

IL-27 Signaling Promotes Th1 Responses and Is Required to Inhibit Fungal Growth in the Lung during Repeated Exposure to *Aspergillus fumigatus*

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ABSTRACT

Aspergillus fumigatus is an opportunistic fungal pathogen that causes a wide spectrum of diseases in humans, including life-threatening invasive infections as well as several hypersensitivity respiratory disorders. Disease prevention is predicated on the host's ability to clear *A. fumigatus* from the lung while also limiting inflammation and preventing allergic responses. IL-27 is an important immunoregulatory cytokine, but its role during *A. fumigatus* infection remains poorly understood. In contrast to most infection settings demonstrating that IL-27 is anti-inflammatory, in this study we report that this cytokine plays a proinflammatory role in mice repeatedly infected with *A. fumigatus*. We found that mice exposed to *A. fumigatus* had significantly enhanced secretion of IL-27 in their lungs. Genetic ablation of IL-27R α in mice resulted in significantly higher fungal burdens in the lung during infection. The increased fungal growth in IL-27R α ^{-/-} mice was associated with reduced secretion of IL-12, TNF- α , and IFN- γ , diminished T-bet expression, as well as a reduction in CD4⁺ T cells and their activation in the lung, demonstrating that IL-27 signaling promotes Th1 immune responses during repeated exposure to *A. fumigatus*. In addition, infected IL-27R α ^{-/-} mice displayed reduced accumulation of dendritic cells and exudate macrophages in their lungs, and these cells had a lower expression of MHC class II. Collectively, this study suggests that IL-27 drives type 1 immunity and is indispensable for inhibiting fungal growth in the lungs of mice repeatedly exposed to *A. fumigatus*, highlighting a protective role for this cytokine during fungal infection. *ImmunoHorizons*, 2022, 6: 78–89.

INTRODUCTION

Aspergillus fumigatus is a ubiquitously distributed saprophytic fungus and the most common of its genus to cause disease worldwide. It produces small spores, known as conidia, that readily become airborne and disseminate throughout indoor and outdoor environments (1–5). It is estimated that humans inhale a few hundred to a couple of thousand conidia each day (3, 6). Due to their small size (2–3 μ m), conidia can circumvent pulmonary clearance mechanisms and enter deep into alveolar

spaces (1, 3, 7). In most immunocompetent hosts, these conidia are rapidly eliminated without incident. Occasionally though, noninvasive colonization or the formation of aspergillomas can occur. In addition, otherwise healthy individuals can also develop hypersensitivity disorders such as allergic asthma, farmer's lung, and allergic bronchopulmonary aspergillosis. In the case of immunocompromised or immunosuppressed patients, the inability to eliminate *A. fumigatus* can result in the development of aggressive and often fatal invasive infections such as invasive pulmonary aspergillosis (5, 8).

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Abbreviations used in this article: Af293, *Aspergillus fumigatus* strain 293; EBI3, EBV-induced gene 3; GMS, Grocott–Gomori's methenamine silver stain; qRT-PCR, quantitative real-time PCR; ROR γ t, retinoic acid-related orphan receptor γ t; Treg, regulatory T cell; WT, wild-type.

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Although protection against *A. fumigatus* has mainly been attributed to innate immune responses, recent reports have indicated adaptive immune responses, in particular T cells, as contributing to host defense. In fact, practically all T cell subsets have been identified as playing a role during *A. fumigatus* infection (9). To illustrate, Th1 responses are protective and indispensable for fungal clearance, whereas Th2 responses promote fungal persistence and disease progression (10–12). The role of Th17 responses, alternatively, remains controversial. Although Th17 responses are important for the recruitment and activation of protective neutrophils, they have also been reported to suppress Th1 responses, as well as drive Th2 responses and the development of airway hyperresponsiveness (9, 13–16). If any of these T cell responses are too weak or too strong, then disease can also occur (8). For example, a weak Th1 or Th17 response would be insufficient to remove fungi, whereas uncontrolled inflammation can cause tissue damage and impair fungal clearance (9, 17). Conversely, an overwhelming Th2 response suppresses protective T cells and enables fungi to persist (12). Th2 responses have also been associated with the development of allergic diseases (8, 13, 18). For these reasons, the intensity and duration of each of immune response must be tightly regulated, and a fine balance between the protective and detrimental responses must also be established to ensure that fungi are successfully eliminated while also minimizing immunopathology and preventing the development of allergic disease.

Interestingly, repeated exposure to *A. fumigatus*, such as what occurs on a daily basis, results in the codevelopment of Th1, Th2, and Th17 responses in the lung (6). The inherent resistance to *A. fumigatus* in immunocompetent hosts, despite recurring exposure, suggests that there are regulatory mechanisms in place that balance these responses so that disease does not develop. Regulatory T cells (Tregs) have been identified as contributing to immune homeostasis during *Aspergillus* infection and were found to be induced by repeated exposure to *A. fumigatus* (6, 19). Similar to helper T cells, though, these cells must also be carefully regulated, as excessive Treg activity can lead to immunosuppression and too little can enable immunopathology (20). This indicates the possible existence of yet another immunomodulatory pathway in the lungs of healthy individuals. In fact, a non-T cell regulatory mechanism was indeed reported to be involved in dampening immune responses during repeated exposure to *A. fumigatus* (6).

IL-27 is an important regulatory cytokine that is best known for its pleiotropic effects on T lymphocytes. It is composed of IL-27p28 and EBV-induced gene 3 (EBI3) subunits, and it signals through a receptor consisting of IL-27R α and gp130 (21–23). IL-27 was initially described as proinflammatory because it upregulated T-bet expression in CD4⁺ T cells and promoted Th1 cell differentiation (24–26). Later, IL-27 was reported to be anti-inflammatory and could instead suppress Th1, Th2, and Th17 responses as well as dendritic cell development (21, 27–33). In terms of Th2 and Th17 cells, IL-27 can directly inhibit their development through downregulation of GATA3 and retinoic acid-related orphan receptor γ t (ROR γ t), respectively (25, 32,

34). IL-27 also possesses broader suppressive activities and can antagonize the production of IL-2 and stimulate T cell production of IL-10 (21, 35, 36). IL-27 also interferes with the development and function of Tregs via the suppression of Foxp3 (37–39). This ability to dampen immune responses has been reported to be essential for preventing the development of T cell-mediated pathologies (27, 29, 40, 41). Furthermore, IL-27 can influence T cell proliferation, prevent activation-induced T cell death, and regulate T cell recruitment through enhancing the expression of various chemokines, chemokine receptors, and adhesive molecules (42–47).

To date, not much is known about the role of IL-27 during fungal infections. Some fungi such as *A. fumigatus* and select *Candida* species, including *C. parapsilosis*, *C. glabrata*, and *C. tropicalis*, but not *C. albicans*, can induce host expression of IL-27 (48, 49). During systemic infection with *C. parapsilosis*, IL-27 suppressed inflammatory responses and, in its absence, infected mice had improved fungal clearance and increased production of IFN- γ and IL-17 (48). Likewise, during acute aspergillosis, IL-27 negatively regulated the production of IFN- γ , which compromised fungal clearance in wild-type (WT) mice (33). In contrast, IL-27 expression by IFN- β -conditioned dendritic cells was shown to result in increased Th1-mediated IFN- γ production in vitro (49, 50). In addition, IL-27 has also been implicated in the maintenance of immune homeostasis during anti-*Aspergillus* responses through the development of type 1 Tregs (Tr1s) (49).

Despite these findings, the role of IL-27 during repeated exposure to *A. fumigatus* has yet to be investigated. In this study, we found that during repeated exposure to *A. fumigatus*, IL-27R α ^{−/−} mice displayed enhanced growth of the fungus in their lungs associated with reduced numbers and activation of CD4⁺ T cells, lower expression of T-bet, and diminished production of IL-12, IFN- γ , and TNF- α in the lung, as compared with infected WT mice. These data support a proinflammatory role for IL-27, rather than an anti-inflammatory role, during repeated exposure to *A. fumigatus*.

MATERIALS AND METHODS

Ethics approval

All animal studies were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Maryland, College Park.

Animals

Six- to 8-wk-old WT C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). IL-27R α ^{−/−} (WSX-1^{−/−}) mice in the C57BL/6 background (stock no. 018078) were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in the animal facilities at the University of Maryland, College Park. Mice were maintained in specific pathogen-free conditions in individually ventilated cages with a standard 12-h light/12-h dark cycle. For all experiments, age- and sex-matched

6- to 8-wk-old WT and knockout mice were used and cohoused during the experiments.

Fungal strain and culture

A. fumigatus strain 293 (Af293) is a clinical isolate and was provided by Dr. Stuart M. Levitz (University of Massachusetts Medical School) and grown as previously described (51). Briefly, fungi were cultured from frozen stocks on Sabouraud dextrose agar (Difco) slants for 2–3 d at 37°C. Conidia were harvested by vortexing slants with sterile PBS containing 0.01% Tween 20 (Amresco). Fungal suspensions were filtered through a 40- μ m mesh cell strainer and washed three times with and then resuspended in 0.01% Tween 20-PBS. Conidia were counted using a hemocytometer, and the concentration was adjusted to 8.33×10^8 conidia/ml in 0.01% Tween 20-PBS.

Infection models

For acute *A. fumigatus* infection, age- and sex-matched 6- to 8-wk-old C57BL/6 mice were anesthetized using a mix of ketamine (200 mg/kg) and xylazine (10 mg/kg) and infected intranasally with a single dose of 2.5×10^7 Af293 in 30 μ l of 0.01% Tween 20-PBS and were sacrificed at 1, 3, and 5 d postinfection for pulmonary CFU quantification. For repeated *A. fumigatus* exposure, age- and sex-matched 6- to 8-wk-old C57BL/6 WT and IL-27 $\alpha^{-/-}$ mice were anesthetized using a mix of ketamine (200 mg/kg) and xylazine (10 mg/kg) and infected intranasally with 2.5×10^7 Af293 in 30 μ l of 0.01% Tween 20-PBS every other day for 28 d and were sacrificed 1 d following the final instillation to examine immune responses in the lung.

Histology and quantification

Lungs from repeatedly infected WT and IL-27 $\alpha^{-/-}$ mice were harvested 1 d following the final instillation of fungi and fixed in 10% neutral buffered formalin. Samples were sent to American HistoLabs (Gaithersburg, MD) for processing and staining. Lung sections were stained with Grocott–Gomori’s methenamine silver stain (GMS) to visualize *A. fumigatus* or with H&E stain to visualize cellular infiltrates. Fungi were contained to the airways in all mice, and cell infiltration was closely associated with airways containing fungi. Images were taken to include as much of the airway and surrounding areas as possible, and for airways too large to fit in a single frame, additional images were taken to image the entire area. Slides were imaged at a magnification of $\times 10$ and $\times 63$ on a Zeiss Axio Lab.A1 microscope. For subsequent analyses, five to eight images were analyzed for each mouse.

The amount of *A. fumigatus* in the lungs was calculated as the % GMS⁺ area. To calculate % GMS⁺ areas, images were opened in ImageJ (National Institutes of Health) and colors were split into red, green, and blue channels (Image > Color > Split Channels). The green channel was then selected for subsequent analyses, as it had the most contrast between GMS⁺ and GMS[−] areas. A threshold of 35 was set and used for each GMS image analyzed (Image > Adjust > Threshold) to ensure that only GMS⁺ fungi

were highlighted. To measure the highlighted GMS⁺ areas, “area” was selected (Analyze > Set Measurements) and measured (Analyze > Measure) for each image. Individual values for each image were entered into GraphPad Prism 5 for analysis (GraphPad Software, San Diego, CA). The amount of cell infiltration in the lungs was calculated as the % H&E⁺ area. % H&E⁺ areas were calculated similar to % GMS⁺ areas for H&E-stained images, with a threshold value of 70.

To calculate the depth of *A. fumigatus* growth in the lungs, an image containing a scale bar with a known length was opened in ImageJ. Using the straight-line tool, a line with the same length as the scale bar was drawn and its known distance and unit of length of 20 μ m was set (Analyze > Set Scale) and applied globally (check Global box). Fungal cell lengths were then measured for GMS images by drawing a straight line from one end of the cell to the other and calculated (Analyze > Measure). Between 5 and 10 measurements were made for each image, and fungal cells measured were randomly spaced out around the airways to avoid bias. Measurements from images of the same magnification were entered into GraphPad Prism 5 for analysis.

Lung leukocyte isolation

One day following the final instillation of Af293, lungs from repeatedly infected C57BL/6 WT and IL-27 $\alpha^{-/-}$ mice were harvested into 1 ml of sterile RPMI 1640 medium in a 24-well plate and minced with surgical scissors. One hundred microliters of each lung sample was reserved on ice for RNA extraction for use in gene expression studies. The remaining tissues were digested with collagenase IV (Worthington Biochemical) at a final concentration of 1 mg/ml and incubated for 45 min at 37°C with gentle shaking. Samples were then manually homogenized on ice through a 70- μ m cell strainer and centrifuged at $500 \times g$ for 5 min at 4°C, and the supernatants were collected and stored at -80°C for use in cytokine analyses. Pelleted cells were resuspended in 37% Percoll (GE Healthcare Biosciences) and subjected to gradient centrifugation at $1200 \times g$ for 15 min at room temperature with the brake off. The supernatants were decanted, and cell pellets were resuspended and incubated in ACK (ammonium-chloride-potassium) lysing buffer (Lonza BioWhittaker) for 5–10 min at room temperature to lyse RBCs. Cells were then washed once with sterile PBS and resuspended in flow cytometry staining buffer (1% BSA in PBS with 0.05% sodium azide). A 10- μ l portion of the isolated cells were diluted and stained with trypan blue (Sigma-Aldrich), and live cells were enumerated using a hemocytometer.

Flow cytometry

Following isolation, 1×10^6 isolated lung leukocytes were resuspended in 100 μ l of flow cytometry staining buffer. Fc receptors were blocked using anti-CD16/32 mAb (93) for 20 min at 4°C. Cells were then stained with fluorophore-conjugated Abs including anti-CD45 (30-F11), anti-CD11c (N418), anti-MHC class II (M5/114.15.2), anti-Ly6G (1A8), anti-Ly6C (HK1.4), anti-CD11b (M1/70), anti-F4/80 (BM8), anti-SiglecF (E50-2440), anti-CD3 ϵ (17A2), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD44 (IM7),

anti-CD62L (MEL-14), anti-CD80 (16-10A1), and anti-CD86 (GL-1). Stained cells were detected with a FACSCanto II flow cytometer (BD Biosciences), and data were analyzed using FlowJo v7.6 (Tree Star). Gating strategies are shown in Supplemental Figs. 1 and 2. All Abs used for flow cytometry were purchased from BioLegend, unless otherwise stated.

Quantification of fungal burdens

The lungs of acutely infected mice were collected into 2 ml of sterile PBS in 15-ml tubes on ice and were homogenized with a hand-held tissue homogenizer (Omni International). Serial dilutions were performed using sterile water and were plated on Sabouraud dextrose agar. Plates were then incubated overnight at 37°C and CFU were counted and the total fungal burden was calculated.

Gene expression

Total RNA was extracted from 100- μ g portions of lung tissues collected during leukocyte isolation using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA

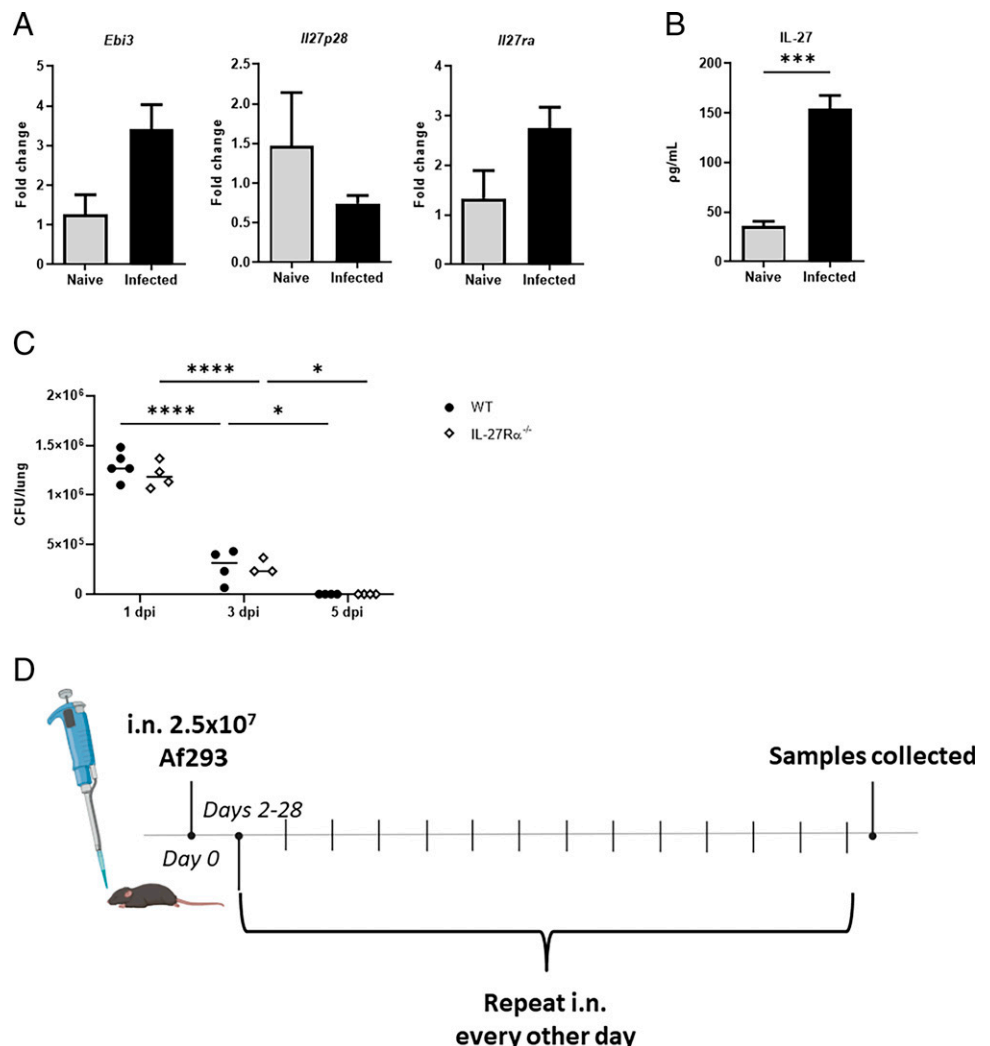
was transcribed using the SuperScript IV first-strand synthesis system (Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed with appropriate primers on a Bio-Rad CFX96 real-time system using SYBR Green PCR master mix (Applied Biosystems). The gene expressions for each sample were normalized against the housekeeping gene GAPDH and fold change was calculated using the $\Delta\Delta$ Ct method. The primers used are listed in Supplemental Table I.

Cytokine quantification

The supernatants from lung homogenates collected during leukocyte isolation were stored at -80°C prior to use. Samples were then thawed and centrifuged to pellet any debris. The remaining suspensions were used to measure cytokine concentrations. BD OptEIA IL-4, IL-5, IL-10, IL-12p40, and IFN- γ ELISA kits were purchased from BD Biosciences, and TNF- α , IL-1 β , IL-17A, and IL-27 ELISA kits were purchased from Thermo Fisher Scientific, and all were used according to the manufacturers' protocols. ELISA plates were read using a BioTek Synergy HTX plate reader.

FIGURE 1. Enhanced IL-27 expression in the lung following infection with *A. fumigatus* and establishment of an infection model for repeated exposure to *A. fumigatus*.

(A and B) Six- to 8-wk-old C57BL/6 wild-type (WT) mice were infected intranasally with 2.5×10^7 Af293 for 14 d. (A) Gene expression of the IL-27 cytokine subunits EBI3 and IL-27p28 as well as the IL-27-specific receptor subunit IL-27R α for naive WT ($n = 3$) and infected WT ($n = 5$) mice. (B) Protein levels of IL-27 in the supernatant of lung homogenates from naive ($n = 3$) and infected WT ($n = 5$) mice. Data are expressed as mean \pm SEM. *** $p < 0.001$. (C) Age- and sex-matched 6- to 8-wk-old WT and IL-27R $\alpha^{-/-}$ mice were infected intranasally with 2.5×10^7 Af293. The pulmonary CFU of WT and IL-27R $\alpha^{-/-}$ mice ($n = 3$ –5) on days 1, 3, and 5 postinfection. Data are expressed as mean \pm SEM and are representative of two independent experiments. * $p < 0.05$, **** $p < 0.0001$. (D) Graphic representation of the experimental model of repeated infections. Age- and sex-matched 6- to 8-wk-old WT and IL-27R $\alpha^{-/-}$ mice were infected intranasally (i.n.) with 2.5×10^7 Af293 every other day for 28 d and were sacrificed 1 d following the final instillation of fungi (29 d after initial infection) to assess immune responses.



Statistical analysis

All data were expressed as mean \pm SEM. Statistical tests were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). For datasets containing two groups, the unpaired Student *t* test was used to determine significance. For comparisons of more than two groups, one-way ANOVA was performed followed by a Tukey post hoc test. A *p* value <0.05 was considered to be significant.

RESULTS

IL-27 is upregulated in the lung during *A. fumigatus* infection

To determine whether IL-27 is involved in the immune response to *A. fumigatus*, we first examined the expression of this cytokine and its receptor in the lungs of mice following infection. WT C57BL/6 mice were infected intranasally with 2.5×10^7 Af293 and sacrificed at 14 d postinfection. The expression of the IL-27 cytokine subunits EBI3 and IL-27p28, as well as the IL-27-specific receptor subunit IL-27R α , were then measured by qRT-PCR. The expression of both EBI3 and IL-27R α were increased following *Aspergillus* infection, whereas the expression of IL-27p28 was unexpectedly decreased (Fig. 1A). To confirm that the upregulation of EBI3 correlated to an increased production of IL-27, we next measured the cytokine concentrations in the supernatants of lung homogenates and found a 3-fold increase in IL-27 levels following infection (Fig. 1B). These data provide evidence that IL-27 may be involved in immune responses to *A. fumigatus*.

Establishing an infection model for repeated exposure to *A. fumigatus*

To examine the immune responses that protect immunocompetent hosts from developing disease during repeated exposure to *A. fumigatus*, we sought to create an infection model in which a robust adaptive immune response was elicited. To this end, we modified a previously described model of repeated *Aspergillus* exposure to ensure that fungi were consistently present in the lungs, as it is rapidly cleared in immunocompetent hosts (6). For this, we determined the frequency with which fungi needed to be instilled. WT and IL-27R $\alpha^{-/-}$ mice were infected intranasally with 2.5×10^7 Af293 and fungal burdens in the lungs were assessed at days 1, 3, and 5 postinfection. One day following infection, less than half of the original inoculum was detected as CFU in the lungs of both WT and IL-27R $\alpha^{-/-}$ mice. By 3 d postinfection the CFU had decreased an additional 5-fold, and by 5 d there were no detectable CFU in either strain of mouse (Fig. 1C). With this in mind, we developed a model in which mice were infected intranasally with 2.5×10^7 Af293 every other day during the course of 28 d, for a total of 15 instillations. We selected this duration for our model because T helper responses were previously reported to peak at 4 wk

during repeated *Aspergillus* exposure (6). Mice were then sacrificed 1 d following the final administration of fungus to examine immune responses in the lung (Fig. 1D).

IL-27 signaling is required to suppress *A. fumigatus* growth in the lung

To assess fungal growth, lung sections from repeatedly infected WT and IL-27R $\alpha^{-/-}$ mice were stained with GMS to visualize *A. fumigatus*. The overall amount of fungi was determined as the % GMS $^{+}$ area. We selected this method to quantify fungal loads, as traditional culture methods are often unreliable for *A. fumigatus*, especially at later time points when fungi have germinated into hyphae and form aggregates within tissues that are often difficult to disperse (52, 53). In addition, these methods fail to take into account how much fungi have grown, which is especially important to consider for a filamentous fungus such as *Aspergillus*. In general, fungi were confined to the airway epithelium in both strains of mice, but IL-27R $\alpha^{-/-}$ mice had twice the amount of GMS $^{+}$ *A. fumigatus* in their lungs compared with WT mice (Fig. 2A). In addition, fungi in IL-27R $\alpha^{-/-}$ mice had grown longer, which correlated to a significantly higher average fungal length (Fig. 2B). These data demonstrate that IL-27 signaling is required to suppress *A. fumigatus* growth in the lung.

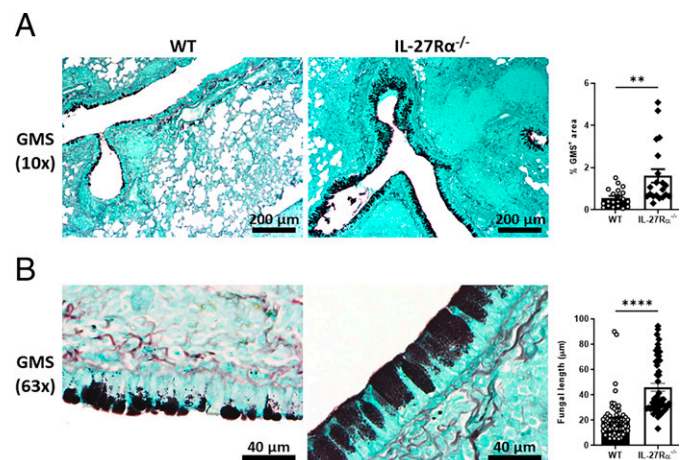


FIGURE 2. IL-27 is required to suppress *A. fumigatus* growth in the lung.

Age- and sex-matched 6- to 8 wk-old WT and IL-27R $\alpha^{-/-}$ mice were infected intranasally with 2.5×10^7 Af293 every other day for 28 d and were sacrificed 1 d following the final instillation of fungi. (A) Representative images of GMS-stained lung sections from infected WT (*n* = 3) and IL-27R $\alpha^{-/-}$ (*n* = 3) mice and quantification of % GMS $^{+}$ areas. *A. fumigatus* can be visualized as black (original magnification, $\times 10$). (B) Representative images of *Aspergillus* growth in the lungs of repeatedly infected WT (*n* = 3) and IL-27R $\alpha^{-/-}$ (*n* = 3) mice and the quantification of fungal cell lengths (original magnification, $\times 63$). Each data point represents an individual measurement. Data are expressed as mean \pm SEM. ***p* < 0.01 , *****p* < 0.0001 .

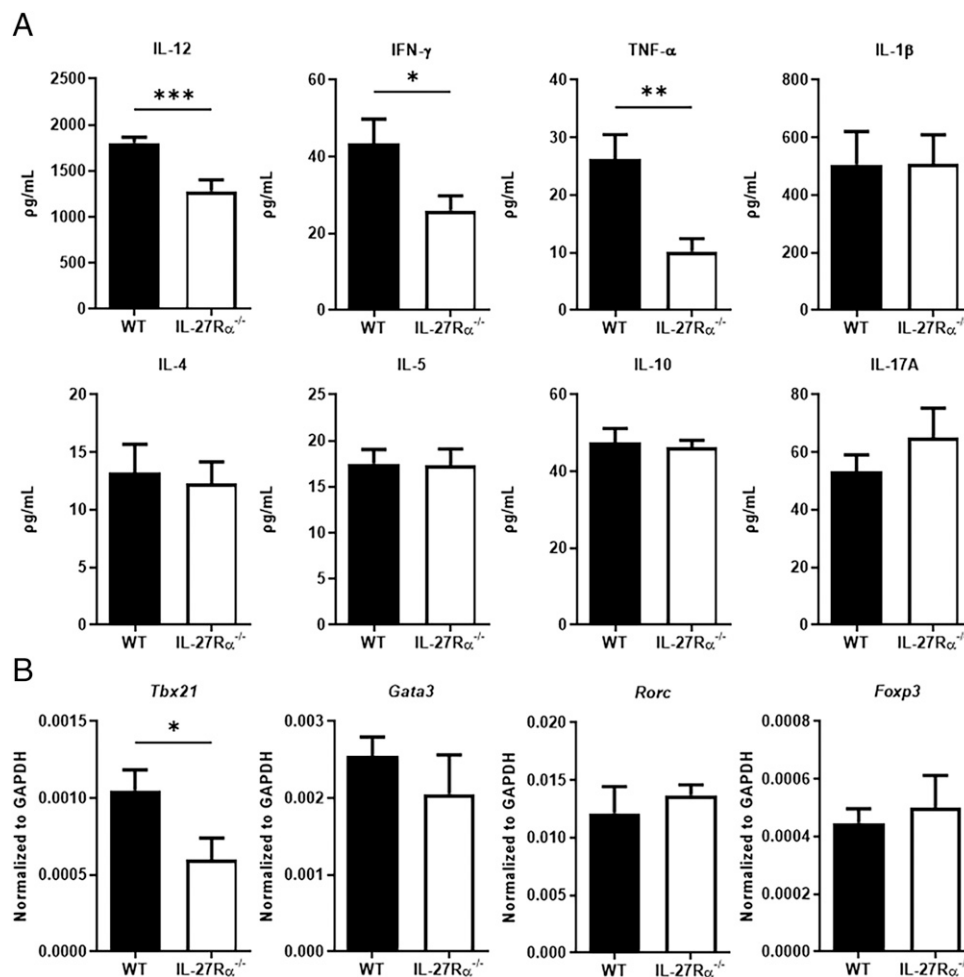


FIGURE 3. IL-27 signaling promotes Th1 responses in the lung during repeated exposure to *A. fumigatus*.

(A) Concentrations of various cytokines in the lungs of repeatedly infected WT ($n = 15$) and IL-27Rα^{-/-} ($n = 12$) mice were measured 1 d following the final instillation of fungi. (B) Gene expression of T cell subset inducing transcription factors in repeatedly infected WT ($n = 7$) and IL-27Rα^{-/-} ($n = 7$) mice 1 d following the final instillation of fungi. Data are expressed as mean \pm SEM and are pooled from two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

IL-27 promotes Th1 immune responses in the lung during repeated exposure to *A. fumigatus*

IL-27 is best known for its effects on T cell polarization. For example, IL-27 can promote the development of Th1 cells, as well as inhibit Th1, Th2, and Th17 cells and Tregs (42). To gain insight into how IL-27 might influence T cell responses during *Aspergillus* infection, we measured the concentrations of various cytokines associated with these responses in the lungs of repeatedly infected mice. Of note, infected IL-27Rα^{-/-} mice had significantly lower concentrations of the Th1 cytokines IL-12, IFN-γ, and TNF-α compared with infected WT mice. This decrease in Th1 responses was not due to enhanced Th2 or Treg responses, as WT and IL-27Rα^{-/-} mice had comparable levels of IL-4, IL-5, and IL-10. Likewise, Th17 responses were also minimally affected by the absence of IL-27 signaling, as IL-17A concentrations were similar in these mice (Fig. 3A). Based on these results, IL-27 promotes Th1 cytokine responses during repeated *A. fumigatus* infection.

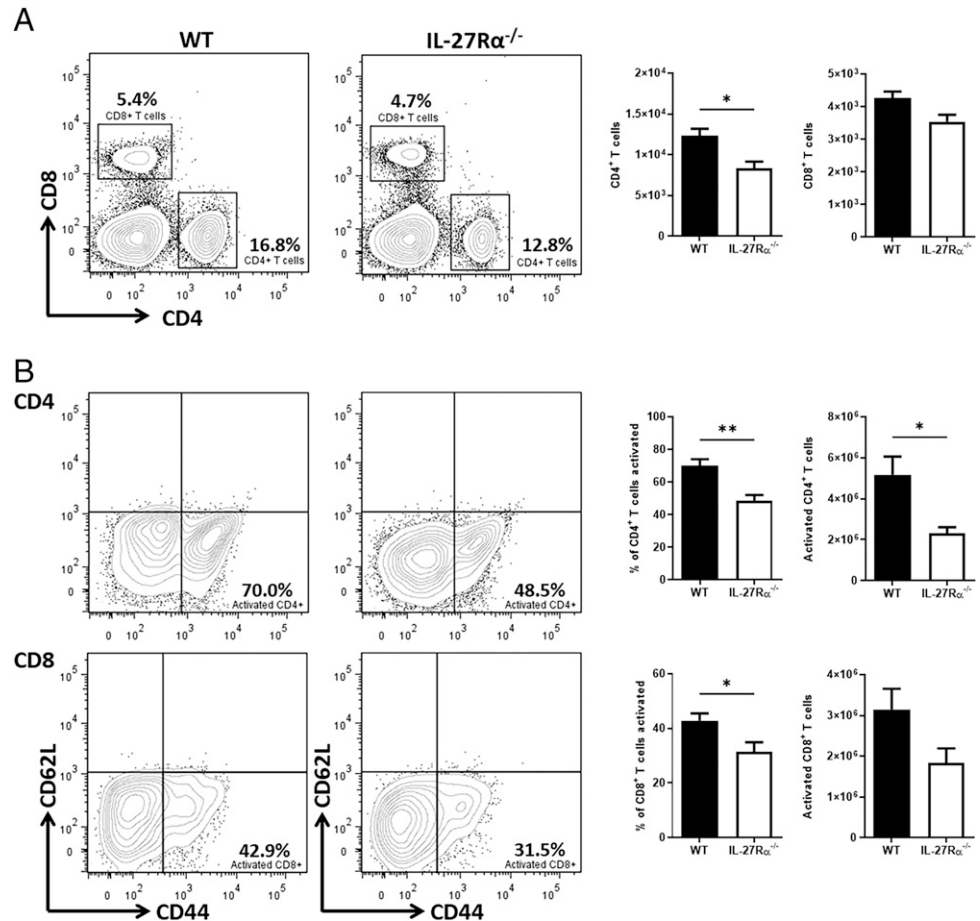
One way in which IL-27 regulates the development of T cell subsets is through regulating the expression of transcription factors. For instance, IL-27 promotes Th1 cell development via inducing the expression of T-bet, and it suppresses Th2, Th17,

and Treg development by inhibiting expression of GATA3, RORγt, and Foxp3, respectively (21, 36, 37). To determine whether IL-27 signaling affects the expression of these transcription factors during repeated exposure to *A. fumigatus*, we next measured their expression in the lung using qRT-PCR. Compared to WT mice, IL-27Rα^{-/-} mice had significantly reduced expression of *Tbx21* (T-bet), whereas there were no differences between their expressions of *Gata3*, *Rorc* (RORγt), or *Foxp3* (Fig. 3B). This supports the notion that IL-27 drives Th1 polarization by enhancing T-bet expression.

To determine whether IL-27 affects T cell numbers during *Aspergillus* infection, CD4⁺ and CD8⁺ T cell populations in the lungs of repeatedly infected mice were analyzed using flow cytometry (Supplemental Fig. 1). IL-27Rα^{-/-} mice had significantly fewer CD4⁺ T cells compared with WT mice, but they had similar numbers of CD8⁺ T cells (Fig. 4A). Importantly, these CD4⁺ and CD8⁺ T cells in the lungs of IL-27Rα^{-/-} mice were less activated than those in WT mice (Fig. 4B). These data indicate that IL-27 signaling leads to increased T cell, especially CD4⁺ T cell, numbers and activation in the lungs during *Aspergillus* infection. Overall, these data suggest that IL-27 signaling drives Th1 immune responses in the lung during repeated exposure to *A. fumigatus*.

FIGURE 4. IL-27 enhances T cell numbers and activation in the lung of mice repeatedly infected with *A. fumigatus*.

(A) Representative flow cytometry dot plots and quantification of CD4⁺ and CD8⁺ T cell populations in the lung of repeatedly infected WT ($n = 7$) and IL-27R $\alpha^{-/-}$ ($n = 7$) mice 1 d following the final instillation of fungi. (B) Representative flow cytometry dot plots and quantification of activated (CD44⁺CD62L⁻) CD4⁺ and CD8⁺ T cells in the lung of repeatedly infected WT ($n = 7$) and IL-27R $\alpha^{-/-}$ ($n = 7$) mice. The gating strategy is shown in Supplemental Fig. 1. Data are expressed as mean \pm SEM and are representative of two independent experiments. * $p < 0.05$, ** $p < 0.01$



IL-27 signaling promotes dendritic cell and macrophage accumulation and activation in the lungs of mice repeatedly infected with *A. fumigatus*

An important function of Th1 cells is to recruit and activate effector cells (54). Two of the most important effector cells against *A. fumigatus* are macrophages and neutrophils (2, 4, 55). To determine whether recruitment of these and other myeloid cells was altered in the absence of IL-27 signaling, we examined their populations in the lungs of repeatedly infected mice using flow cytometry (Supplemental Fig. 2). Overall, infected WT and IL-27R $\alpha^{-/-}$ mice had comparable numbers of total leukocytes, neutrophils, eosinophils, inflammatory monocytes, patrolling monocytes, and alveolar macrophages (Fig. 5A). Interestingly, though, infected IL-27R $\alpha^{-/-}$ mice had less than half the number of dendritic cells as well as exudate (monocyte-derived) macrophages as did infected WT mice (Fig. 5A). Furthermore, dendritic cells and exudate macrophages of infected IL-27R $\alpha^{-/-}$ mice exhibited lower expression of MHC class II compared with those of infected WT mice, although the expression levels of costimulatory molecules CD80 and CD80 were not affected by the deficiency of IL-27R α (Fig. 6). The reduced accumulation of macrophages and dendritic cells, along with the drop in CD4⁺ T cells (Fig. 4), was reflected in H&E-stained lung sections where cell infiltration, determined as the % H&E⁺ area,

was significantly lower in infected IL-27R $\alpha^{-/-}$ mice, with only 4% of the total areas imaged being positive, as compared with 12% in infected WT mice (Fig. 5B).

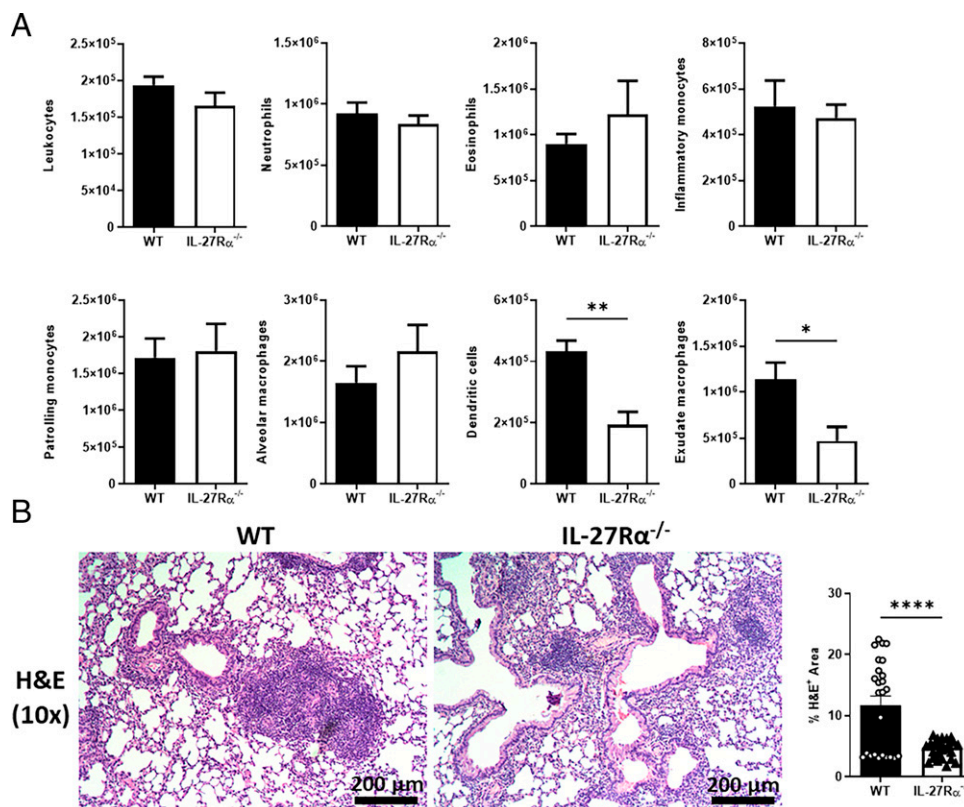
Because macrophages are considered one of the main defenses against *Aspergillus* infection, the reduction of macrophage numbers in the lungs of infected IL-27R $\alpha^{-/-}$ mice may explain the inability for IL-27R $\alpha^{-/-}$ mice to control fungal growth. The fungicidal activity of these cells is typically associated with M1 polarization (56, 57). To assess whether IL-27 influences macrophage polarization during infection with *A. fumigatus*, we examined the gene expression of various M1 and M2 macrophage markers and found that IL-27R $\alpha^{-/-}$ mice had a tendency toward lower M1 marker expression, including lower *Nos2*, *Ifng*, and *Tnf* expression, although the difference of these molecules did not reach statistically significant levels (Fig. 7). Collectively, these data demonstrate that IL-27 signaling promotes accumulation and activation of dendritic cells and macrophages in the lung during repeated exposure to *A. fumigatus*.

DISCUSSION

On average, humans inhale somewhere between a few hundred and a couple of thousand *A. fumigatus* spores each day (3, 6). Repeated exposure to this fungal pathogen results in the

FIGURE 5. IL-27 promotes dendritic cell and macrophage accumulation in the lung during repeated *A. fumigatus* infection.

(A) The cell numbers of leukocytes, neutrophils, eosinophils, inflammatory monocytes, patrolling monocytes, alveolar macrophages, dendritic cells, and exudate macrophages in the lung of repeatedly infected WT ($n = 7$) and IL-27R $\alpha^{-/-}$ ($n = 7$) mice 1 d following the final instillation of fungi. The gating strategy is shown in Supplemental Fig. 2. Data are expressed as mean \pm SEM and are representative of two independent experiments. * $p < 0.05$, ** $p < 0.01$. (B) Representative images of H&E-stained lung sections of repeatedly infected WT ($n = 3$) and IL-27R $\alpha^{-/-}$ ($n = 3$) mice and quantification of % H&E $^{+}$ areas. Cell infiltrates can be visualized as dark purple. Each data point represents an individual measurement. Data are expressed as mean \pm SEM. **** $p < 0.0001$



codevelopment of Th1, Th2, and Th17 responses in the lung (6). The way in which these responses influence the outcome of *Aspergillus* infection varies greatly. For example, Th1 and Th17 responses confer protection, whereas Th2 responses are detrimental. Moreover, if one of these responses is too strong or too weak, then disease can occur. The fact that most immunocompetent individuals do not develop *Aspergillus*-associated diseases

suggests that these responses are effectively regulated so that fungi are successfully cleared while minimizing immunopathology and preventing the development of allergic disease. Although Tregs have been identified as playing an important role in suppressing excessive immune responses during *A. fumigatus* infection, these cells must also be carefully regulated to prevent the development of disease (19, 20). IL-27 is a cytokine that can

FIGURE 6. Reduced expression of MHC class II in lung dendritic cells and macrophages in the absence of IL-27 signaling during repeated *A. fumigatus* infection.

The expression of MHC class II, determined as the median fluorescence intensity, was measured in dendritic cells (DCs) and exudate macrophages (ExMs) in the lung of repeatedly infected IL-27R $\alpha^{-/-}$ ($n = 7$) mice and WT ($n = 7$) mice 1 d following the final instillation of fungi. Data are expressed as mean \pm SEM and are representative of two independent experiments. *** $p < 0.001$, **** $p < 0.0001$

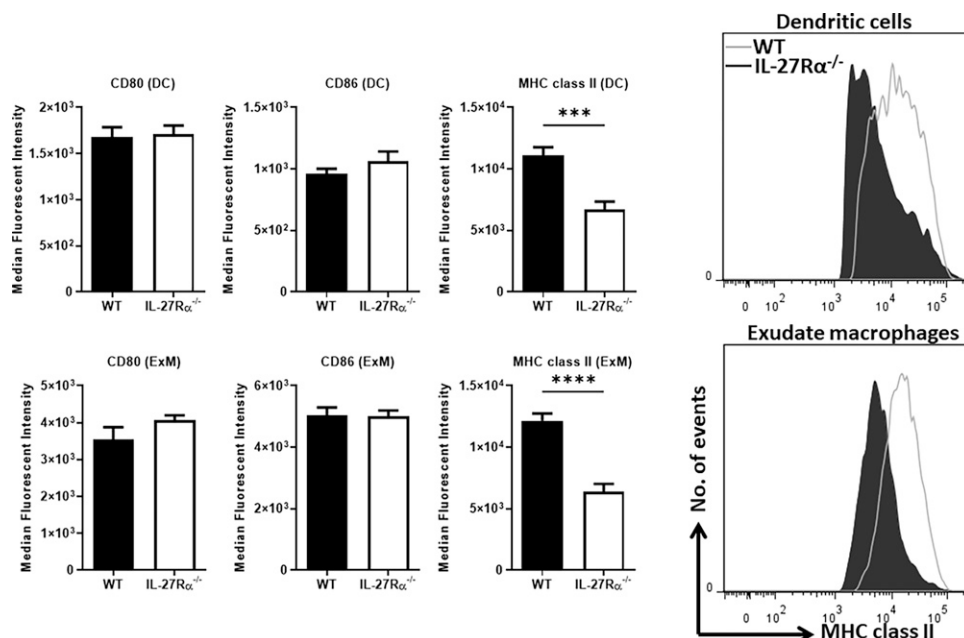
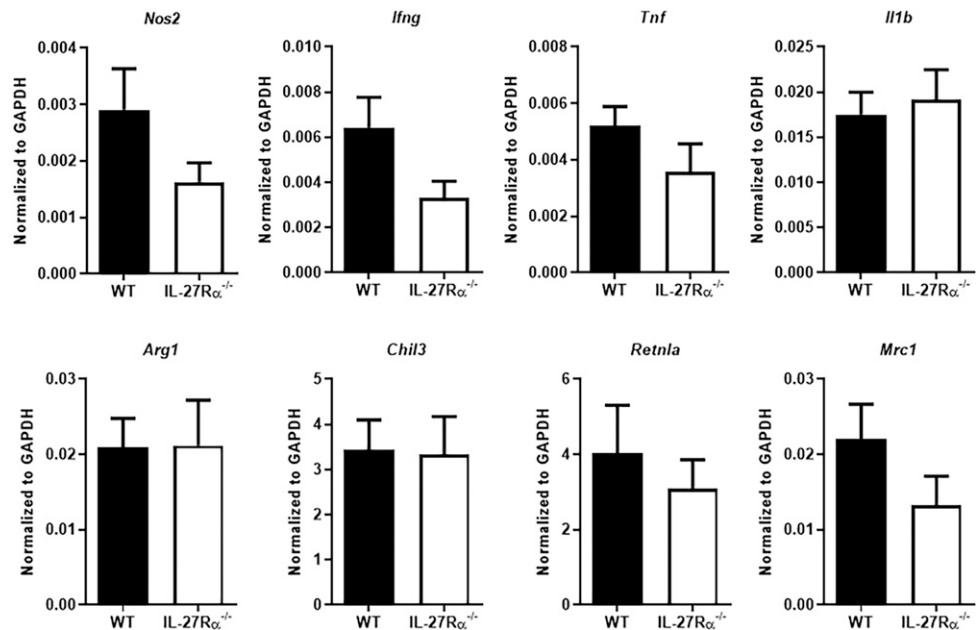


FIGURE 7. Characterization of M1 macrophage polarization in WT and IL-27R $\alpha^{-/-}$ mice during repeated *A. fumigatus* infection.

Gene expression of M1 and M2 macrophage markers in the lung of repeatedly infected WT ($n = 7$) and IL-27R $\alpha^{-/-}$ ($n = 7$) mice 1 d following the final instillation of fungi as determined by qRT-PCR. Data are expressed as mean \pm SEM.



regulate immune responses through promoting the development of the various T cell subsets. For this reason, we hypothesized that IL-27 may contribute to immunity during repeated *A. fumigatus* exposure.

Supporting this hypothesis, IL-27 production in the lungs of WT mice was significantly increased following *A. fumigatus* infection. This went hand in hand with increased expression of the IL-27 subunit EBI3. In contrast, IL-27p28 expression was unexpectedly decreased. The fact that these subunits can be secreted independently of one another and possess unique biological functions outside of IL-27 cytokine formation may account for this discrepancy (58). For example, IL-27p28 can act as a gp130 receptor antagonist, and its decreased expression likely represents a downregulation of this function rather than of IL-27 production, especially as IL-27 protein levels did not reflect this decrease (21, 42).

The increased fungal growth in IL-27R $\alpha^{-/-}$ mice was associated with reduced concentrations of IL-12, TNF- α , and IFN- γ , all of which have been shown to be required for protection against *A. fumigatus*, and were accompanied by lower expression of the Th1-inducing transcription factor T-bet (10, 11, 59–62). These data support the notion that IL-27 drives T-bet expression in CD4 $^{+}$ T cells and promotes the development of Th1 cells in mice (24). In addition, we showed that infected IL-27R $\alpha^{-/-}$ mice displayed reduced CD4 $^{+}$ T cell numbers in the lung, which is consistent with previous studies showing that IL-27 promoted the proliferation of CD4 $^{+}$ T cells as well as prevented their activation-induced cell death (42–46, 63).

On top of fewer CD4 $^{+}$ T cells, IL-27R $\alpha^{-/-}$ mice also had less T cell activation compared with WT mice, which could further explain the lower Th1 cytokine production. Due to its pleiotropic effects on T cells, it is conceivable that IL-27 directly influences T cell activation. However, we could not exclude the possibility

that IL-27 also acts on APCs, which would in turn affect T cell activation. For instance, IL-27 has been shown to upregulate MHC class II expression on APCs, which is not only required to prime and activate T cells (64–67), but is also required for APC survival (68). In this regard, we found that MHC class II expression on dendritic cells and exudate macrophages was indeed dramatically lower in IL-27R $\alpha^{-/-}$ mice and that infected IL-27R $\alpha^{-/-}$ mice had fewer of these cells, suggesting this as a potential cause for their reduced T cell activation.

The lower numbers of dendritic cells and exudate macrophages observed in infected IL-27R $\alpha^{-/-}$ mice are likely the direct cause of impaired fungal clearance in these mice, because these are well-known effector cells that can directly kill *A. fumigatus* through the production of reactive oxygen species (4, 69). It is well established that Th1 immune responses and IFN- γ , the signature Th1 cytokine, are essential for clearance of *Aspergillus* infections (10–12, 59–62, 70). It is also known that IFN- γ as well as TNF- α promote monocyte differentiation to dendritic cells and macrophages (23, 71–74). Thus, it is conceivable that disruption of IL-27 signaling reduces Th1 polarization and IFN- γ secretion, hence negatively influencing dendritic cell and exudate macrophage development, which leads to impaired *Aspergillus* clearance.

Taken together, our data suggest a protective role for IL-27 during repeated exposure to *A. fumigatus*. Interestingly, IL-27 does not appear to be anti-inflammatory, but instead promotes inflammatory responses in the lung that are essential to clear fungi. This is in conflict with a recent study showing that IL-27 was detrimental during an acute model of invasive pulmonary aspergillosis, as it negatively regulated the production of IFN- γ , which was required for fungal clearance and improved survival (33).

In this study, acutely infected WT mice succumbed within 8 d, whereas IL-27R $\alpha^{-/-}$ mice survived significantly longer (33).

This contrasts with our repeated exposure model where neither strain of mice died. These differences in survival could stem from the different dosages of *A. fumigatus* used to infect mice in each model. In the acute model, mice were infected once intratracheally with 5×10^7 Af293 (33). For repeated exposure, mice were infected every other day intranasally with 2.5×10^7 Af293. It is possible that the higher dosage of *A. fumigatus* used for acute infection could cause death in WT mice, which would be in line with a previous report that found that mortality was positively correlated with the fungal dose used to infect mice (75). This difference in susceptibility is also reflected as differences in the fungal burdens between both studies. In the acute model of aspergillosis, IL-27R $\alpha^{-/-}$ mice had lower pulmonary CFU compared with WT mice, whereas IL-27R $\alpha^{-/-}$ mice repeatedly exposed to *A. fumigatus* had more fungi in their lungs.

The decreased fungal burdens in the lungs of acutely infected IL-27R $\alpha^{-/-}$ mice were likely the result of increased IFN- γ at days 1 and 3 postinfection (33). This differs from repeatedly infected IL-27R $\alpha^{-/-}$ mice, whose production of IFN- γ was significantly lower than that of WT mice at 29 d after initial infection. Although the source of IFN- γ during acute aspergillosis was not identified, it is likely that it is produced by a subset of innate immune cells such as NK cells rather than T cells. This is reasonable, as NK cells were identified as the major source of IFN- γ during early *Aspergillus* infection in neutropenic mice (70). Furthermore, IL-27 was also reported to influence IFN- γ production in these cells (21). At later stages of *A. fumigatus* infection, the major source of IFN- γ was identified as CD4 $^{+}$ T cells, which is in agreement with our results that show that IL-27R $\alpha^{-/-}$ mice with dramatically fewer CD4 $^{+}$ T cells had significantly lower levels of IFN- γ (70).

Based on these results, it is possible that IL-27 suppresses the early production of IFN- γ by innate immune cells/NK cells, which prevents fungal clearance during acute aspergillosis, but at later time points mediates protective responses through the promotion of IFN- γ -producing Th1 cells, which are required to suppress *Aspergillus* growth following repeated exposure. Whether IL-27 is anti-inflammatory or proinflammatory during *A. fumigatus* infection may be dependent on the cell types in question, the infection stages, and/or the infection models. Future studies are needed to better understand what factors dictate IL-27 activity, and how IL-27 differentially shapes immune responses in the lung at different times of *A. fumigatus* infection.

In summary, IL-27 signaling plays a protective role during repeated exposure to *A. fumigatus* through promotion of type 1 immune responses. This finding is surprising, because the function of IL-27 is currently considered to be mostly immunosuppressive in infectious diseases, although IL-27 was initially identified as a proinflammatory cytokine.

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DISCLOSURES

The authors have no financial conflicts of interest.

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