 13). Mutations in analogous genes also increase the susceptibility of experimental mice to infection (14–16). Mouse experiments also identified Th17 responses as being important for resistance (17). However, very few people who develop disseminated coccidioidomycosis have mutations in cytokine genes or genes that are known to affect the development of Th1 or Th17 cells, but instead, dissemination appears to be associated with non-Mendelian genetic traits, especially in African-Americans and Filipinos (18). Those traits must affect the immune response, as people who develop disseminated infections make high titers of IgG antibodies but do not develop positive skin test reactions to fungal antigens, the converse of the response of patients who do not have clinically evident dissemination (19).

The pathology of coccidioidomycosis is characterized by granulomas that are infiltrated by neutrophils that congregate in and around rupturing spherules. We and others have been struck by the plethora of neutrophils in the lungs of genetically susceptible mice (20, 21) (Fig. 1). Neutrophils contribute to innate defense responses, but they can also influence adaptive immunity. Since neutrophils are prominent in the early host response to spherules (21), we wanted to understand how neutrophils enter the infected lung and how they affected the immune response coccidioidomycosis. The chemokines that attract neutrophils to rupturing spherules are unknown, but Galgiani et al. showed that spherules interact with normal human serum to generate a chemotactic signal (22). This was recently confirmed by Lee et al., who found both a complement-dependent chemoattractant and a heat-stable chemotactic activity in normal serum that promoted the attraction of neutrophils to endospores and spherules (23). We found that macrophages that are stimulated by spherules (the tissue form of *Coccidioides*) secrete the CXC chemokine macrophage inflammatory protein 2 (MIP-2) (24), which suggested that CXC chemokines could be involved in the passage of neutrophils into the infected lungs. We tested this hypothesis by infecting IL-8R2 knockout (KO) mice, also known as CXCR2 KO mice, on a BALB/c background. Mice do not make IL-8 (CXCL8), but they make MIP-2 and KC (kerotinocyte-derived chenotactic factor), two other ELR+ CXC chemokines that are ligands for CXCR2.

RESULTS

IL-8R2 KO mice have fewer white blood cells (WBC) in the infected lungs. To determine the importance of CXC chemokines in the migration of neutrophils into *C.*

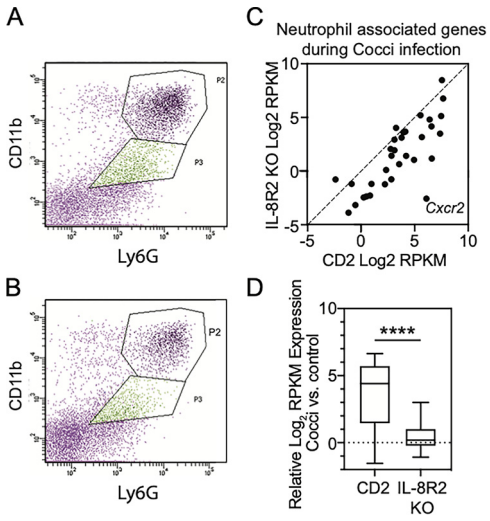

FIG 2 PMN are recruited to the lungs in an IL-8R2 (CXCR2)-dependent manner. (A and B) Percentage of Ly6G-high/CD11b-high cells in C.D2 control mice (A) and in IL-8R2 KO mice (B) as determined by flow cytometry. Cells were collected from the BAL fluid of 4 mice in each group at time of necropsy, 14 days after infection. P2 cells had the morphology of neutrophils. P3 cells were not further characterized. (C) Scatterplot depicting the expression of 31 mouse neutrophil-associated genes and *Cxcr*2 (Table 1 in reference 25; 26) in WT (C.D2) and IL-8R2 KO lungs infected with *C. immitis*. (D) Relative expression of 31 mouse neutrophil-associated genes after infection in C.D2 and IL-8R2 KO lung tissue. The difference in relative expression was analyzed using a paired *t* test. Asterisks indicate statistically significant differences (****, $P < 0.0001$).

immitis-infected lungs, we compared the number of neutrophils in the lungs of IL-8R2 KO and control (C.D2) mice. We collected cells by lung lavage (BAL) from 4 mice per group and pooled them for analysis. C.D2 mice had 3 times more total cells than the IL-8R2 KO mice and double the percentage of Ly6G/CD11b-high cells (neutrophils) in the bronchoalveolar lavage fluid (BALF), so there were approximately 6 times as many neutrophils in the C.D2 mice (Fig. 2A) as in the IL-8R2 KO mice (Fig. 2B). We also used transcriptome sequencing (RNA-seq) to compare the expression of 31 genes that are specific to neutrophils or neutrophil subsets (25, 26), and these genes were mostly expressed at higher levels in infected C.D2 mouse lungs than in infected IL-8R2 KO lungs (Fig. 2C). Additionally, their change in expression upon infection increased significantly more in the lungs of C.D2 mice than in IL-8R2 KO mice (Fig. 2D). As expected, there was virtually no expression of *Cxcr2* in the lungs of the mutant mice (Fig. 2C).

IL-8R2 KO mice are more resistant to pulmonary infection with *C. immitis*. To compare the severity of infection, we did quantitative mycology at necropsy. IL-8R2 KO mice are difficult to breed as homozygous mutants and have high WBC counts, so we treated them as if they were functionally neutropenic and kept them on oral co-trimoxazole from birth until 3 days prior to infection (PTI; see Materials and Methods). That antimicrobial combination has no activity against the fungus, but unexpectedly, the IL-8R2 KO mice had 20-fold fewer viable fungi in their lungs. However, because the drug might have affected immune response indirectly, we repeated the experiment, keeping both the IL-8R2 KO and control mice on oral co-trimoxazole from birth until 3 days PTI. Since there was no significant difference in the results of the two experiments, we combined the results, and the IL-8R2 KO mice had 10-fold fewer organisms in their lungs (Fig. 3). Despite that significant difference in geometric mean CFU in the lungs of the two experimental groups, there was no significant difference in the numbers of fungi in the spleens.

Because there is some genetic variability among BALB/c-derived strains (27), and the control (C.D2) and IL-8R2 KO mice used in this study were derived from different BALB/c lines, we compared the severity of infection in both of the parental lines to the C.D2

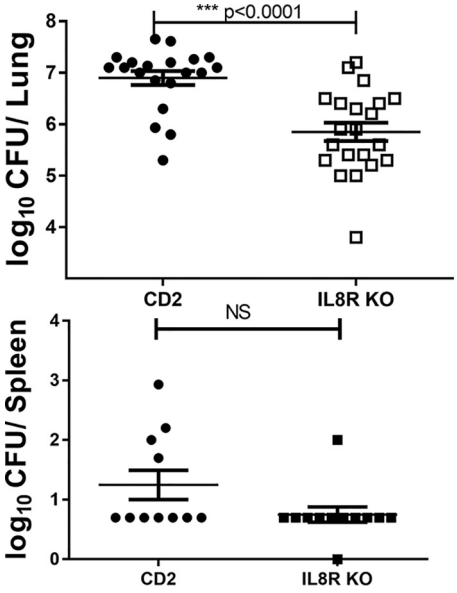

FIG 3 CFU in the lungs and spleens of IL-8R2 KO mice and BALB/c.D2 (C.D2) controls 14 days after infection. The geometric mean of CFU recovered from IL-8R2 KO mice was >20-fold lower than that in the C.D2 controls. Despite that, there was no significant difference in the number of fungi recovered from the spleens of the two strains (Student's *t* test).

mice that had been bred in our laboratory for a decade. As shown in Fig. S1 in the supplemental material, the geometric mean lung CFU from C.D2 mice was slightly lower than the mean from the other two strains, but the differences were not statistically significant. We concluded that whatever the genetic drift was between the BALB/c lines, it did not affect their susceptibility to this infection and that the C.D2 mice were a valid control for the IL-8R2 KO mice.

IL-8-R2 knockout mice upregulate Th1- and Th17-associated transcriptional programs in the lung during *C. immitis* infection. We used RNA-seq to compare gene expression in the lungs of uninfected and infected IL-8R2 KO and C.D2 mice. In the absence of infection, gene expression in the two strains of BALB/c mice was similar (Fig. 4A); only 55 genes were differentially expressed in uninfected lungs, 16 of which were expressed at higher levels and 39 of which were expressed at lower levels in C.D2 compared to IL-8R2 KO mice (Tables S1 and S2). On day 14 after *C. immitis* infection, more than 3,500 genes were differentially expressed (up- or downregulated with a fold change [FC] of >2 and a false-discovery rate [FDR] of <0.01) compared to the respective uninfected controls (Fig. 4B and C; Tables S3 to S6). There was substantial overlap in the transcriptional programs elicited in C.D2 and IL-8R2 KO lung tissue during *C. immitis* infection (Fig. 4B and C). Over 64% of the genes whose expression was significantly changed after infection (FC, >2; FDR, <0.01) were similarly regulated in C.D2 and IL-8R2 KOs lungs (Fig. 4B and C). To identify genes specifically upregulated in C.D2 or IL-8R2 KO mice during infection, we identified genes that were upregulated compared to those of uninfected controls (infection-induced genes) in both experimental groups after infection. Then we identified the subset of those induced genes that were more highly expressed in either C.D2 or IL-8R2 KO mice during *C. immitis* infection (genotype-specific upregulation) (Fig. 4D and E; Tables S7 and S8). C.D2-specific upregulated genes were enriched for gene ontology (GO) categories related to leukocyte activation, such as inflammatory response, leukocyte migration, and neutrophil degranulation (Fig. 4D and F), consistent with the higher numbers of neutrophils that were in their lungs. In contrast, IL-8R2 KO-specific upregulated genes were enriched for categories related to lymphocyte activation, including Th17 differentiation, T cell activation, and adaptive immune responses (Fig. 4E and G). Genes associated with Th17 responses, including the Th17 transcription factor (TF) *Rorc* and Th17 effector

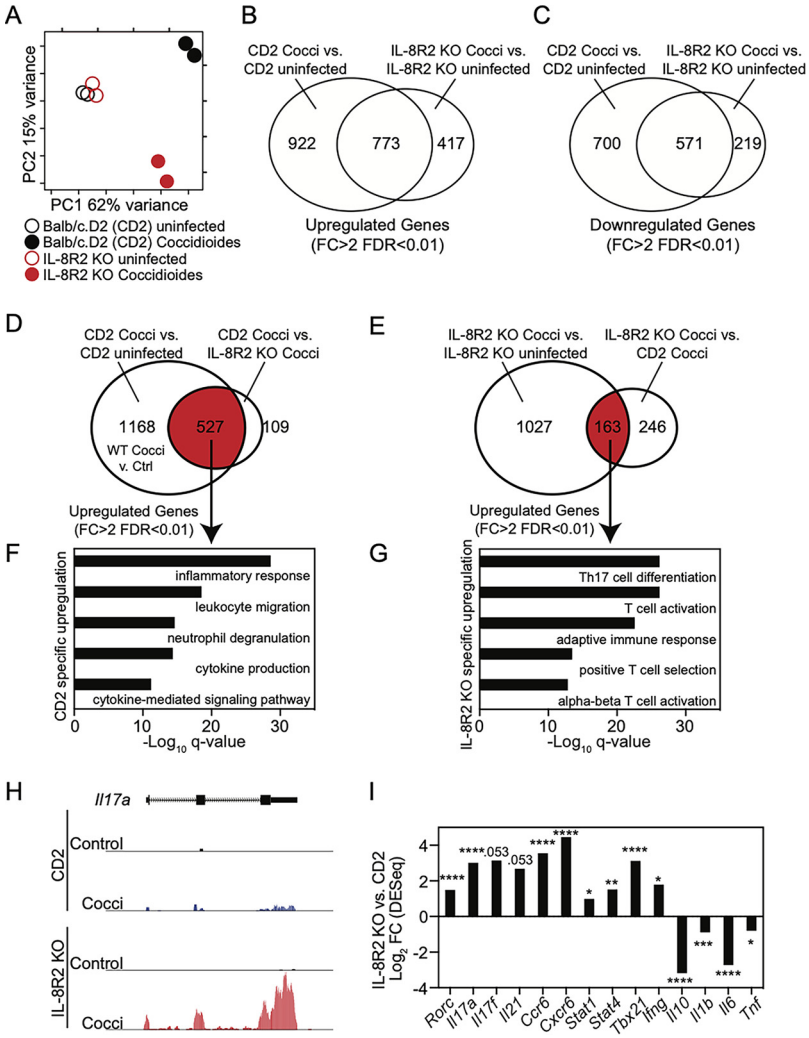

FIG 4 Gene expression analysis of whole lungs during *C. immitis* infection. (A) PCA biplot of the first two principal-component dimensions comparing RNA-seq of uninfected or *C. immitis*-infected lung tissue from WT (C.D2) or IL-8R2 KO mice (2 animals per group). (B and C) Venn diagrams showing the number of (B) upregulated or (C) downregulated genes and their overlap in WT or IL-8R2 KO mice during *C. immitis* infection compared to their uninfected controls. Genes with a fold change (FC) of >2 and FDR of <0.01 compared to uninfected mice as determined by DESeq2 are included. (D) Venn diagram of upregulated (FC, >2; FDR, <0.01) genes in lungs of WT mice during *C. immitis* infection compared to those of WT uninfected controls (Ctrl) and infected IL-8R2 KO mice. Genes upregulated compared to both groups (WT-specific) are highlighted in red. (E) Venn diagram of upregulated (FC, >2; FDR, <0.01) genes in lungs of IL-8R2 KO mice during *C. immitis* infection compared to those of IL-8R2 KO uninfected controls (Ctrl) and infected WT (C.D2) mice. Genes upregulated compared to both groups (IL-8R2 KO-specific) are highlighted in red. (F and G) The top enriched functional annotations of (panel C) WT-specific and (panel D) IL-8R2 KO-specific genes. -Log_{10} Benjamini-corrected *P* values are shown. (H) UCSC browser visualization of the *Il17a* gene locus in WT and IL-8R2 KO lungs under control and *C. immitis* infection conditions. Panels display transcription as defined by RNA-seq for WT uninfected (black), WT infected (blue), IL-8R2 KO uninfected (black), and IL-8R2 KO infected (red) mice. (I) Expression of selected individual genes in IL-8R2 KO lung tissue relative to C.D2. *P* values were determined with DESeq2. Asterisks indicate statistically significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

cytokines *Il17a*, *Il17f*, and *Il21*, and the Th17 chemokine receptors *Ccr6* and *Cxcr6* (28) were more highly expressed in the lungs of IL-8R2 KO mice. Genes that are associated with Th1 responses were also more highly expressed in the IL-8R2 KO mice, including the TFs *Stat1*, *Stat4*, and *Tbx21* and the effector cytokine IFN-γ (Fig. 4H and I). The inhibitory cytokine gene *Il10* and the proinflammatory cytokine genes *Il1b*, *Tnf*, and *Il6* were preferentially upregulated in C.D2 lungs (Fig. 4I).

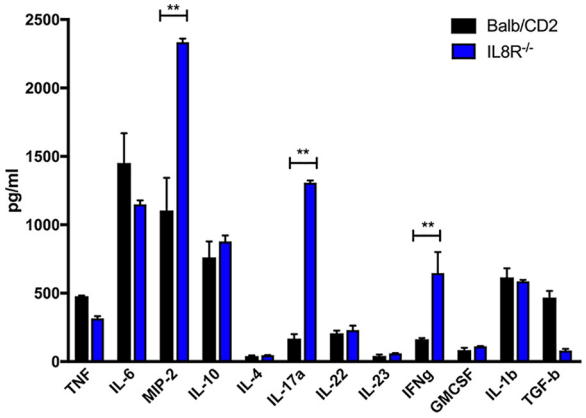

FIG 5 Cytokine concentrations in the BALF of infected lungs. Mice were sacrificed 14 days after infection. We collected and pooled BALF from four mice from each group (BALB/c.D2, C.D2 [the WT control]; IL-8R$^{-/-}$, IL-8R2 KO). Each assay was done in duplicate, and the experiment was done twice. The graph shows the means of the combined experiments. The error bars show the standard error of the mean (SEM) of those determinations. Statistical significance was determined using the discovery-determined two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli, with Q = 1%. Each row was analyzed individually, without assuming a consistent standard deviation (SD) (GraphPad Prism 7.04). **, $P \leq 0.01$. The difference in transforming growth factor β (TGFβ) was almost significant ($P = 0.06$).

To further assess the cytokine responses in infected lungs, we measured the amounts of selected cytokines in BALF (Fig. 5). We focused on cytokines that have been shown to be relevant to immunity to coccidioidomycosis and other pathogenic dimorphic fungi. The BALF from IL-8R2 KO mice contained significantly higher concentrations of IL-17A and IFN-γ, consistent with the RNA-seq results. We did not detect IL-12p70 in either group. The levels of IL-1β, tumor necrosis factor alpha (TNF-α), IL-6, and IL-10 in BALF were not significantly different in the two groups.

The IL-8R2KO mice had more MIP-2 (CXCL2) in the BALF than wild-type (WT) mice, even though they had fewer neutrophils and less expression of neutrophil-specific mRNA in their lungs. The explanation for the discrepancy between mRNA expression and protein levels of MIP-2 is not clear, but one possibility is that in WT mice, the secreted MIP-2 was largely bound to IL-8Rs on neutrophils, and only unbound MIP-2 was washed out of the lungs in the BALF. We did not detect KC (CXCL1) in the BALF, although expression was upregulated ~40-fold in both groups of mice. KC is 10-fold less potent than MIP-2 in activating mouse neutrophils (29).

***In vitro* production of IL-10 by neutrophils.** We previously reported that IL-10 production in the lungs of susceptible strains of mice is higher than that in a resistant strain and that is detrimental to the host response to this infection (30), and transcriptional analysis showed that IL-10 mRNA was less abundant in IL-8R2 KO mice. This led us to consider that neutrophils could be a source of IL-10 in this infection. It has been reported that β-glucan can act synergistically with a Toll-like receptor (TLR) agonist to stimulate neutrophils to secrete IL-10 and IL-1Ra (31), so we isolated neutrophils from the bone marrow of C.D2 mice using positive selection for Ly6G+ cells, and we stimulated them with the optimal concentration of the TLR1/2 agonist Pam3cSK4, various numbers of FKS, or a combination of the two stimuli to measure IL-10 secretion by C.D2 neutrophils. As shown in Fig. 6, Pam3CSK4 and some doses of FKS induced the production of small amounts of IL-10. However, the combination of the two stimuli synergized to significantly increase the amount of IL-10 produced. In the same experiments, Pam3CSK4 but not FKS induced secretion of IL-1Ra, and the combination was inhibitory (Fig. S2).

DISCUSSION

The principle findings in this paper were that *C. immitis*-infected IL-8R2 KO BALB/c mice had fewer neutrophils in their lungs and fewer fungi in their lungs than control

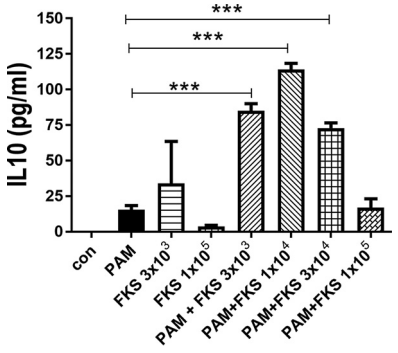

FIG 6 Bone marrow neutrophils from C.D2 mice secrete IL-10 in response to the combination of FKS and an optimal concentration of Pam3CSK4. Higher numbers of FKS are less stimulatory. Control (con) was unstimulated cells. All conditions were done in triplicate. There was no detectable IL-10 released after stimulation with 1 and 3×10^4 FKS alone, so those bars were left off the graph to simplify the figure. Note that even though some doses of FKS alone failed to stimulate IL-10 secretion alone, they still acted synergistically with Pam3CSK4 (PAM3CYs) to stimulate IL-10 production. The highest dose of FKS did not show synergy. One-way ANOVA with Dunnett's multiple-comparison test; ***, $P < 0.001$ compared to Pam3CSK4 alone.

mice 14 days after infection. BALB/c mice are one of the inbred strains most susceptible to infection with *C. immitis* (32), and our finding that BALB/c IL-8R2 KO mice are more resistant implies that neutrophils infiltrating the lung are responsible for some of their heightened susceptibility.

Using RNA-seq to compare gene expression in the lungs of infected IL-8R2 KO mice and controls, we discovered that the *Cxcr2* deletion changed the response of the host so that there was more expression of the protective cytokines IFN-γ and IL-17A and -F and less expression of the detrimental cytokine IL-10 in their lungs (30). There was also the expected increase in the expression of the transcription factors that promote Th1 and Th17 differentiation. This alteration in the adaptive immune response was largely confirmed by measurement of the concentrations of some of those cytokines in the BALF of the infected mice at the time of necropsy. The implication is that infiltrating neutrophils skewed the adaptive immune response toward a Th10 and away from protective Th1/Th17 immune response (33). As yet, we cannot account for the discrepancy between the differences in IL-10 mRNA in the lungs and the similar concentrations of IL-10 in the BALF from the two mouse strains.

The transit of neutrophils from bone marrow and spleen, through the blood to tissues, is a complex process. Infection induces several neutrophil chemotactic factors that act in concert, probably sequentially, to draw cells into the infected tissue. This study shows that ERL+ CXC ligands (CXCL) are induced in the lung and are very important for neutrophil migration during *C. immitis* infection, as there was a reduced number of neutrophils in the BALF in the absence of their receptor, IL-8R2. Injection of IL-8 directly into the airway of normal mouse lungs is sufficient to cause neutrophils to enter the lung parenchyma from the blood, but the infiltrate is diffuse, whereas in infections it is focused in the area of the lung that is infected (34). Ordinarily, the CXCLs are made by epithelial cells, myeloid cells, including macrophages, and neutrophils themselves, which creates a positive feedback loop, but CXCLs may not be sufficient to focus the infiltrate at areas of infection. Other host chemokines could be involved in neutrophil trafficking, such as prostaglandins and C5a that is generated by complement interacting with microbes. We cannot exclude the possibility that rupturing spherules also generate a locally active chemotactic factor.

The mechanism whereby neutrophils alter the immune response in this infection is not clear, but importantly, Hung et al. showed that neutrophils are the predominant inflammatory cells in the lungs of nonimmunized C57BL/6 (B6) mice infected with *C. posadasii* (17), so the predominance of polymorphonuclear leukocytes (PMN) in coccidioidomycosis is not peculiar to BALB/c mice or to *C. immitis*. They also found that

neutrophils from infected lungs were a major source of IL-10 in infected B6 mice and that IL-10-producing neutrophils but not neutrophils from IL-10 KO mice are associated with impaired resistance to pulmonary coccidioidomycosis in nonvaccinated mice (35). They speculated that spherules could induce production of IL-10 by PMN, and in this paper we show that FKS acted synergistically with the TLR2 agonist Pam3CSK4 to induce IL-10 production. We did not investigate other possible neutrophil immunosuppressive products such as arginase 1, which is secreted by immunosuppressive immature granulocytes (36). *Arg1* was expressed at higher levels in WT mice, but the FC was less than 2-fold. *Arg1* was highly upregulated in both mouse strains, possibly because it is also a marker for M2a macrophages (37).

In recent years, it has become clear that neutrophils are not simply short-lived killing machines, but they also are able to secrete several cytokines and chemokines that can be either proinflammatory or immunosuppressive (38). In the last decade, immunosuppressive neutrophils have been described in malignancies and chronic infection (39). Although there is some controversy about the finding, Zhang et al. showed that a combination of TLR2 agonist and a Dectin-1 agonist activate fluorescence-activated cell sorter (FACS)-purified neutrophils to secrete IL-10 (31). Other model infections have found lung neutrophils to be detrimental. For instance, depletion of neutrophils during the chronic phase of infection increases the resistance of mice to *Mycobacterium tuberculosis* (40), and IL-10 is known to suppress both Th1 and Th17 immune responses in tuberculosis (41). Romani et al. found that a virulent strain of *C. albicans* stimulates neutrophils to secrete IL-10 and that anti-IL-10 treatment made mice more resistant to that infection (42, 43).

There is debate about whether human peripheral blood neutrophils can make IL-10. *In vitro* assays have been called into question because there are histone modifications of DNA that make the IL-10 promoter inaccessible to transcription factors, even after treatment with TLR agonist (44). However, the transcriptional pattern of neutrophils changes throughout their life cycle, so it is possible that under certain conditions human neutrophils could make IL-10 (45).

Our findings suggest that in mice that are susceptible to *C. immitis* infection, there is a negative feedback loop that adversely affects the acquired immune response. Rupturing spherules in the lung attract neutrophils in a process that is partially dependent on signaling through the IL-8R. The infiltrating neutrophils make the immunosuppressive cytokine IL-10, which in turn prevents the development of Th1 and Th17 $CD4^+$ T cells, which in turn allows the growth of more spherules that rupture and attract more neutrophils.

Although the IL-8R2 KO mice had fewer organisms in their lungs than did the WT controls, there was no significant difference between the mutant and wild-type mice in the numbers of CFU in the spleen, a site of hematogenous dissemination (Fig. 2). In nearly all studies of coccidioidomycosis in mice, there is a strong correlation between the severity of the lung infections and the amount of extrapulmonary dissemination. In these experiments, lung CFU were relatively low in both groups, and hematogenous dissemination to the spleens was minimal in the WT controls, thereby making it difficult to detect any differences between the mice.

Our results are somewhat in conflict with those of Hung et al., who found that induced neutropenia did not affect the severity of the infection in unimmunized mice but interfered with vaccine-induced immunity (46). It is possible that experimental differences account for the discrepancy between their results. They infected a different mouse strain with a different species of *Coccidioides*, and the lung infection they induced was more severe, which may have obscured the modest but significant adverse effect of neutrophils in the lung that we observed. Although there has not been a direct comparison between the mouse virulence of *C. immitis* RS and *C. posadasii* C735, the Silvera strain of *C. posadasii* induces more inflammation and grows faster in BALB/c mice (5). Furthermore, they produced neutropenia using a rat monoclonal anti-Ly6G. It is possible that the clearance of millions of dead neutrophils had a suppressive effect on macrophage function.

There are some limitations in this study, including the fact that IL-8R2 is not

exclusively expressed on mature neutrophils but is also expressed by immature myeloid cells that include immature cells that can be either suppressive neutrophils or suppressive monocyte-like myeloid cells (38), by virtue of producing IL-10 and Arg2, which could have contributed to the susceptibility of the control mice. It is also possible that there were fewer exudative macrophages in the IL-8R2 KO mice (47), but one might expect that those macrophages would help control fungal growth, not impair resistance to the infection. We also did not use littermate controls. However, we knew that despite extensive backcrossing the IL-8R2 KO mice retained the WT *Slc11a*, which encodes Nramp1, from the ES cell line (48). Although there is no evidence that Nramp1 plays a role in resistance to *C. immitis*, we felt that the congenic C.D2 strain that also has a WT *Slc11a1* from DBA/2 mice was a better control. Because of the concern that there could be other genetic differences that might affect their resistance to this infection, we compared the infection in the three pertinent BALB/c strains and found that there was no significant difference between them as measured by CFU in their lungs. Therefore, we believe that the C.D2 control that we used was valid.

MATERIALS AND METHODS

Animals. Female BALB/c/HeJ mice were purchased from Jackson Laboratories, and BALB/c/AnNCr females were purchased from Charles River, both at 6 to 8 weeks of age. We also used female BALB/c.D2^{SLC11A1} congenic mice (C.D2) that were bred at our facility and were originally a gift from B. A. Mock at the NIH. BALB/c^{CXCR2tm} IL-8R2 KO breeding pairs were purchased from Jackson Laboratory (38); at Jackson Laboratory they breed homozygous KO males to heterozygous KO females. We found that by keeping the breeding pairs on co-trimoxazole in their drinking water, the females could be bred as homozygotes. Because this antimicrobial was expected to change the normal flora, which could affect immune responses, in a second experiment we kept the both the mutant and the control C.D2 mice on co-trimoxazole prophylaxis.

Infection. We removed the co-trimoxazole from the drinking water 3 days before we infected the mice intranasally (i.n.) with 50 to 100 arthroconidia from *C. immitis* RS, as previously described (49). Mice were sacrificed 14 to 15 days postinfection, and we removed and homogenized the left lungs and spleens using Tissuelyser II. Homogenized tissues were serially diluted 10-fold in 0.9 ml of sterile saline, and 0.1 ml of each of the three highest dilutions was plated on Sabouraud dextrose agar plates that were then taped shut and incubated for 3 days at ~27°C before being examined for growth inside the biosafety hood. Those plates that had between 10 and 200 CFU were used to determine colony counts for the organ. If two dilutions were both in that range, we averaged the results. Plates with no visible growth after 3 days were stored at room temperature for another 24 h before they were considered sterile. If the only growth was from an undiluted tissue sample, we used that number to establish CFU/organ, even if it was <20 CFU. One lobe of the left lung was snap-frozen in liquid nitrogen and stored at −70°C for subsequent RNA extraction.

Bronchial alveolar lavage (BAL). BAL fluid was obtained by gentle intratracheal installation of 0.2 ml of sterile saline and aspiration of as much injected fluid as possible after each rinse, a process that was repeated four times for each mouse. All the aspirated fluid from a mouse was combined and centrifuged in closed plastic tubes to pellet cells and any organisms that were washed out of the lungs. The pelleted cells were resuspended in flow cytometry staining buffer (eBioscience, San Diego, CA) and preincubated with anti-mouse CD16/CD32 (Ebioscience) for 10 min on ice to block nonspecific Fc-mediated interactions. The cells were then incubated for 30 min on ice with anti-mouse CD11b PE (eBioscience) and anti-Ly6G brilliant violet 421 (Biolegend) monoclonal antibodies before being suspended in 1% formaldehyde in PBS for flow cytometry (*C. immitis* is a BSL3 agent, so all cells from infected mice had to be fixed before undergoing analysis).

The supernatant BAL fluid was used to measure cytokine chemokine levels by enzyme-linked immunosorbent assay (ELISA). The ELISA kits for measuring tumor necrosis factor alpha (TNF-α), IL-6, IL-4, IL-10, IL-17a, IL-23, and gamma interferon (IFN-γ) were purchased from eBioscience. The kits for IL-1β and granulocyte-macrophage colony-stimulating factor (GM-CSF) were from BD Biosciences; the macrophage inflammatory protein 2 (MIP-2), IL-22, and IL-1Ra assay kits were from R&D.

RNA extraction from mouse lung tissue. Snap-frozen lung tissue was thawed in the presence of TRIzol (Life Technologies) and homogenized using Tissuelyser II (Qiagen). RNA was isolated with TRIzol (Life Technologies), DNase-treated using TURBO DNase (Ambion) according to the manufacturer's instructions, and ethanol-precipitated. For RNA-seq library preparation and sequencing, libraries for RNA sequencing were generated as previously described (41). PolyA-RNA was selected from total RNA using the MicroPoly(A)Purist kit (Ambion) according to the manufacturer's instructions. Isolated RNA was hydrolyzed in a total volume of 20 μl with 2 μl RNA fragmentation buffer (Ambion) for 10 min at 70°C. The reaction was stopped with stop buffer, and buffer was exchanged to Tris, pH 8.5, using P30 size exclusion columns (Bio-Rad). The fragmented RNA (30 ng) was 5′-decapped in a total volume of 21 μl containing 0.5 μl tobacco acid pyrophosphatase (TAP; Epicentre), 2 μl 10× TAP buffer, and 1 μl SUPERase-IN and incubated for 2 h at 37°C. To dephosphorylate RNA 3′ ends, 0.5 μl 10× TAP buffer, 1.5 μl water, 0.5 μl of 0.25 M $MgCl_2$ (4.17 mM final; 1 mM EDTA for maximum phosphatase activity), and 0.5 μl of 10 mM ATP (0.2 μM final to protect PNK [polynucleotide kinase]) were added, and the reaction mixture was incubated with 1 μl PNK (Enzymatics) for 50 min at 37°C. RNA fragments were 5′-

phosphorylated by adding 10 μl 10× T4 DNA ligase buffer, 63 μl water, and 2 μl PNK and incubated for 60 min at 37°C. RNA fragments were isolated using TRIzol LS, precipitated in the presence of 300 mM sodium acetate and 2 μl GlycoBlue (Ambion), washed twice with 80% ethanol, and dissolved in 4.5 μl water. To prepare sequencing libraries, 0.5 μl of 9 μM 5′-adenylated 3′MPX adaptor/5Phos/AGATCGGAAGAGCACACGTCTGA/3AmMO (IDT; desalted; adenylated with Mth ligase [NEB] according to the manufacturer's instructions, phenol-chloroform/chloroform-extracted, ethanol-precipitated with glycogen, and dissolved in water at 9 μM) was heat-denatured together with the RNA for 2 min at 70°C and ligated with 100 U truncated T4 RNA ligase 2 K227Q (NEB) in 10 μl 1× T4 RNA ligase buffer without ATP, containing 10 U SUPERase-In and 15% PEG8000 for 2 h at 16°C. To reduce adaptor dimer formation, 0.5 μl of 10 μM MPX_RT primer 5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3′ (IDT; desalted) was added and annealed to the ligation product by incubating it at 75°C for 2 min, at 37°C for 30 min, and then at 25°C for 15 min. Finally, 0.5 μl of 5 μM hybrid DNA/RNA sRNA 5′h adaptor 5′-GTTCAGAGTTCTACArGrUrCrCrGrArCrGrArUrC-3′ (IDT) was ligated to previously capped RNA 5′ ends by adding 2 μl T4 RNA ligase buffer, 6 μl 50% PEG8000 (15% final), 1 μl of 10 mM ATP, 9.5 μl water, and 0.5 μl (5 U) T4 RNA ligase 1 for 90 min at 20°C. To 15 μl of ligation reaction, an additional 0.5 μl 10 μM MPX_RT primer was added; reactions were denatured at 70°C for 1 min and then placed on ice. RNA was reverse-transcribed by adding 3 μl 10× first-strand buffer (4.5 μl water, 1.5 μl of 10 mM dNTP, 3 μl of 0.1 M DTT, 1.5 μl RNaseOUT, and 1 μl Superscript III reverse transcriptase [Invitrogen]), and incubating for 30 min at 50°C. cDNA was isolated by adding 35 μl AMPure XL beads (Beckman), binding and washing according to the manufacturer's instructions, and dissolving in 40 μl TET (0.1% Tween 20 in TE buffer). Libraries were PCR-amplified for 12 to 15 cycles with 0.75 μM oNTI201 primer and TruSeq-compatible indexed primers using Phusion Hot Start II in Phusion HF buffer (Thermo Scientific) containing 0.5 M betaine (98°C, 30 s; 12× [98°C, 10 s; 57°C, 25 s; 72°C, 20 s]; 72°C, 1 min; stored at 4°C), and 225- to 400-bp fragments were size-selected on 10% PAGE gels. Libraries were diluted with TET buffer and quantified using a Qubit double-stranded DNA (dsDNA) high-sensitivity (HS) assay kit (Thermo Fisher Scientific), pooled, and sequenced on a Hi-Seq 2000 sequencer (Illumina) using single-end 50-bp reads.

RNA-seq data analysis. Fastq files from RNA-seq experiments were mapped to the Genome Reference Consortium Mouse Build 38 (GRCm38 or mm10) using STAR (50) with default parameters and analyzed using HOMER (51) (Table S9). To measure gene expression, analyzeRepeats was used with the option rna and the parameters -pc 3 -condenseGenes -count exons. Subset-specific expression was defined with a 2-fold difference in expression between two experimental groups or time points and a false-discovery rate (FDR) less than 0.01. For gene ontology and pathway analysis, differentially expressed genes were analyzed using Metascape (52). Cluster analysis of all differentially expressed genes was performed using Cluster 3 (53) and Java Tree View (54). Principal-component analysis (PCA) was performed in R (55). All RNA-seq data described in the manuscript are available at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) series GSE146594.

***In vitro* neutrophil stimulation.** Neutrophils were positively selected from bone marrow cells using anti-Ly6G magnetic coated beads (Miltenyi Biotec, San Diego, CA) according to manufacturer's instructions. Wright-Giemsa stain of these cells confirmed that ≥95% were neutrophils. The remainder were large cells that may have been early myeloid precursors. We added 5×10^5 Ly6G+ cells/well in 96-well plates. They were incubated with various numbers of *in vitro*-grown formalin-killed spherules (FKS) harvested after 48 h in culture, a gift from John Galgiani (University of Arizona), and/or 1 μg/ml of the TLR2 agonist Pam3CSK4 (InvivoGen, San Diego, CA) in a total volume of 200 ml of RPMI with added glutamine, 5% low-endotoxin fetal calf serum (FCS), and gentamicin 10 mg/liter. After overnight incubation at 37°C, the supernatants were removed and 1 μl of proteinase inhibitor cocktail set IV (Calbiochem) was added, and the material was assayed for IL-10 (R&D) and IL-1RA (eBioscience) by ELISA.

Statistics. Geometric means were compared using a *t* test (GraphPad Prism 7). If there were more than 2 groups, a one-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison test was performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA. USA).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

SUPPLEMENTAL FILE 2, XLS file, 0.03 MB.

SUPPLEMENTAL FILE 3, XLS file, 0.03 MB.

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