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# Requisite Role for the Dectin-1 $\beta$ -Glucan Receptor in Pulmonary Defense against *Aspergillus fumigatus*<sup>1</sup>

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Immune suppression increases the incidence of invasive fungal infections, particularly those caused by the opportunistic mold *Aspergillus fumigatus*. Previous investigations revealed that members of the TLR family are not absolutely required for host defense against *A. fumigatus* in nonimmunosuppressed hosts, suggesting that other pattern recognition receptors are involved. We show in this study that naive mice (i.e., not pharmacologically immunosuppressed) lacking the  $\beta$ -glucan receptor Dectin-1 (Dectin-1<sup>-/-</sup>) are more sensitive to intratracheal challenge with *A. fumigatus* than control mice, exhibiting >80% mortality within 5 days, ultimately attributed to a compromise in respiratory mechanics. In response to *A. fumigatus* challenge, Dectin-1<sup>-/-</sup> mice demonstrated impaired IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ , CCL3/MIP-1 $\alpha$ , CCL4/MIP-1 $\beta$ , and CXCL1/KC production, which resulted in insufficient lung neutrophil recruitment and uncontrolled *A. fumigatus* lung growth. Alveolar macrophages from Dectin-1<sup>-/-</sup> mice failed to produce proinflammatory mediators in response to *A. fumigatus*, whereas neutrophils from Dectin-1<sup>-/-</sup> mice had impaired reactive oxygen species production and impaired killing of *A. fumigatus*. We further show that IL-17 production in the lung after *A. fumigatus* challenge was Dectin-1 dependent, and that neutralization of IL-17 significantly impaired *A. fumigatus* clearance. Collectively, these results support a requisite role for Dectin-1 in *in vivo* defense against *A. fumigatus*. *The Journal of Immunology*, 2009, 182: 4938–4946.

Patients with a history of solid organ or hematopoietic cell transplantation are at high risk for developing invasive fungal infections (1). Among these, invasive pulmonary aspergillosis (IPA)<sup>3</sup> caused by the opportunistic fungal pathogen *Aspergillus fumigatus* is associated with an exceptional mortality rate. Predisposition to IPA is associated with impairments in innate immunity, primarily neutropenia (2, 3). In patients with hematologic malignancies, infections caused by *A. fumigatus* have increased from 0.9 to 2.9% between 1989 and 2003 (4). However, data are beginning to uncover an increase in IPA in nonneutropenic intensive care unit patients who do not have classic risk factors. Recent studies report an appreciable incidence of IPA in patients with chronic obstructive pulmonary disease (5, 6). Additionally, a recent study indicated the development of IPA in patients that were not neutropenic and had underlying diseases other than hematologic cancer, highlighting an increasing role for *A.*

*fumigatus* as a cause of hospital-acquired pneumonia in seemingly immunocompetent patients (7).

Upon inhalation of *A. fumigatus* conidia into the lungs, initial recognition is the responsibility of the alveolar macrophage. Organisms that escape the effector functions of alveolar macrophages are subsequently targeted by neutrophils. An early landmark study showed that *A. fumigatus* conidia were more efficiently handled by macrophages, whereas neutrophils were the most effective against *A. fumigatus* germinating conidia/hyphae (8). Recognition of *A. fumigatus* by myeloid cells is reported to involve TLRs (9) and Dectin-1 (10–12). We have previously reported that interruption of *A. fumigatus* recognition by the  $\beta$ -glucan receptor Dectin-1 attenuated alveolar macrophage-inflammatory responses to *A. fumigatus* *in vitro* (10). Dectin-1 is a 43-kDa, type II transmembrane receptor containing a single cytoplasmic immunoreceptor tyrosine activation motif and a single extracellular C-type lectin recognition domain, and is the predominant receptor for  $\beta$ -1,3 glucans (13). In both humans (14) and mice (15), Dectin-1 is highly expressed on macrophages, neutrophils, and dendritic cells.

Significant effort has been placed on delineating the role of innate receptors, particularly TLRs, in host defense against *A. fumigatus*. *In vitro* studies have primarily focused on the role of TLR2 and TLR4, with most studies showing a more prominent role for TLR2 (9). However, we have previously reported that TLR2-deficient alveolar macrophages have an intact inflammatory response to *A. fumigatus* (10). *In vivo*, MyD88-, TLR2-, or TLR4-deficient mice (16) have each been reported to have higher susceptibility to *A. fumigatus* lung infection, but only in the setting of neutropenia, because nonimmunosuppressed MyD88-, TLR2-, and TLR4-deficient mice do not succumb to infection (17). However, a subsequent study has reported delayed lung clearance of *A. fumigatus* in nonimmunosuppressed MyD88-deficient mice (18). These results suggest that TLRs are essential in the absence of neutrophils, but not in an immunocompetent host, and further suggest that other pattern recognition receptors (PRRs), such as

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<sup>3</sup> Abbreviations used in this paper: IPA, invasive pulmonary aspergillosis; BAL, bronchoalveolar lavage; PRR, pattern recognition receptor; ROS, reactive oxygen species; WT, wild type.

Dectin-1, may be more critical for innate defense against *A. fumigatus*. In our previous study, and confirmed by others (11, 12), Dectin-1 expressed by alveolar macrophages recognized unmasked  $\beta$ -glucan moieties in *A. fumigatus* swollen and germinating conidia, which led to a potent inflammatory response (10). We further showed that an attempt to block Dectin-1 in vivo after *A. fumigatus* challenge using soluble Dectin-1 resulted in moderate reductions in inflammatory mediator levels and a 30% increase in *A. fumigatus* lung burden, suggesting that Dectin-1 may have a role in the control of this organism in vivo (10). Therefore, in this study, we determined the consequences of Dectin-1 deficiency on lung host defense against invasive fungal disease caused by *A. fumigatus*.

## Materials and Methods

### Mice

Male 129/SvEv mice, 6–8 wk of age, were purchased from Taconic Farms. Dectin-1<sup>-/-</sup> mice were generated on the 129/SvEv background, as previously described (19), and bred at Taconic Farms. All mice were maintained in a specific pathogen-free environment in microisolation cages within an American Association for Laboratory Animal Science-certified animal facility in the Lyons Harrison Research Building at the University of Alabama Birmingham. Animal studies were reviewed and approved by the University of Alabama Birmingham Institutional Animal Care and Use Committee.

### Preparation of *A. fumigatus* conidia

*A. fumigatus* isolate 13073 (American Type Culture Collection) was maintained on potato dextrose agar for 5–7 days at 37°C. Conidia were harvested by washing the culture flask with 50 ml of sterile PBS supplemented with 0.1% Tween 20. The conidia were then passed through a sterile 40- $\mu$ m nylon membrane to remove hyphal fragments and enumerated on a hemacytometer.

### In vivo *A. fumigatus* challenge, tissue burden assessment, and histology

Mice were lightly anesthetized with isoflurane and administered 5–7  $\times$  10<sup>7</sup> *A. fumigatus* conidia in a volume of 50  $\mu$ l intratracheally. For survival studies, mice were monitored every 6 h post-*A. fumigatus* challenge and sacrificed when appearing moribund. For lung fungal burden analysis, the left lungs were collected and homogenized in 1 ml of PBS. Total RNA was extracted from 0.1 ml of unclarified lung homogenate using the MasterPure yeast RNA purification kit (Epicentre Biotechnologies), which includes a DNase treatment step to eliminate genomic DNA, as previously reported (20). Lung *A. fumigatus* burden was analyzed with real-time PCR measurement of the *A. fumigatus* 18S rRNA (GenBank accession B008401) (21) and quantified using a standard curve of *A. fumigatus* conidia (10<sup>1</sup>–10<sup>9</sup>), as previously described (20). Specifically, total RNA was isolated using the MasterPure kit from serial 1/10 dilutions of *A. fumigatus* conidia beginning with 10<sup>9</sup>, and real-time PCR amplification of *A. fumigatus* 18S rRNA was performed on each dilution. As a validation of the real-time PCR method, heat-killed *A. fumigatus* did not yield a signal by real-time PCR and were unable to grow on potato dextrose agar plates. In addition, no amplification controls (i.e., no reverse transcriptase included in the cDNA reaction) yielded a signal of <0.001% by real-time PCR, indicating that the DNase treatment step was efficient at eliminating contaminating *A. fumigatus* DNA. For lung neutrophil recruitment, mice were challenged with *A. fumigatus*, and a bronchoalveolar lavage (BAL) was performed 24 h thereafter, as previously described (10). Lavage cells were enumerated on a hemacytometer and then stained with the neutrophil marker Gr-1. Flow cytometry was performed to quantify the percentage of Gr-1-positive cells. For lung histology, the left lungs were collected and fixed in 4% formalin. The fixed lungs were paraffin embedded and then processed and stained by the Comparative Pathology Laboratory at the University of Alabama. Imaging was performed using a Nikon Eclipse 90i microscope and Nikon NIS-Elements imaging analysis software.

### Pulmonary function assessment

A tracheostomy was performed on individual anesthetized *A. fumigatus*-exposed wild-type (WT) and Dectin-1<sup>-/-</sup> mice. Each animal was then attached to a computer-controlled volume ventilator (flexiVent; SCIREQ). Regular breathing was set at 150 bpm, with volume and pressure controlled by the flexiVent system based on individual animal weights. Positive end-

expiratory pressure was set to 2 cm H<sub>2</sub>O and measured during each breath stroke. Respiratory input impedance (Zrs) was measured using the forced oscillation technique controlled by the flexiVent system (22). The single-compartment model was used to describe whole lung resistance (R), and the constant phase model was used to describe Newtonian resistance (R<sub>N</sub>). All measurements were collected at baseline and after a linear dose response with methacholine challenge (10–40 mg/ml). No significant differences were observed in baseline and methacholine-challenged R<sub>N</sub> or R between WT and Dectin-1<sup>-/-</sup> mice in the absence of infection (*p* values for R<sub>N</sub> were 0.936 and 0.171 for 0 and 40 mg/ml methacholine; *p* values for R were 0.601 and 0.26 for 0 and 40 mg/ml methacholine; *n* = 3–4 mice per group).

### Alveolar macrophage isolation and culture

For alveolar macrophage isolation, WT or Dectin-1<sup>-/-</sup> mice were anesthetized with i.p. ketamine/xylazine and sacrificed by exsanguination. Thereafter, lungs were lavaged through an intratracheal catheter with prewarmed (37°C) calcium and magnesium-free PBS supplemented with 0.6 mM EDTA. A total of 10 ml was used in each mouse in 0.5-ml increments with a 30-s dwell time. The lavage fluids were pooled and centrifuged at 300  $\times$  g for 10 min, and the cells were collected for *A. fumigatus* coculture. Alveolar macrophages (2–3  $\times$  10<sup>6</sup>) were cocultured with *A. fumigatus* conidia at a ratio of 1:1 for 24 h in a 96-well plate at 37°C, 5% CO<sub>2</sub>. Controls for spontaneous cytokine and chemokine production included alveolar macrophages cultured in medium alone. Thereafter, the contents of each well were collected and the supernatants were stored at –80°C until Bio-Plex cytokine and chemokine analysis.

### Neutrophil isolation and culture

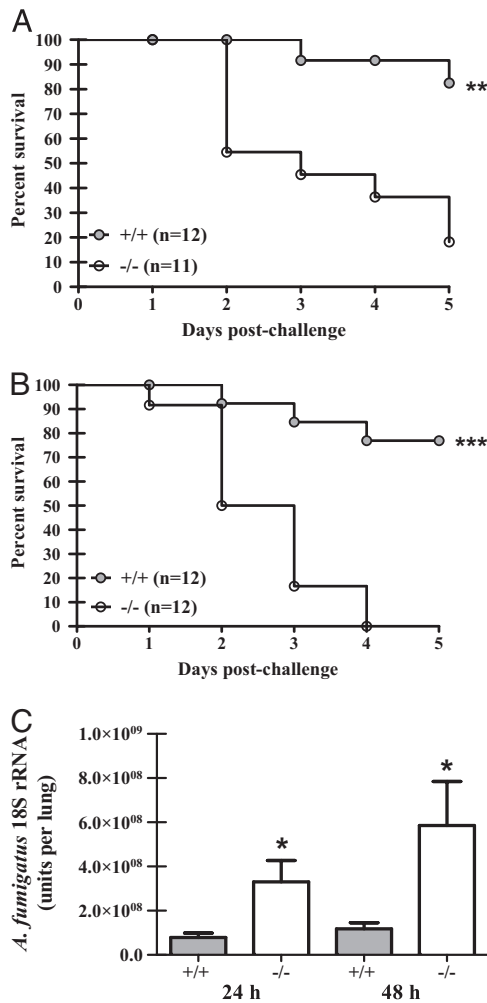
Thioglycolate-elicited peritoneal exudate cells were collected after 14 h from at least two mice per group by peritoneal lavage with ice-cold RPMI 1640 medium plus 10% heat-inactivated FCS. Pooled cells were used as a source of inflammatory granulocytes (mainly neutrophils) and monocytes. Inflammatory leukocytes (2  $\times$  10<sup>6</sup>) were mixed with live *A. fumigatus* swollen conidia (1  $\times$  10<sup>7</sup>), which were kept for 60 min at 4°C to allow the cells to settle, before being transferred to an incubator at 37°C for an additional 60 min. For analysis of hydrogen peroxide generation, inflammatory cells were loaded with dihydrorhodamine 123 at a final concentration of 1  $\mu$ M. After incubation with live *A. fumigatus* swollen conidia, the conversion of dihydrorhodamine 123 was assessed by flow cytometry and was expressed as mean fluorescent intensity (19). Cells loaded with dihydrorhodamine 123, but not treated with conidia, were used to assess background hydrogen peroxide production. For *A. fumigatus* killing in vitro, inflammatory leukocytes (1  $\times$  10<sup>5</sup>) were cocultured at a 1:1 E:T ratio with live *A. fumigatus* swollen/germinating conidia (10) for 2–4 h, followed by RNA isolation with the MasterPure yeast RNA purification kit, as described above. Controls included *A. fumigatus* swollen/germinating conidia cultured in the absence of inflammatory leukocytes for 2–4 h. To generate a standard curve, total RNA was isolated from serial 1/10 dilutions of *A. fumigatus* swollen/germinating conidia beginning at 10<sup>6</sup> that were simultaneously cultured for 2–4 h in the cell-killing assay, and real-time PCR amplification of *A. fumigatus* 18S rRNA was performed on each dilution.

### Analysis of IL-17

WT and Dectin-1<sup>-/-</sup> mice were challenged intratracheally with 7  $\times$  10<sup>7</sup> *A. fumigatus* conidia, and IL-17 levels were assessed in lung homogenates at 24 h by Bio-Plex. For in vivo IL-17 neutralization, mice were challenged intratracheally with 7  $\times$  10<sup>7</sup> *A. fumigatus* conidia in 50  $\mu$ l, and 6 h thereafter, mice were administered 50  $\mu$ g of rat anti-mouse IL-17A (clone 50104; R&D Systems) (23) or rat IgG2a isotype control Ab. Twenty-four or 48 h after challenge, mice were sacrificed, and the left lungs were collected and homogenized in 1 ml of PBS. Total RNA was extracted from 0.1 ml of unclarified lung homogenate using the MasterPure yeast RNA purification kit, and lung *A. fumigatus* burden was analyzed with real-time PCR measurement of the *A. fumigatus* 18S rRNA.

### Cytokine and chemokine quantification

For lung homogenates, the right lung was homogenized in PBS supplemented with Complete Mini protease inhibitor tablets (Roche), clarified by centrifugation, and stored at –80°C. Samples were analyzed for protein levels of a panel of cytokines and chemokines using Luminex-based Bio-Plex multiplex suspension protein array (Bio-Rad Laboratories), according to the manufacturer's instructions, as previously described (10). Concentrations of each cytokine and chemokine were determined using Bio-Plex Manager version 4.1.1 software. In specific experiments, levels of IL-23 in lung homogenates were quantified by ELISA (R&D Systems).



**FIGURE 1.** Enhanced susceptibility of Dectin-1-deficient mice after challenge with *A. fumigatus*. WT and Dectin-1<sup>-/-</sup> mice were challenged intratracheally with  $5 \times 10^7$  conidia in a volume of 50  $\mu$ l (A), or  $7 \times 10^7$  conidia in a volume of 50  $\mu$ l (B), and survival was monitored for 5 days. This figure illustrates cumulative results from two independent studies per inoculum dose. \*\*\*, Represents a *p* value of <0.001 (log rank Mantel-Cox test). C, Real-time PCR analysis of *A. fumigatus* 18S rRNA levels in the lungs of WT and Dectin-1<sup>-/-</sup> mice challenged intratracheally with  $7 \times 10^7$  conidia and collected at 24 and 48 h postchallenge. This figure illustrates cumulative data from three independent studies (*n* = 5 mice/group for each study). Data are expressed as mean *A. fumigatus* 18S rRNA levels + SEM. \*, Represents a *p* value of <0.05 (unpaired two-tailed Student's *t* test).

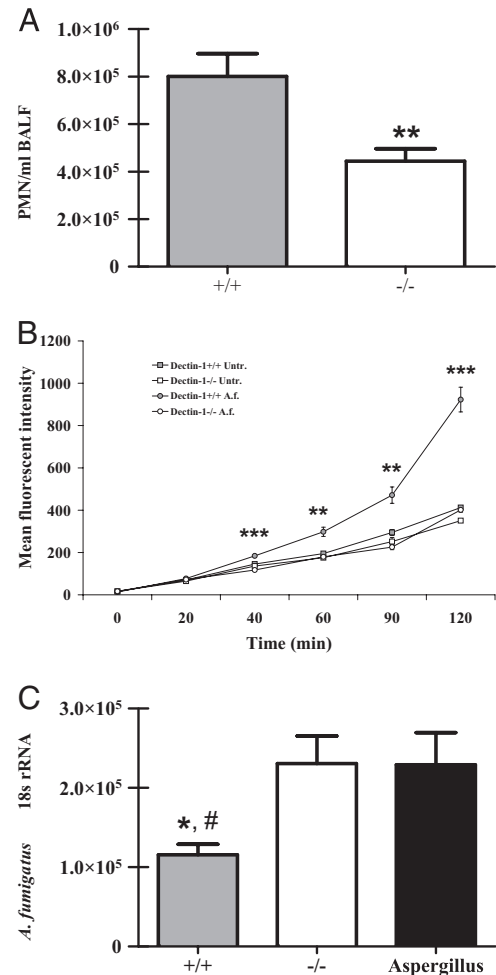
### Statistics

Data were analyzed using GraphPad Prism version 5.0 statistical software. Comparisons between groups when data were normally distributed were made with Student's *t* test. Comparisons among nonparametric data were made with the Mann-Whitney *U* test. Survival analysis was performed using an asymmetrical 95% confidence interval for the Gehan-Breslow-Wilcoxon and Mantel-Cox log rank tests. Significance was accepted at a value of *p* < 0.05.

## Results

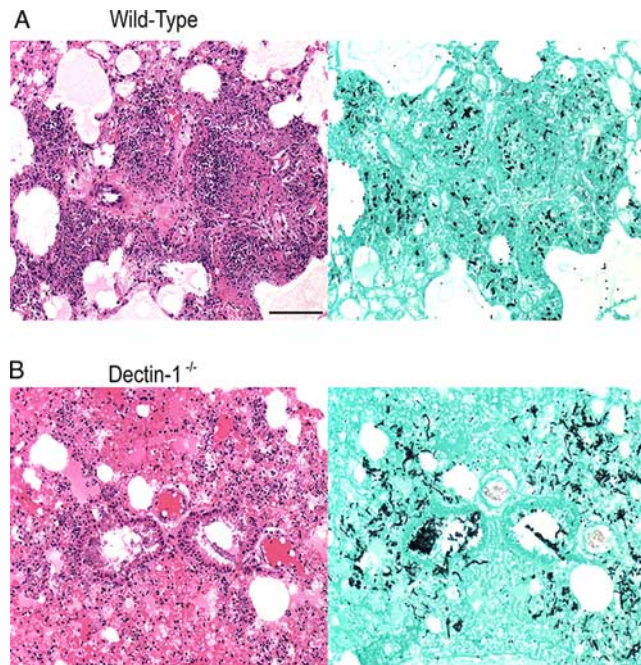
### Enhanced susceptibility of Dectin-1-deficient mice after challenge with *A. fumigatus*

We first examined whether Dectin-1 was required for defense against the opportunistic mold *A. fumigatus*. Intratracheal administration of  $5 \times 10^7$  *A. fumigatus* conidia resulted in rapid mortality in Dectin-1<sup>-/-</sup> mice, with greater than 60% of the mice succumbing by 4 days postchallenge (Fig. 1A). Increasing the inoculum to



**FIGURE 2.** Defective *A. fumigatus* recognition by Dectin-1-deficient neutrophils. A, Neutrophil levels in BAL fluid were assessed by flow cytometry of individual mice 24 h after intratracheal challenge with  $7 \times 10^7$  conidia. This figure illustrates data from one of two independent studies (*n* = 5 mice/group). Data are expressed as neutrophils/ml BAL fluid + SEM. \*\*, *p* < 0.01 (unpaired two-tailed Student's *t* test). B, Neutrophils were isolated from the peritoneum of WT and Dectin-1<sup>-/-</sup> mice challenged with 3% thioglycolate for 14 h and cocultured with live *A. fumigatus* swollen conidia for 2 h. ROS production was assessed using dihydrorhodamine 123 conversion. This figure illustrates cumulative data in triplicate wells from one of two independent studies. Data are expressed as mean fluorescent intensity ± SEM. \*\* and \*\*\*, Represent *p* values of <0.01 and 0.001, respectively (unpaired two-tailed Student's *t* test). C, Peritoneal neutrophils were isolated and cocultured with live *A. fumigatus* swollen conidia at a cell to organism ratio of 1:1 for 2–4 h. Controls included live *A. fumigatus* swollen conidia cultured in medium alone for 2–4 h. Thereafter, RNA was isolated from WT, Dectin-1<sup>-/-</sup>, and *A. fumigatus* cultures, and *A. fumigatus* 18S rRNA levels were quantified by real-time PCR. This figure illustrates cumulative results from two independent studies, each containing triplicate wells for WT, Dectin-1<sup>-/-</sup>, and *A. fumigatus* cultures. Data are expressed as mean *A. fumigatus* 18S rRNA levels + SEM. \*, Represents a *p* value of <0.05 comparing WT with *Aspergillus*; #, represents a *p* value of <0.05 comparing WT with Dectin-1<sup>-/-</sup> (unpaired two-tailed Student's *t* test).

$7 \times 10^7$  resulted in 100% mortality in Dectin-1<sup>-/-</sup> mice by 4 days (Fig. 1B). Real-time PCR measurement of *A. fumigatus* 18S rRNA in lung tissue, recently demonstrated as the most sensitive method for determination of lung fungal burden in experimental aspergillosis (24), showed >4-fold higher *A. fumigatus* burden in Dectin-1<sup>-/-</sup> mice at 24 and 48 h (Fig. 1C). Analysis of kidney and liver

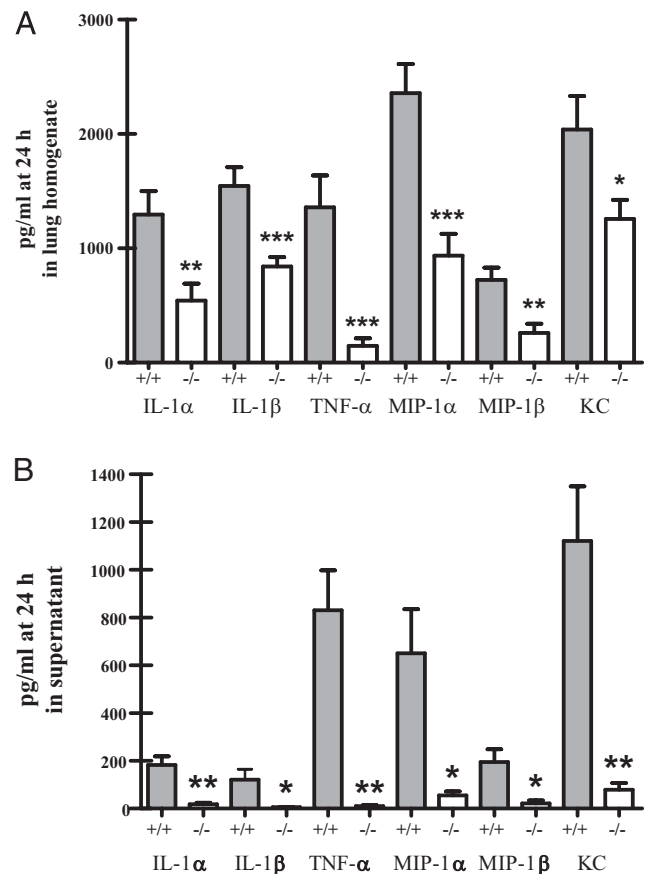


**FIGURE 3.** Histological evidence for *A. fumigatus* invasion and lung damage in Dectin-1-deficient mice. Representative H&E-stained (left) and Grocott's methenamine silver-stained (right) lung sections from WT mice (A) and Dectin-1<sup>-/-</sup> mice (B) challenged intratracheally with  $7 \times 10^7$  conidia for 48 h. Original magnification  $\times 200$ . Scale bar in A, left image, represents 100  $\mu\text{m}$ .

tissue in these animals indicated little *A. fumigatus* burden ( $<10^2$  in either organ) and no difference between WT and Dectin-1<sup>-/-</sup> mice (data not shown). Thus, Dectin-1 deficiency results in enhanced *A. fumigatus* growth in the lungs, but does not predispose to systemic infection that might otherwise explain the increased mortality.

#### Defective *A. fumigatus* recognition by Dectin-1-deficient neutrophils

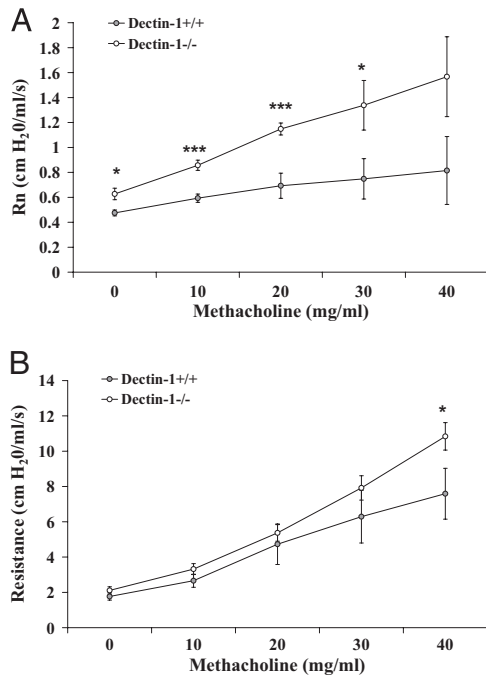
Neutrophil deficiency/dysfunction is the hallmark predisposing factor for the development of invasive pulmonary aspergillosis (2, 3). Because Dectin-1<sup>-/-</sup> mice demonstrated increased mortality and higher lung burden after *A. fumigatus* challenge, we questioned whether defects existed in neutrophil recruitment and/or neutrophil function. WT and Dectin-1<sup>-/-</sup> mice were challenged with *A. fumigatus* ( $7 \times 10^7$ ), and neutrophil levels in bronchoalveolar fluid were assessed at 24 h. Results in Fig. 2A show that a 50% reduction in neutrophil recruitment to the lungs was observed in *A. fumigatus*-challenged Dectin-1<sup>-/-</sup> mice. Previous studies have shown that mice deficient in NADPH oxidase (gp91<sup>phox</sup>-deficient mice) are highly susceptible to infection with *A. fumigatus* (25), despite having no impairment in killing of *A. fumigatus* by alveolar macrophages (26). More recent studies have shown extensive *A. fumigatus* germination in neutrophil aggregates from gp91<sup>phox</sup>-deficient mice, but negligible germination in alveolar macrophages (27), suggesting that oxidative responses by neutrophils are critical for the prevention of *A. fumigatus* germination. A correlate of this is the observation that thioglycolate-elicited peritoneal neutrophils from uninfected Dectin-1<sup>-/-</sup> mice failed to activate NADPH oxidase in response to live *A. fumigatus* swollen/germinating conidia in vitro (Fig. 2B). The lack of reactive oxygen species (ROS) production further correlated with an inability of Dectin-1<sup>-/-</sup> neutrophils to kill *A. fumigatus* in vitro (Fig. 2C).



**FIGURE 4.** Requirement for Dectin-1 in the inflammatory response to *A. fumigatus* in vivo and in vitro. A, WT and Dectin-1<sup>-/-</sup> mice were challenged intratracheally with  $7 \times 10^7$  conidia, and 24 h after exposure, IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ , CCL3/MIP-1 $\alpha$ , CCL4/MIP-1 $\beta$ , and CXCL1/KC levels were quantified in lung homogenates by Bio-Plex. This figure illustrates cumulative data from four independent studies ( $n = 5$  mice/group for each study). Data are expressed as mean pg/ml  $\pm$  SEM. \*, \*\*, and \*\*\*, Represent  $p$  values of  $<0.05$ ,  $0.01$ , and  $0.001$ , respectively (unpaired two-tailed Student's  $t$  test). B, Alveolar macrophages were isolated via BAL from WT or Dectin-1<sup>-/-</sup> mice and cultured with live *A. fumigatus* conidia for 24 h at a macrophage to conidium ratio of 1:1. Cytokine/Chemokine concentrations in coculture supernatants were determined by Bio-Plex. Unstimulated levels have been subtracted from each column. This figure illustrates cumulative results from four independent studies. Data are expressed as mean pg/ml  $\pm$  SEM. \* and \*\*, Represent  $p$  values of  $<0.05$  and  $0.01$ , respectively (unpaired two-tailed Student's  $t$  test).

Thus, one possible mechanism of the susceptibility observed in Dectin-1<sup>-/-</sup> mice may be impaired neutrophil oxidative killing of *A. fumigatus*.

H&E staining of lung tissue sections revealed a profound, intense inflammatory response in WT mice (Fig. 3A, left; 48 h). In contrast, inflammatory cell recruitment was significantly lower in Dectin-1<sup>-/-</sup> mice, despite the increased growth of *A. fumigatus* growth (Fig. 3B, left). Moreover, dramatic alveolar edema, capillary congestion, and proteinaceous exudate deposition in the alveolar space were observed in Dectin-1<sup>-/-</sup> mice (Fig. 3B, left; 48 h). Grocott's methenamine silver staining of lung tissue at 48 h revealed considerable *A. fumigatus* germinating conidia and hyphal masses in Dectin-1<sup>-/-</sup> mice (Fig. 3B, right). In contrast, fewer germinating conidia and relatively few hyphae were observed in WT mice (Fig. 3A, right). Germinating *A. fumigatus* was observed in Dectin-1<sup>-/-</sup> mice in areas containing inflammatory cells, such as neutrophils, albeit significantly fewer in number compared with



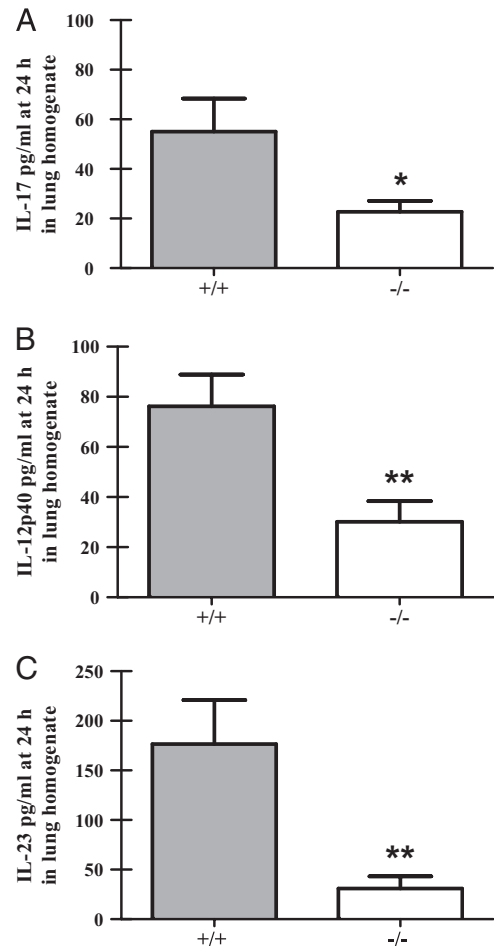
**FIGURE 5.** Compromised lung function in *A. fumigatus*-challenged Dectin-1-deficient mice. WT and Dectin-1<sup>-/-</sup> mice were challenged intratracheally with  $4 \times 10^7$  conidia, and 72 h after exposure, respiratory mechanics were analyzed via mechanical ventilation using the flexiVent system. Pulmonary function measurements were airway resistance ( $R_N$ ) (A) and whole lung resistance (R) (B). This figure illustrates cumulative data from two independent studies ( $n = 5$  mice/group for each study). Data are expressed as mean airway or whole lung resistance  $\pm$  SEM. \* and \*\*\*, Represent  $p$  values of  $<0.05$  and  $<0.001$ , respectively (unpaired two-tailed Student's  $t$  test).

WT mice, which supports the *in vitro* data demonstrating impaired responses to *A. fumigatus* by Dectin-1<sup>-/-</sup> neutrophils.

#### Requirement of Dectin-1 for the inflammatory response to *A. fumigatus* *in vivo* and *in vitro*

We next investigated proinflammatory cytokine and chemokine levels in lung homogenates to better understand inflammatory responses occurring early after exposure. Results showed that *A. fumigatus* elicited a robust inflammatory response in the lungs of WT mice at 24 h after challenge, characterized by the production of IL-1 $\alpha$ , IL-1 $\beta$ , and TNF- $\alpha$  (Fig. 4A). Each of these was significantly reduced in the lungs of Dectin-1<sup>-/-</sup> mice. The response to *A. fumigatus* in WT mice was also associated with significant production of CCL3/MIP-1 $\alpha$ , CCL4/MIP-1 $\beta$ , and CXCL1/KC, chemokines essential for neutrophil recruitment during *A. fumigatus* lung infection (28, 29) (Fig. 4A). Again, Dectin-1<sup>-/-</sup> mice had significant reductions in these mediators in response to *A. fumigatus*. Thus, Dectin-1 is required for optimal lung proinflammatory cytokine and chemokine production after *A. fumigatus* challenge.

Because alveolar macrophages are a critical source for proinflammatory cytokine and chemokine production in response to *A. fumigatus* (30), we isolated alveolar macrophages from uninfected WT and Dectin-1<sup>-/-</sup> mice and assessed cytokine and chemokine production in response to live *A. fumigatus* *in vitro*. *A. fumigatus* elicited robust production of IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ , CCL3/MIP-1 $\alpha$ , CCL4/MIP-1 $\beta$ , and CXCL1/KC from WT alveolar macrophages (Fig. 4B). In stark contrast, Dectin-1<sup>-/-</sup> alveolar macrophages were virtually unresponsive to *A. fumigatus*, despite undergoing stimulation with *A. fumigatus* for 24 h. Similar results

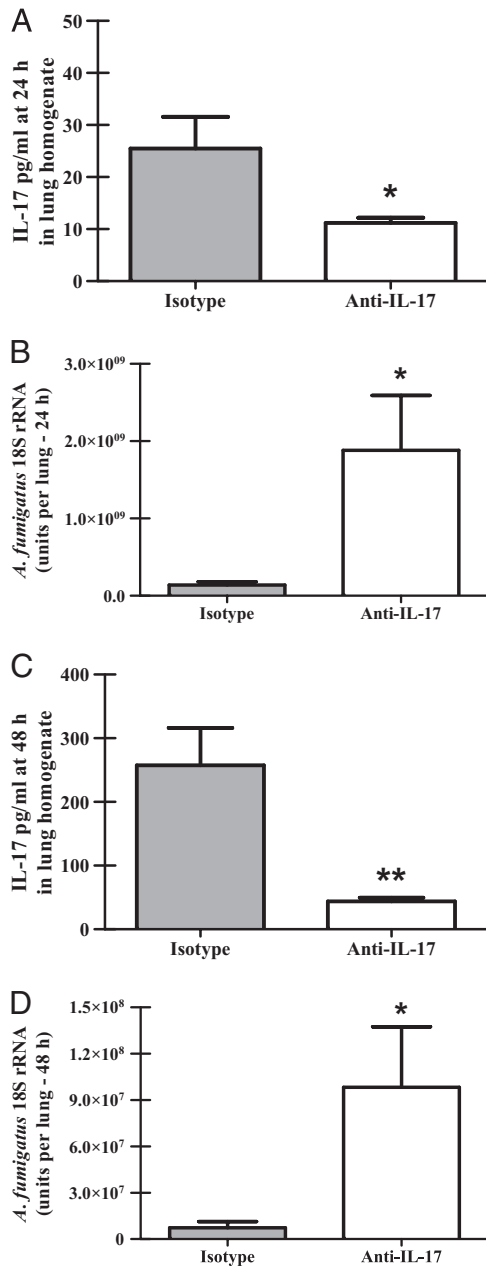


**FIGURE 6.** IL-17 levels in the lungs of Dectin-1-deficient mice exposed to *A. fumigatus*. WT and Dectin-1<sup>-/-</sup> mice were challenged intratracheally with  $7 \times 10^7$  conidia, and 24 h after exposure, IL-17 (A), IL-12p40 (B), and IL-23 (C) levels were quantified in lung homogenates. This figure illustrates cumulative data from three independent studies ( $n = 5$  mice per group per time point). Data are expressed as mean pg/ml  $\pm$  SEM. \* and \*\*, Represent  $p$  values of  $<0.05$  and  $0.01$ , respectively (unpaired two-tailed Student's  $t$  test).

were observed when coculturing *A. fumigatus* swollen conidia (10) with Dectin-1<sup>-/-</sup> alveolar macrophages for 6 h (data not shown). The hyporesponsiveness of Dectin-1<sup>-/-</sup> macrophages is specific to  $\beta$ -glucan containing ligands/organisms because responses to ligands for TLR2 and TLR4 have been shown to be unaffected in Dectin-1<sup>-/-</sup> macrophages (19) (data not shown). These results suggest that alveolar macrophages are a likely source of proinflammatory cytokine and chemokine production during *A. fumigatus* infection *in vivo*.

#### Compromised lung function in *A. fumigatus*-challenged Dectin-1-deficient mice

Histological assessment of Dectin-1<sup>-/-</sup> mice indicated *A. fumigatus* overgrowth and invasiveness in conjunction with severe alveolar damage. We therefore assessed measurements of respiratory mechanics to determine whether histological changes observed in Dectin-1<sup>-/-</sup> mice after *A. fumigatus* challenge correlated with a worsening in pulmonary function. For this, mice were challenged with  $4 \times 10^7$  *A. fumigatus* conidia to allow for enough survival through 3 days postchallenge, and pulmonary function was determined using the flexiVent system. Measurement of airway resistance indicated a functional compromise in the airways of *A. fumigatus*-challenged Dectin-1<sup>-/-</sup> mice at baseline, which was



**FIGURE 7.** Neutralization of IL-17 impairs early *A. fumigatus* lung clearance. WT and Dectin-1<sup>-/-</sup> mice were challenged intratracheally with  $7 \times 10^7$  conidia. Six hours after challenge, mice were administered 50  $\mu$ g of rat anti-mouse IL-17 or rat IgG2A Abs intratracheally. IL-17 levels were quantified in lung homogenates 24 (A) and 48 (C) h after challenge by Bio-Plex. This figure illustrates cumulative data from two independent studies ( $n = 5$  mice per group per time point). Data are expressed as mean pg/ml + SEM. \* and \*\*, Represent  $p$  values of <0.05 and 0.01, respectively (unpaired two-tailed Student's  $t$  test). Real-time PCR analysis of *A. fumigatus* 18S rRNA levels in the lungs of WT and Dectin-1<sup>-/-</sup> mice administered anti-IL-17 or isotype control Abs 24 (B) and 48 (D) h after challenge. This figure illustrates cumulative data from two independent studies ( $n = 5$  mice per group per time point). Data are expressed as mean *A. fumigatus* 18S rRNA + SEM. \*, Represents a  $p$  value of <0.05 (unpaired two-tailed Student's  $t$  test).

amplified upon treatment with the bronchoconstrictor methacholine (Fig. 5A). Whole lung resistance was also found to be elevated in *A. fumigatus*-challenged Dectin-1<sup>-/-</sup> mice (Fig. 5B). No differences were observed in baseline and methacholine-challenged airway resistance or whole lung resistance between WT and Dec-

tin-1<sup>-/-</sup> mice in the absence of infection (data not shown). Thus, *A. fumigatus* infection in Dectin-1<sup>-/-</sup> mice is associated with increases in lung resistance.

#### *IL-17 production in Dectin-1-deficient mice exposed to A. fumigatus*

Recent studies using mice deficient in CARD9, a component in the signaling cascade of many immunoreceptors (31), including Dectin-1 (32, 33), have implicated a role for CARD9/Dectin-1 in generating protective Th17 responses during fungal infection (33). However, recent studies suggest an immunopathogenic, rather than protective, role for Th17/IL-17 during *A. fumigatus* infection (23, 34). Because IL-17 production has yet to be specifically characterized in Dectin-1<sup>-/-</sup> mice, we assessed IL-17 levels in WT and Dectin-1<sup>-/-</sup> mice 24 h after *A. fumigatus* levels in lung homogenates. Results in Fig. 6A show that Dectin-1<sup>-/-</sup> mice have lower IL-17 levels in the lungs 24 h after *A. fumigatus* challenge. IL-12p40 (Fig. 6B), a subunit of IL-23 (35), and IL-23 (Fig. 6C), which is required for Th17 development (35), were also observed to be significantly lower in Dectin-1<sup>-/-</sup> mice. Thus, Dectin-1<sup>-/-</sup> mice have impaired Th17 responses during fungal infection, putatively as a result of impaired IL-23 production.

#### *Neutralization of IL-17 impairs early A. fumigatus lung clearance*

Neutralizing IL-17 in vivo has been shown to augment clearance of *A. fumigatus* in both normal and p47<sup>phox</sup>-deficient mice (34). Our data indicate a requirement for Dectin-1 in IL-17 production early after *A. fumigatus* challenge (Fig. 6A). We speculate that the lower levels of IL-17 in Dectin-1<sup>-/-</sup> mice early after challenge, in conjunction with the lower levels of other proinflammatory mediators (Fig. 4A), may allow *A. fumigatus* infection to thrive. To address the role of IL-17 early, we sought to determine whether neutralizing IL-17 affected the development of *A. fumigatus* lung infection in WT mice. For this, we challenged mice with  $7 \times 10^7$  *A. fumigatus* conidia, and 6 h postchallenge, administered 50  $\mu$ g of rat anti-mouse IL-17 Ab intratracheally (23). At 24 h postchallenge, mice were sacrificed and assessed for *A. fumigatus* lung burden. Treatment of mice with neutralizing Abs significantly lowered IL-17 levels in the lungs at 24 h (Fig. 7A) and resulted in a dramatic increase in *A. fumigatus* lung burden (Fig. 7B). Similar results were also observed at 48 h (Fig. 7, C and D). Thus, IL-17 is involved in clearance of *A. fumigatus* from the lung.

## Discussion

When exposed to *Pneumocystis carinii*, mice deficient in Dectin-1 (Dectin-1<sup>-/-</sup>) show an early impairment in lung clearance, yet clear the organism similar to WT mice (36). Furthermore, Dectin-1<sup>-/-</sup> mice are not more susceptible to lung infection with the fungal organism *Cryptococcus neoformans* (37). These results may likely be explained by the  $\beta$ -glucan content of these organisms, because *P. carinii* cysts have a much higher  $\beta$ -glucan content than the more numerous trophozoites (38, 39) and *C. neoformans* not only possesses a large capsule, but has much lower  $\beta$ -1,3-glucan content than most Dectin-1-dependent fungal organisms (37). Nevertheless, because these studies suggest a moderate role for Dectin-1 in lung defense against fungal pathogens, we questioned whether Dectin-1 was required for defense against a fungal pathogen acknowledged for its invasiveness, *A. fumigatus*. We found that mice deficient in Dectin-1 rapidly succumbed to invasive pulmonary disease caused by *A. fumigatus* as a result of functional defects in specific cell populations universally acknowledged to be

required for clearing the organism from the lung. Dectin-1<sup>-/-</sup> alveolar macrophages had compromised production of proinflammatory cytokines and chemokines that were essential for neutrophil mobilization to the lung. Neutrophils in Dectin-1<sup>-/-</sup> mice were recruited to the lung in lower numbers and displayed defective antifungal defenses in vitro, which most likely promoted invasion of *A. fumigatus*, leading to dramatic changes in lung architecture and subsequently impaired lung function.

The phenotype of Dectin-1<sup>-/-</sup> mice challenged with *A. fumigatus* was dramatic, with these mice having 80–100% mortality within 5 days after exposure. One potential caveat of our study is using high inocula of *A. fumigatus* to achieve mortality differences in Dectin-1<sup>-/-</sup> mice. Our mortality experiments were performed with inocula of  $5\text{--}7 \times 10^7$ , based on published reports using inocula at this level when assessing mortality and fungal clearance in animals that are not dually immunosuppressed with cyclophosphamide and cortisone. For example, several publications assessing the role of PRRs, such as TLRs or pentraxin 3, in defense against *A. fumigatus* administered inocula of  $4\text{--}6 \times 10^7$  *A. fumigatus* conidia (16, 40) and even as high as  $2 \times 10^8$  (41). It is important to note that our experiments were not conducted in the presence of immunosuppressive drugs (cyclophosphamide, cortisone acetate, etc.) or during transient neutropenia (i.e., Ab-mediated depletion of neutrophils). Some studies using a variety of genetically deficient mice have reported dramatic mortality phenotypes after *A. fumigatus* challenge under immunosuppressive conditions; however, our position was that induction of immunosuppression before infection may exaggerate the role of Dectin-1. A surprising observation in our studies was that despite these inocula, Dectin-1<sup>-/-</sup> mice did not have significant extrapulmonary dissemination of *A. fumigatus*. These results suggest that the rapid mortality of Dectin-1<sup>-/-</sup> mice was primarily associated with events occurring in the lung. Assessing pulmonary function supports this hypothesis because Dectin-1<sup>-/-</sup> mice suffering from *A. fumigatus* demonstrated significant increases in airway and whole lung resistance. These increases were specifically associated with *A. fumigatus* infection, because no differences were observed between WT and Dectin-1<sup>-/-</sup> mice in the absence of infection (data not shown). The significance of this correlates with clinical observations because studies have reported that individuals who are diagnosed with IPA often initially present with dyspnea (42, 43). Thus, uncontrolled *A. fumigatus* lung infection in Dectin-1<sup>-/-</sup> mice results in dramatic structural changes in lung architecture, which impairs respiratory mechanics. Furthermore, our data strongly support a fundamental role for Dectin-1 in lung clearance of *A. fumigatus* in immunocompetent mice.

The necessity for Dectin-1-mediated recognition of *A. fumigatus* was understandable when analyzing the responses of alveolar macrophages and neutrophils. Alveolar macrophages are considered first-line defenders against inhaled *A. fumigatus* and are tasked with providing the initial wave of inflammatory mediator production and inflammatory signaling (30). Alveolar macrophages from Dectin-1<sup>-/-</sup> mice were not responsive to stimulation with *A. fumigatus*, producing ~15% of the IL-1 $\alpha$ , IL-1 $\beta$ , TNF- $\alpha$ , CCL3/MIP-1 $\alpha$ , CCL4/MIP-1 $\beta$ , and CXCL1/KC levels elicited by *A. fumigatus* from WT alveolar macrophages. We further note that proinflammatory mediators produced by alveolar macrophages in a Dectin-1-dependent manner were also significantly lower in the lungs of Dectin-1<sup>-/-</sup> mice challenged with *A. fumigatus*. Previous studies investigating these cytokines and chemokines have provided critical insight into their role in lung defense against *A. fumigatus*. Neutralization of TNF- $\alpha$  has been shown to blunt lung neutrophil recruitment during *A. fumigatus* infection, resulting in delayed fungal clearance and increased mortality (44). Mice lack-

ing CCR1, the receptor for CCL3/MIP-1 $\alpha$ , are more susceptible to *A. fumigatus* lung infection, also through impaired neutrophil recruitment (45), as are mice administered CCL3/MIP-1 $\alpha$ -neutralizing Abs (29). Similarly, neutralization of CXCL1/KC also results in severe susceptibility to *A. fumigatus* infection (28). IL-1 is an understudied cytokine in lung defense against *A. fumigatus*; however, ongoing studies in our laboratory indicate that mice deficient in both IL-1 $\alpha$  and IL-1 $\beta$  are more susceptible to *A. fumigatus* (our unpublished data). Therefore, because mediators such as TNF- $\alpha$ , CCL3/MIP-1 $\alpha$ , and CXCL1/KC are involved in neutrophil recruitment to the lung during *A. fumigatus* infection (28, 44, 45) and Dectin-1 is essential for these mediators to be produced at optimal levels in vivo (Fig. 4), we can predict that Dectin-1 is involved in neutrophil mobilization to the lungs during fungal infection.

Neutrophil deficiency or dysfunction is the hallmark predisposing factor for developing IPA (46). Neutrophils kill *A. fumigatus* through a variety of different mechanisms, although the most well-studied include ROS (25), myeloperoxidase (47), and lactoferrin (48). Of these, ROS, specifically superoxide, is the most acclaimed for being indispensable for the killing of *A. fumigatus* by neutrophils. Indeed, NADPH oxidase deficiency in humans is uniquely associated with the development of IPA (49). Moreover, mice deficient in NADPH oxidase are arguably the most susceptible mouse strain for the development for experimental IPA (25, 26). We found that the oxidative burst by neutrophils in response to *A. fumigatus* swollen conidia, a morphological state that we have previously shown to have  $\beta$ -glucans unmasked at the highest levels (10), was dependent on Dectin-1. The inability of Dectin-1<sup>-/-</sup> neutrophils to produce superoxide/hydrogen peroxide correlated with an inability to kill *A. fumigatus*, suggesting that  $\beta$ -glucan recognition via Dectin-1 on neutrophils results in oxidative killing of *A. fumigatus*. Neutrophils possess additional nonoxidative antimicrobial mechanisms, such as serine proteases and antimicrobial peptides (50); therefore, we cannot eliminate the possibility that Dectin-1 is involved in the release of nonoxidative antimicrobial factors that also contribute to the killing of *A. fumigatus* observed in this study. Nevertheless, we propose that  $\beta$ -glucan recognition via Dectin-1 is a central event in eliciting alveolar macrophage and neutrophil-mediated anti-fungal defense against *A. fumigatus*.

Recent studies have identified a major component of the Dectin-1 signaling pathway as CARD9 (32). Mice deficient in CARD9 have a similar phenotype as Dectin-1<sup>-/-</sup> mice after challenge with *Candida albicans* (19, 32). First, our results partially support the finding of reduced IL-17 production in splenocyte cultures from *C. albicans*-challenged CARD9<sup>-/-</sup> mice (33), because Dectin-1<sup>-/-</sup> mice exposed to *A. fumigatus* for 24 h had lower levels of IL-17 protein in lung homogenates. We hypothesize this is due to a dependency on Dectin-1 for IL-23 production, based on the impaired lung production of IL-23 in *A. fumigatus*-challenged Dectin-1<sup>-/-</sup> mice. In fact, this is the first report to not only show impaired IL-17 production in the absence of Dectin-1, but also the first to specifically measure a defect in IL-23 production (rather than its surrogate marker, IL-12p40). With regard to the role of IL-17 in *A. fumigatus* host defense, IL-17 surprisingly appears to hamper neutrophil-mediated killing of *A. fumigatus* as well as in vivo clearance of the organism (23). Moreover, IL-17 appears to be a contributing factor to mortality observed in *A. fumigatus*-exposed p47<sup>phox</sup>-deficient mice and to a lesser extent, Tir8-deficient mice (51). In the case of p47<sup>phox</sup>-deficient mice, deficient neutrophil function leads to high *A. fumigatus* burden in the lungs, which itself may promote enhanced Ag-driven Th17 develop and subsequent immunopathology (34). These data challenge the current



dogma of IL-17 as an essential mediator for effective immune responses against microorganisms that cause infections at the mucosa (52). Furthermore, it is somewhat surprising that a proinflammatory cytokine such as IL-17 has a pronounced negative role against a pathogen, *A. fumigatus*, in which neutrophils are required for its elimination (2, 3) and neutrophil deficiency/dysfunction is the dominant predisposing factor for the incidence of disease (46). In our study, we were unable to show that neutralizing IL-17 in WT mice was beneficial for clearing *A. fumigatus* from the lungs, but rather observed that neutralizing IL-17 significantly worsened the infection. These results suggest that IL-17 is involved, at some level, in protective responses to *A. fumigatus*. Support for this comes from a recent study showing that *A. fumigatus*-sensitized TLR9<sup>-/-</sup> mice challenged with swollen conidia had higher lung fungal burden 14 days postchallenge that correlated with lower Dectin-1 mRNA levels and lower IL-17 protein levels in the lung (53). Finally, clinical data, although limited, also suggest a protective role for Th17/IL-17 in defense against *A. fumigatus*. Studies have recently demonstrated that individuals with hyper-IgE syndrome (Job's syndrome), who have mutations in STAT3 and are unable to produce Th17 cells (54), are severely susceptible to a lung infection caused by *A. fumigatus*, most often when cavitory lung lesions are present (55). However, it remains to be determined whether *A. fumigatus* infection in hyper-IgE syndrome is the result of an inherent susceptibility of these patients, cavitory lung disease, or both.

In summary, we have identified a signaling PRR that is essential for host defense against *A. fumigatus* in an immunocompetent host. Moreover, we show a comprehensive role for Dectin-1 in multiple aspects of lung immunity against *A. fumigatus*, including lung clearance, alveolar macrophage cytokine and chemokine production, neutrophil-mediated killing, and in vivo lung inflammatory responses. We further provide experimental evidence for a protective role of IL-17 in host defense against *A. fumigatus* and that Dectin-1-mediated  $\beta$ -glucan recognition is involved in the induction of Th17/IL-17 responses in the lung. We therefore feel these studies lay the foundation for further examination of Dectin-1 expression and function in individuals who are at risk for IPA, which may provide valuable insight into the role of Dectin-1 in susceptibility to IPA in immunosuppressed patients.

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## Disclosures

The authors have no financial conflict of interest.

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