Eosinophil Deficiency Compromises Lung Defense against *Aspergillus fumigatus*

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Exposure to the mold *Aspergillus fumigatus* may result in allergic bronchopulmonary aspergillosis, chronic necrotizing pulmonary aspergillosis, or invasive aspergillosis (IA), depending on the host's immune status. Neutrophil deficiency is the predominant risk factor for the development of IA, the most life-threatening condition associated with *A. fumigatus* exposure. Here we demonstrate that in addition to neutrophils, eosinophils are an important contributor to the clearance of *A. fumigatus* from the lung. Acute *A. fumigatus* challenge in normal mice induced the recruitment of CD11b⁺ Siglec F⁺ Ly-6G^{lo} Ly-6C^{neg} CCR3⁺ eosinophils to the lungs, which was accompanied by an increase in lung *Epx* (eosinophil peroxidase) mRNA levels. Mice deficient in the transcription factor *dblGATA1*, which exhibit a selective deficiency in eosinophils, demonstrated impaired *A. fumigatus* clearance and evidence of germinating organisms in the lung. Higher burden correlated with lower mRNA expression of *Epx* (eosinophil peroxidase) and *Prg2* (major basic protein) as well as lower interleukin 1 β (IL-1 β), IL-6, IL-17A, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and CXCL1 levels. However, examination of lung inflammatory cell populations failed to demonstrate defects in monocyte/macrophage, dendritic cell, or neutrophil recruitment in *dblGATA1*-deficient mice, suggesting that the absence of eosinophils in *dblGATA1*-deficient mice was the sole cause of impaired lung clearance. We show that eosinophils generated from bone marrow have potent killing activity against *A. fumigatus* *in vitro*, which does not require cell contact and can be recapitulated by eosinophil whole-cell lysates. Collectively, our data support a role for eosinophils in the lung response after *A. fumigatus* exposure.

Exposure to the opportunistic mold *Aspergillus fumigatus* may result in a wide range of infectious diseases, with the type and severity largely determined by the status of the host immune system. Among these aspergillosis infections, individuals may develop allergic bronchopulmonary aspergillosis, chronic pulmonary aspergillosis (chronic necrotizing [CNPA] or chronic cavitary pulmonary [CCPA] aspergillosis), and invasive aspergillosis (IA). Development of the last infection (IA) may be a result of multiple predisposing factors, yet immunosuppression leading to neutropenia remains the predominant risk factor (1, 2). Collectively, the most susceptible populations for the development of IA include those undergoing solid-organ or hematopoietic stem cell (HSC) transplants (3). The most recent data from the Transplant Associated Infections Surveillance Network (March 2001 to March 2006) reflect incidence rates for invasive fungal infections (IFIs) of ~7% in both solid-organ (4) (19% due to IA) and HSC (5) (43% due to IA) transplants. Although IA is a known infectious complication of these conditions, there is a growing concern for the development of nosocomial IA in nonneutropenic patients in intensive care units (ICUs), particularly those on high-dose steroids (those with chronic obstructive pulmonary disease [COPD], for instance) and those with cirrhosis and solid cancers (6). Subsequent studies have identified “new” nonneutropenic conditions associated with the development of IA as COPD, chronic steroid use, and liver failure (7, 8, 9, 10).

Chronic pulmonary aspergillosis (CNPA and CCPA), which may be referred to as aspergilloma in some cases, is thought to be a result of an underlying or preexisting pulmonary disease, such as tuberculosis, pneumoconiosis, or COPD (11). Diagnosis of CNPA and CCPA relies heavily on histologic and radiographic examinations. The extra scrutiny regarding the diagnosis of chronic pulmonary aspergillosis has led to the discovery of an eosinophilic component in some cases (12). Fungal pneumonia and IA in

chronic granulomatous disease (CGD) can also sometimes present as an eosinophilic pneumonia (13, 14).

Allergic bronchopulmonary aspergillosis (ABPA) is a hypersensitivity disorder caused by *A. fumigatus* (and other *Aspergillus* species) and is often marked by an eosinophilic immune response in humans (15). Moreover, in experimental repetitive-exposure models of *A. fumigatus*, we (16) and others (17, 18) have demonstrated that chronic exposure to *A. fumigatus* resulted in substantial recruitment of eosinophils to the airways. Assessment of the role allergic inflammatory responses play in fungal clearance has shown that mice deficient in the Th2 cytokine interleukin 13 (IL-13) or mice in which the Th2 and proeosinophil cytokine IL-5 was neutralized resulted in attenuated lung clearance of *Aspergillus niger* (19). Collectively, these results unexpectedly suggest that Th2 responses and eosinophils play a role in immune-mediated clearance of *Aspergillus* spp. from the lungs during asthma. As eosinophils have been identified in clinical cases of chronic pulmonary aspergillosis and in some cases of IA in CGD patients, the goal of the current study was to further evaluate the exact role of eosinophils during lung exposure to *A. fumigatus*. Employing mice deficient in the eosinophil lineage and *in vitro* eosinophil cultures, we found that this cell type is a potent lung effector cell against *A. fumigatus*.

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MATERIALS AND METHODS

Mice. Wild-type (WT) BALB/c and dblGATA1-deficient mice, 6 to 8 weeks of age, were obtained from The Jackson Laboratory (Bangor, ME) and bred at the University of Alabama at Birmingham (UAB). All animals were housed in a specific-pathogen-free, Association for Assessment and Accreditation of Laboratory Animal Care-certified facility and handled according to Public Health Service Office of Laboratory Animal Welfare policies after review by the UAB Institutional Animal Care and Use Committee.

Preparation of *A. fumigatus*, in vivo challenge, and lung fungal burden assessment and lung histology. *A. fumigatus* isolate ATCC 13073 (ATCC, Manassas, VA) was maintained on potato dextrose agar for 5 to 7 days at 37°C. Conidia were harvested by washing the culture flask with 50 ml of sterile phosphate-buffered saline (PBS) supplemented with 0.1% Tween 20. The conidia were then passed through a sterile 40- μ m nylon membrane to remove hyphal fragments and enumerated on a hemacytometer. For challenge, mice were lightly anesthetized with isoflurane and administered 7×10^7 *A. fumigatus* conidia in a volume of 50 μ l intratracheally as previously described (20, 21). Briefly, mice were held in a vertical, upright position and the tongue was withdrawn from the mouth using forceps. A pipette was used to deliver the 50- μ l inoculum to the caudal oropharynx, in which normal breathing results in fluid aspiration into the lungs (22). For lung fungal burden analysis, the left lungs were collected at 24 or 48 h postexposure and homogenized in 1 ml of PBS. Total RNA was extracted from 0.1 ml of unclarified lung homogenate using a MasterPure yeast RNA purification kit (Epicentre Biotechnologies, Madison, WI), which includes a DNase treatment step to eliminate genomic DNA as previously reported (23). Total RNA was also extracted from serial 1:10 dilutions of live *A. fumigatus* conidia (10^1 to 10^9) and DNase treated to form a standard curve. Lung *A. fumigatus* burden was analyzed with real-time PCR measurement of the *A. fumigatus* 18S rRNA (GenBank accession number AB008401 [24]) and quantified using a standard curve of *A. fumigatus* conidia as previously described (23). As a validation of the real-time PCR method, heat-killed *A. fumigatus* did not yield a signal by real-time PCR and was unable to grow on potato dextrose agar plates (23). 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 (as DNA is not predicative of organism viability [25]). 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 at Birmingham. Imaging was performed using a Nikon Eclipse 90i microscope and Nikon NIS-Elements imaging analysis software.

Bronchoalveolar lavage cell surface marker flow cytometry. Bronchoalveolar lavage cell isolations were performed as previously described (26, 27). Cells were washed and Fc receptors blocked with Mouse BD Fc Block (BD Biosciences, San Diego, CA) at 4°C for 20 min. Thereafter, cells were stained with a single-color LIVE/DEAD fixable dead cell stain (Invitrogen), followed by labeling with specific immune cell surface markers. The following staining parameters were employed: macrophages were identified as CD11b^{lo/neg} CD11c⁺, eosinophils as CD11b⁺ Siglec F⁺ Ly-6G^{lo} Ly-6C^{neg} CCR3⁺, neutrophils as CD11b⁺ Ly-6G⁺, and dendritic cells as CD11b⁺ CD11c⁺ (all antibodies purchased from eBiosciences or BD Biosciences). Samples were acquired using a four-laser, 20-parameter analytic BD LSR II flow cytometer, and data were analyzed using the FlowJo software program (Tree Star, Ashland, OR).

Real-time PCR analysis for *Epx*, *Prg2*, *Ear1*, and *Ear2* expression in lung tissue. WT BALB/c and dblGATA1-deficient mice were challenged with *A. fumigatus* as described above; 24 h after exposure, lungs were collected and homogenized in TRIzol reagent (Invitrogen), and total RNA was isolated as per the manufacturer's instructions. Thereafter, RNA was transcribed to cDNA (iScript cDNA synthesis kit; Bio-Rad), and real-time PCR for *Epx* (Mm00514768_m1; Applied Biosystems), *Prg2*

(Mm00435905_m1; Applied Biosystems), *Ear1* (Mm03059811_g10; Applied Biosystems), and *Ear2* (Mm04207376_gH; Applied Biosystems) was performed (iQ Supermix; Bio-Rad). mRNA levels were normalized to *Gapdh* mRNA levels (primers and probe from Applied Biosystems) using the threshold cycle [$2^{-\Delta\Delta CT}$] method.

Whole-lung cytokine and chemokine analysis. BALB/c WT and dblGATA1-deficient mice were challenged with *A. fumigatus* as described above; 24 or 48 h after exposure, the right lungs were isolated and homogenized in PBS supplemented with cComplete Mini protease inhibitor tablets (Roche), clarified by centrifugation, and stored at -80°C . Supernatants from lung homogenates were analyzed for protein levels of 23 cytokines and chemokines using a Bio-Plex multiplex suspension cytokine array (Bio-Rad Laboratories), according to the manufacturer's instructions (20, 21). The data were analyzed using Bio-Plex Manager software (Bio-Rad Laboratories).

Derivation of eosinophils from bone marrow and assessment of *A. fumigatus* killing and cytokine production. Bone marrow-derived eosinophils were generated using a previously described protocol (28). Briefly, bone marrow was isolated from naive BALB/c mice and cells plated at 1×10^6 cells/ml in RPMI 1640 containing 20% fetal bovine serum (FBS; Irvine Scientific, Santa Ana, CA), 2 mM glutamine, 25 mM HEPES, $1 \times$ minimal essential medium (MEM) nonessential amino acids, 1 mM sodium pyruvate (all from Life Technologies BRL, Rockville, MD), 50 μ M β -mercaptoethanol (Sigma-Aldrich, St. Louis, MO), and 100 ng/ml of stem cell factor (SCF) and 100 ng/ml of FLT3-L (both from Peprotech). After 4 days, cells were replated in the above medium supplemented with 10 ng/ml of IL-5. After 10 days, bone marrow cells were fully differentiated into eosinophils. As previously reported (28), samples of 1×10^5 cells were taken for RNA analysis each time medium was changed for real-time PCR analysis of *Epx* (Mm00514768_m1; Applied Biosystems) for eosinophil development and *Mpo* (Mm01298424_m1; Applied Biosystems) for neutrophil development. In addition, cells were cytospun onto glass slides, Giemsa stained, and analyzed for morphology and purity by a murine pathologist in the Comparative Pathology Laboratory at the University of Alabama at Birmingham. On the 10th day, bone marrow-derived eosinophils were enumerated and utilized in experiments. To assess eosinophil-mediated killing of *A. fumigatus*, 1×10^5 eosinophils in a volume of 50 μ l were cultured in triplicate with 3×10^5 *A. fumigatus* conidia in a volume of 50 μ l for 24 h at 37°C and 5% CO₂. Controls included 3×10^5 *A. fumigatus* conidia cultured alone in triplicate in a volume of 100 μ l for 24 h. Thereafter, total RNA was extracted from each well using a MasterPure yeast RNA purification kit (Epicentre Biotechnologies, Madison, WI) and real-time PCR was performed as described above. In other studies, the eosinophil-*A. fumigatus* cocultures were performed in Corning Transwell plates (24 wells; 0.4- μ m pore size) to assess the requirement for cell contact. For this, 1×10^6 eosinophils in a volume of 100 μ l were cultured in the top chamber in the presence of 3×10^6 eosinophils in a volume of 300 μ l cultured in the bottom chamber. Controls included 100 μ l of medium in the top chamber in the presence of 3×10^6 eosinophils in a volume of 300 μ l cultured in the bottom chamber. Parallel studies determined the effects of eosinophil whole-cell lysates on *A. fumigatus* viability (as assessed by real-time PCR). For this, 1×10^6 bone marrow-derived eosinophils were lysed in 100 μ l of PhosphoSafe extraction buffer (Novagen/EMD/Millipore) for 10 min at room temperature, followed by centrifugation at $12,000 \times g$ for 10 min. Lysates (100 μ l) were added in triplicate to 3×10^5 *A. fumigatus* conidia in a volume of 100 μ l for 24 h at 37°C and 5% CO₂. Controls included PhosphoSafe extraction buffer (100 μ l) added in triplicate to 3×10^5 *A. fumigatus* conidia in a volume of 50 μ l for 24 h at 37°C and 5% CO₂. To assess eosinophil cytokine and chemokine production, eosinophils and *A. fumigatus* were cultured at the ratio above (except that 1×10^6 eosinophils and 3×10^6 conidia were employed). Twenty-four hours after culture, the contents of each well were collected and centrifuged, and clarified supernatants were assessed using the Bio-Plex multiplex suspension cytokine array (Bio-Rad Laboratories) as detailed above.

FIG 1 Eosinophils are recruited to lung after *A. fumigatus* challenge. (A) BALB/c wild-type mice were challenged intratracheally with 7×10^7 *A. fumigatus* conidia (AF), and 24 h postexposure, whole lungs were collected, total RNA was isolated and transcribed to cDNA, and quantitative real-time PCR was performed for *Epx*. Gene expression was normalized to *Gapdh*, and fold changes between naive mice (set at 1) and *A. fumigatus*-exposed BALB/c mice were determined using the $2^{-\Delta\Delta CT}$ method. Cumulative data from three independent studies ($n = 3$ to 5 mice per group per study) are illustrated. *, P value of <0.05 (paired two-tailed Student t test). (B) BALB/c wild-type mice were challenged intratracheally with 7×10^7 *A. fumigatus* conidia, and 24 or 48 h postexposure, lung cells were isolated via bronchoalveolar lavage, Fc blocked, stained with a LIVE/DEAD staining kit, and thereafter stained with fluorochrome-conjugated CD11c, CD11b, Gr-1, and Siglec F. Cumulative data from three independent studies ($n = 2$ or 3 mice per group per time point per study) are illustrated. Data are expressed as absolute numbers of live cells in lung lavage fluid. * and ***, P values of <0.05 and <0.0001 , respectively (unpaired two-tailed Student t test).

Statistics. Data were analyzed using GraphPad Prism, version 5.0, statistical software (GraphPad Software, San Diego, CA). Comparisons between groups when data were normally distributed were made with the two-tailed unpaired Student t test. In real-time PCR analyses, the two-tailed paired Student t test was employed. Significance was accepted at a P value of <0.05 .

RESULTS

Eosinophils are recruited to the lungs after *A. fumigatus* challenge. Eosinophils are well documented to play a role in allergic disorders, including the allergic form of aspergillosis, allergic bronchopulmonary aspergillosis (15). In contrast, a previous study employing chemically immunosuppressed mice challenged with a low dose of *A. fumigatus* suggested that eosinophils likely played a minimal role in defense (29). Therefore, we sought to clarify their role during *A. fumigatus* exposure in immunocompetent mice. We first show that in normal BALB/c mice challenged with *A. fumigatus*, there is a significant increase in lung expression of the eosinophil marker *Epx* (eosinophil peroxidase) 24 h after exposure (Fig. 1A). We subsequently verified the presence of eosinophils by detecting CD11b⁺ Siglec F⁺ Ly-6G^{lo}Ly-6C^{neg} CCR3⁺ cells in lung lavage fluid 24 and 48 h after *A. fumigatus* exposure (Fig. 1B). The absolute eosinophil numbers are roughly a log less than what we have

previously reported for neutrophils in lung lavage fluid after *A. fumigatus* challenge in C57BL/6 mice (20, 21). Assessment of *Epx* mRNA levels and eosinophils by flow cytometry at 12 h did not reveal a significant presence in the lungs (data not shown). Thus, gene expression and flow cytometric analysis reveal that eosinophils are recruited to the lungs during an *A. fumigatus* infection.

Enhanced susceptibility of eosinophil-deficient mice after challenge with *A. fumigatus*. As eosinophils are recruited to the lungs during infection, we questioned whether they played a role in clearance an *A. fumigatus* challenge from the lung. To determine this, we challenged BALB/c WT and dblGATA1-deficient mice with *A. fumigatus* conidia and assessed fungal burden 24 h and 48 h postinfection. dblGATA1-deficient mice are deficient in a high-affinity GATA-binding site in the GATA-1 promoter and have a selective depletion of eosinophils but not mast cells or platelets (30). dblGATA1-deficient mice demonstrated a nearly 2-log-higher *A. fumigatus* burden 24 h postchallenge (Fig. 2A). Although there was some level of fungal clearance in dblGATA1-deficient mice between 24 and 48 h postchallenge, these mice nevertheless maintained a 2-log-higher burden at 48 h than did *A. fumigatus*-challenged BALB/c WT mice (Fig. 2A). Additional studies carried out for 7 days did not demonstrate increased mortality of dblGATA1-deficient mice (data not shown). However, Gomori-Grocott methenamine silver (GMS) staining of lung tissue from dblGATA1-deficient mice revealed the presence of germinating conidia and some hyphae, which was not observed in BALB/c WT mice (Fig. 2B). Neutrophils are widely acknowledged to be the most important effector cell for clearing *A. fumigatus* from the lungs (31, 32, 33). Assessing cell recruitment via flow cytometry of lung lavage cells 12, 24, and 48 h post-*A. fumigatus* challenge did not demonstrate a difference in neutrophil recruitment between BALB/c WT and dblGATA1-deficient mice (Fig. 2C). Moreover, we verified that thioglycolate-elicited neutrophils from dblGATA1-deficient mice stimulated with *A. fumigatus* conidia did not demonstrate impaired reactive oxygen species (ROS) production (data not shown). Dendritic cells (Fig. 2D) and monocyte/macrophage populations (Fig. 2E) were also not affected by *A. fumigatus* exposure in dblGATA1-deficient mice. Thus, mice deficient in eosinophils demonstrate impaired clearance of *A. fumigatus* from the lungs, which is not a result of defects in inflammatory cell recruitment or function.

Eosinophil-deficient mice challenged with *A. fumigatus* demonstrate reductions in specific eosinophil antimicrobial factors. Like neutrophils, eosinophils are equipped with potent granule proteins that possess antimicrobial activity, including basic protein (MBP), MBP2, eosinophil cationic protein (ECP), eosinophil peroxidase (EPX), and eosinophil-derived neurotoxin (EDN) (34). However, although highly expressed in eosinophils, some eosinophil-associated factors, such as various ribonucleases, may be expressed by additional cell types, such as macrophages and epithelial cells (35). Therefore, we sought to determine which of these eosinophil antimicrobial components were differentially expressed in the lungs of BALB/c WT and dblGATA1-deficient mice after *A. fumigatus* challenge. Results show that despite demonstrating significantly higher *A. fumigatus* lung burden at 24 and 48 h postchallenge (Fig. 2), mRNA levels of eosinophil peroxidase, *Epx*, were significantly lower in dblGATA1-deficient mice (Fig. 3A). Similar results were observed for major basic protein, *Prg2* (Fig. 3B). In contrast, mRNA levels of eosinophil-associated RNase 1, *Ear1*, were significantly elevated in *A. fumigatus*-exposed

FIG 2 Enhanced susceptibility of eosinophil-deficient mice after challenge with *A. fumigatus*. (A) BALB/c WT and *dblGATA1*-deficient (*dblGATA1*) mice were challenged intratracheally with 7×10^7 *A. fumigatus* conidia, and 24 and 48 h after exposure, lung fungal burden was assessed by real-time PCR analysis of *A. fumigatus* 18S rRNA levels. Cumulative data from two independent studies ($n = 4$ or 5 mice per group per time point per study) are illustrated. Data are expressed as means + standard errors of the means. * and ***, P values of <0.05 and <0.0001 , respectively (unpaired two-tailed Student t test). (B) Representative GMS-stained lung sections of two individual WT (top) and *dblGATA1*-deficient (bottom) mice 48 h post-*A. fumigatus* challenge. (C to E) WT and *dblGATA1*-deficient mice were challenged intratracheally with 7×10^7 *A. fumigatus* conidia, and lung cells were isolated via bronchoalveolar lavage, Fc blocked, stained with a LIVE/DEAD staining kit, and thereafter stained with fluorochrome-conjugated antibodies differentiating neutrophils (C), dendritic cells (D), and monocytes/macrophages (E). Cumulative data from three independent studies ($n = 3$ or 4 mice per group per study) are illustrated. Data are expressed as absolute numbers of live cells in lung lavage fluid.

dblGATA1-deficient mice at 24 h but significantly reduced at 48 h (Fig. 3C). mRNA levels of eosinophil-associated RNase 2, *Ear2*, followed the same trend (Fig. 3D). Thus, lower eosinophil peroxidase and major basic protein expression levels, but not eosinophil-associated RNase 1 or 2, correlate with impaired *A. fumigatus*

lung clearance in *dblGATA1*-deficient mice and thus may contribute to antifungal host defense during infection.

Eosinophil-deficient mice challenged with *A. fumigatus* demonstrate differences in lung proinflammatory cytokine and chemokine levels. Despite observing no defects in neutrophil re-

FIG 3 Eosinophil-deficient mice challenged with *A. fumigatus* demonstrate reductions in specific eosinophil antimicrobial factors. BALB/c WT and dblGATA1-deficient (dblGATA1) mice were challenged intratracheally with 7×10^7 *A. fumigatus* conidia, and 24 and 48 h after exposure, whole lungs were collected, total RNA was isolated and transcribed to cDNA, and quantitative real-time PCR was performed for *Epx* (A), *Prg2* (B), *Ear1* (C), and *Ear2* (D). Gene expression was normalized to *Gapdh*, and fold changes between WT mice (set at 1) and dblGATA1-deficient mice were determined using the $2^{-\Delta\Delta CT}$ method. Cumulative data from two independent studies ($n = 4$ or 5 mice per group per time point per study) are illustrated. *, **, and ***, P values of <0.05 , <0.01 , and <0.0001 , respectively (paired two-tailed Student t test).

cruitment to the lungs of dblGATA1-deficient mice (Fig. 2), we nevertheless determined whether the absence of eosinophils during *A. fumigatus* exposure affected the magnitude of the lung inflammatory response. We analyzed the protein levels of 23 cytokines and chemokines in clarified whole-lung homogenates from BALB/c WT and dblGATA1-deficient mice 24 and 48 h postinfection. Results in Fig. 4 show that despite the significantly higher *A. fumigatus* lung burden at 24 h and 48 h postchallenge (Fig. 2), the proinflammatory cytokines IL-1 β (Fig. 4A), IL-6 (Fig. 4B), and IL-17A (Fig. 4C) were significantly lower in dblGATA1-deficient mice at 24 h (IL-1 β) and 48 h (IL-6 and IL-17A) postchallenge, respectively. Likewise, the growth factors granulocyte colony-stimulating factor (G-CSF) (Fig. 4D) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Fig. 4E) and the chemokine CXCL1/KC (Fig. 4F) were significantly lower in dblGATA1-deficient mice at 24 h (G-CSF) and 48 h (GM-CSF and CXCL1) postchallenge, respectively. Thus, attenuated lung cytokine and chemokine production in the absence of eosinophils correlates with impaired *A. fumigatus* lung clearance in dblGATA1-deficient mice.

Eosinophils recognize and respond to *A. fumigatus*. Data thus far have shown that mice deficient in the generation of eosinophils have a profound defect in clearing *A. fumigatus* from the lungs, and despite impairments in proinflammatory responses, there were no defects in neutrophil recruitment or function in the

absence of eosinophils. These results suggest that eosinophils themselves are active inhibitors of *A. fumigatus* growth in the lungs. To address this, we generated eosinophils from the bone marrow of WT BALB/c mice (28) and determined whether they had the ability to affect *A. fumigatus* viability *in vitro*. As previously reported (28), we verified that culturing bone marrow cells in the presence of stem cell factor and FLT3-L for 4 days followed by the addition of IL-5 for an additional 6 days resulted in a significant enhancement of *Epx* mRNA expression, with a >300 -fold increase by day 10 of the culture (data not shown). In contrast, throughout the 10-day culture period, we saw a parallel decrease in mRNA expression of *Mpo* (myeloperoxidase), a widely employed biomarker of neutrophils (data not shown). A representative image of a Giemsa-stained cytopsin preparation of bone marrow-derived eosinophils at day 10 shows characteristic nuclear morphology and the presence of secretory granules (Fig. 5A). We next determined whether eosinophils limited the growth of *A. fumigatus* *in vitro*. The previously mentioned *A. niger* study employed ratios of eosinophils to conidia of 6:1 up to 100:1, which inhibited *A. niger* growth $\sim 25\%$ and 100% , respectively (19). We assessed killing by employing a significantly lower ratio, as the low numbers of eosinophils detected in lung lavage fluid (Fig. 2) suggested a scenario whereby the organisms outnumbered the eosinophils. With a ratio of eosinophils to conidia of 1:3, results showed that culturing *A. fumigatus* in the presence of eosinophils de-

FIG 4 Eosinophil-deficient mice challenged with *A. fumigatus* demonstrate differences in lung proinflammatory cytokine and chemokine levels. BALB/c WT and dbIGATA1-deficient (dbIGATA1) mice were challenged intratracheally with 7×10^7 *A. fumigatus* conidia, and 24 and 48 h after exposure, levels of IL-1 β (A), IL-6 (B), IL-17A (C), G-CSF (D), GM-CSF (E), and CXCL1 (F) were quantified in lung homogenates by Bio-Plex. Cumulative data from two or three independent studies ($n = 4$ to 6 mice per group per time point per study) are illustrated. Data are expressed as mean concentrations + standard errors of the means. * and ***, P values of <0.05 and 0.001 , respectively (unpaired two-tailed Student t test).

creased viability by more than 2 logs after 24 h of culture (Fig. 5B), a killing rate of $>99\%$. This killing was not cell contact dependent, as separating eosinophils from *A. fumigatus* via a $0.4\text{-}\mu\text{m}$ -pore-containing Transwell membrane still resulted in significant killing of *A. fumigatus* (Fig. 5C). As this supports the hypothesis that secreted or soluble factors derived from eosinophils are toxic to *A. fumigatus*, we confirmed that eosinophil lysates containing granule proteins inhibited the growth of *A. fumigatus* (Fig. 5D). As eosinophils were capable of recognizing and killing *A. fumigatus* *in vitro*, we next determined whether this interaction also resulted in the elaboration of cytokines and chemokines. Employing a culture design identical to that in Fig. 5B, we observed significant production of the chemokine CCL4/MIP-1 β (Fig. 5E) and the Th2 cytokine IL-4 (Fig. 5F). Eosinophils cultured in the presence of *A. fumigatus* also produced IL-1 β and CCL2/MCP-1 (Fig. 5E), as well as the Th2 cytokines IL-13 and IL-9 and the well-recognized eosinophil chemoattractant CCL11/eotaxin (Fig. 5F). Thus, eosinophils respond to *A. fumigatus* with the production of proinflammatory cytokines and chemokines as well as Th2 cytokines, an event that also leads to destruction of the organism.

DISCUSSION

Eosinophils are recognized to play important roles in two main immune responses, antiparasitic responses and proallergic or asthmatic responses. However, the development of unique eosinophil-deficient mouse strains (30, 36) and additional mouse strains in which eosinophil recruitment or function is diminished (37, 38, 39) has provided novel insights into the role of eosinophils in T helper responses, interactions with immune cells such as macrophages and dendritic cells, and antimicrobial defense (40). With respect to the last, the identification of Toll-like receptor (TLR) expression and responsiveness by human eosinophils (41, 42) has renewed interest in their host defense capabilities, which is supported by findings such as a role for eosinophils in host defense against the Gram-negative bacterium *Pseudomonas aeruginosa* (43) and respiratory syncytial virus (RSV) (44, 45). Consequently, the goal of the current study was to determine whether the eosinophil antimicrobial activity extended to fungi, specifically *A. fumigatus*.

We have reported that eosinophils are the predominant cell

FIG 5 Eosinophils recognize and respond to *A. fumigatus*. Bone marrow cells were isolated from naive BALB/c mice and cultured with 100 ng/ml of stem cell factor and 100 ng/ml of FLT3-L. After 4 days, cells were replated in medium supplemented with 10 ng/ml of IL-5 for an additional 6 days. (A) Representative Giemsa-stained cytopsin of bone marrow-derived eosinophils at day 10 of culture showing characteristic nuclear structure and secretory granules. (B) Bone marrow-derived eosinophils (1×10^5 cells) were cultured in the presence of *A. fumigatus* conidia (3×10^5) in a total volume of 100 μ l for 24 h (AF + Eos). Controls included *A. fumigatus* conidia (3×10^5) cultured alone in a volume of 100 μ l for 24 h (AF alone). Cumulative data from four independent studies are illustrated. Each dot represents a single well. ***, *P* value of <0.0001 (unpaired two-tailed Student *t* test). (C) Bone marrow-derived eosinophils (1×10^6 cells; 100 μ l) were cultured in the top chamber of a 0.4- μ m-pore-containing Transwell membrane in the presence of *A. fumigatus* conidia (3×10^6 ; 300 μ l) for 24 h [Eos (Top)/AF (Bottom)]. Controls included 100 μ l of medium in the top chamber in the presence of *A. fumigatus* conidia (3×10^6 ; 300 μ l) cultured alone in the bottom chamber for 24 h [Media (Top)/AF (Bottom)]. Cumulative data from three independent studies are illustrated. Each dot represents a single well. ***, *P* value of <0.0001 (unpaired two-tailed Student *t* test). (D) Bone marrow-derived eosinophils (1×10^6 cells) were lysed in 100 μ l of PhosphoSafe extraction buffer (10 min at room temperature, followed by centrifugation at $12,000 \times g$ for 10 min). Lysates (100 μ l) were added in triplicate to 3×10^5 *A. fumigatus* conidia in a volume of 50 μ l for 24 h. Controls included PhosphoSafe extraction buffer (100 μ l) added in triplicate to 3×10^5 *A. fumigatus* conidia in a volume of 50 μ l for 24 h. Cumulative data from three independent studies are illustrated. Each dot represents a single well. ***, *P* value of <0.0001 (unpaired two-tailed Student *t* test). (E and F) Bone marrow-derived eosinophils were cultured as for panel B, and supernatants were collected after 24 h and clarified by centrifugation. IL-1 β , CCL2, and CCL4 (E) and IL-4, IL-13, IL-9, and CCL11 (F) were quantified by Bio-Plex. Controls included bone marrow-derived eosinophils cultured in medium alone (gray columns). Cumulative data from two independent studies are illustrated. ** and ***, *P* values of <0.01 and <0.0001, respectively (unpaired two-tailed Student *t* test). Un, unstimulated.

type observed in the lungs of mice repetitively challenged with *A. fumigatus* in an experimental model of fungal asthma (16). However, we have reported that during acute *A. fumigatus* challenge, neutrophils represent the dominant lung cell type, although eosinophils were present as well (20). Curiously, eosinophils were significantly higher, and neutrophils significantly lower, in mice deficient in the beta-glucan receptor dectin-1, which are highly susceptible to *A. fumigatus* (20). This observation suggested the possibility that the ratio of neutrophils to eosinophils could determine the outcome of *A. fumigatus* infection, i.e., that eosinophils may actually be harmful for host defense. We first confirmed that eosinophils were recruited to the lungs of normal BALB/c mice after acute *A. fumigatus* challenge (as opposed to our previous study with normal 129/SvEv mice [20]). We assessed this via the expression of the gene for eosinophil peroxidase, *Epx*, which showed significant upregulation in the lungs after *A. fumigatus* challenge. Eosinophils are sometimes difficult to identify via flow cytometry, yet we corroborated the *Epx* analysis by employing a thorough staining procedure that identified eosinophils in lung lavage fluid via the absence of CD11c expression and moderate Gr-1 expression, which were subsequently identified by high expression of CD11b, Siglec F, and CCR3 (46). Moreover, this population was absent in dbLGATA1-deficient mice (data not shown).

Having established that eosinophils are a component of the acute inflammatory response after *A. fumigatus* exposure, we next determined their relevance in *A. fumigatus* lung defense. As mentioned earlier, the development of mice with specific deficiencies in eosinophils has afforded the ability to better understand the roles these cells play in immune responses. We chose mice deficient in the dbLGATA1 transcription factor, as they have been extensively employed for assessing eosinophil-mediated responses in models of asthma and infectious diseases (36, 47, 48, 49). Although bone marrow progenitors from dbLGATA1-deficient mice may be forced to differentiate into eosinophils *in vitro* (50) (via various cytokine stimulations and differential promoter usage), this does not occur in dbLGATA1-deficient mice *in vivo* during homeostatic (30) or disease or pathological (51) settings. Moreover, chronic *A. fumigatus* exposure, employed as a model of chronic experimental allergic airway inflammation, showed a >99% reduction in Siglec F⁺ CCR3⁺ cells in lung lavage fluid from dbLGATA1-deficient mice (52). Employing the same dbLGATA1-deficient strain, we observed a profound yet somewhat unexpected impairment in their ability to clear *A. fumigatus* from the lungs. In fact, the difference between WT and eosinophil-deficient mice at 24 and 48 h was more than 2 logs, much greater than the observed differences we have previously reported for *Clec7a*^{-/-} (dectin-1-deficient) mice (20) and *Il22*^{-/-} (IL-22-deficient) mice (21) (both approximately a 5- to 6-fold higher lung burden, although both of these strains are on the C57BL/6 background). Although dbLGATA1-deficient mice had difficulty clearing *A. fumigatus* from the lungs, this did not result in increased mortality in these animals, which may be expected based on our observation of intact neutrophil recruitment in these mice. However, the observation of germinating *A. fumigatus* organisms in the lungs of dbLGATA1-deficient mice implies that eosinophils target inhaled conidia and, along with inflammatory monocytes (53), potentially aid in limiting germination.

The susceptibility of dbLGATA1-deficient mice to *A. fumigatus* lung infection appeared to be solely a result of eosinophil deficiency. However, we cannot exclude the possibility that other eosi-

nophil-dependent or -independent mechanisms may have contributed to our observations. Indeed, eosinophils have been reported to modulate dendritic cell and T cell responses during allergen challenge (54) and have been shown to be required for the maintenance of plasma cells in the bone marrow (55). However, acknowledging that the time points employed in the current body of work are within 48 h after a single *A. fumigatus* exposure, in which innate immune responses are prominently active, it is unlikely that adaptive/T cell/B cell responses contributed significantly to the observed clearance defect in dbLGATA1-deficient mice. We therefore concentrated our efforts on identifying specific eosinophilic inflammatory mediators. Whole-lung mRNA analysis revealed significantly reduced expression of *Epx* and *Prp2* 24 h and 48 h postinfection in dbLGATA1-deficient mice. The eosinophil-associated ribonucleases *Ear1* and *Ear2* were unexpectedly observed to be significantly higher in dbLGATA1-deficient mice 24 h post-*A. fumigatus* exposure, yet the opposite was true at 48 h. These data suggest that during a lung infection with *A. fumigatus*, *Epx* (EPO) and *Prp2* (MBP) may play a role in host defense, whereas *Ear1* and *Ear2* are dispensable. Multiple mechanisms for killing by eosinophils have been proposed, including antibody-mediated release and/or complement-mediated release of eosinophil granule proteins, such as EPO, MBP, and ECP (56, 57, 58, 59). Eosinophils may also produce nitric oxide (NO) and *Ear2* as antiviral factors, as demonstrated for RSV (44, 45). Lipopolysaccharide (LPS) stimulation of IL-5- or gamma interferon (IFN- γ)-primed eosinophils (60) or with platelet activating factor (PAF) in the presence of IL-5 or GM-CSF (61) promote the release of eosinophil extracellular traps (EETs) which contain mitochondrial DNA as well as granule proteins. *Epx* encodes EPO, a heme-containing cationic protein which comprises the bulk of protein found within eosinophilic granules (62) and has been shown to selectively kill bacteria, parasites, and viruses (63, 64). To the best of our knowledge, a potential requirement of EPO in fungal clearance has not been previously reported, and the substrate that drives fungal clearance by EPO remains to be identified. Although very little is known regarding EPO and eosinophil-mediated antifungal responses, a previous study reported that exposing macrophages to eosinophil-derived EPO enhanced their respiratory burst, leading to enhanced killing of the opportunistic yeast *Candida albicans* (65). This study suggests that a function of eosinophils and EPO may be to activate the antimicrobial activity of other innate cell types. Another study reported that stimulating human eosinophils with GM-CSF, IL-1, IL-3, and IL-5 augmented phagocytosis of *C. albicans*, yet this paradoxically did not result in increased killing of the organism (66). In contrast, while these studies suggest a possible role for eosinophils in anti-*Candida* defense, studies with the pathogenic yeast *Cryptococcus neoformans* are decidedly opposite. Mice deficient in scavenger receptor A demonstrate increased resistance to *C. neoformans* lung infection, which correlated with reduced Th2 responses and significantly lower eosinophil numbers in the lungs (67). In fact, *C. neoformans* infection in dbLGATA-deficient mice is associated with lower lung fungal burden in the presence of enhanced Th1 and Th17 responses (68).

Our data suggest that the susceptibility of dbLGATA1-deficient mice to *A. fumigatus* is directly due to the specific loss of eosinophils; thus, we felt it was imperative to demonstrate that eosinophils are capable of recognizing and responding to *A. fumigatus* conidia. Indeed, eosinophils derived from bone marrow cells with

SCF, FLT3-L, and IL-5 were adept at inhibiting *A. fumigatus* viability *in vitro*, an interaction that also led to the production of proinflammatory mediators, chemokines, and Th2 cytokines. These observations indicate the use of pattern recognition receptors (PRRs) in eosinophil anti-*Aspergillus* responses. Studies with human eosinophils have identified expression of numerous TLRs, including TLR1, TLR4, TLR7, TLR9, and TLR10 (69), whereas murine eosinophils have been documented to express TLR2, TLR4, and TLR7 (45, 70). With respect to fungus-associated PRRs, the beta-glucan receptor dectin-1 has been observed in human eosinophils (71, 72). Identifying which PRR is utilized for *A. fumigatus* recognition is the subject of current investigations. Our data showing that cell contact was not required for eosinophil-mediated killing of *A. fumigatus* coupled with potent antifungal activity of eosinophil cell lysates suggest that innate recognition of *A. fumigatus* leads to the rapid degranulation of eosinophils and subsequent antifungal responses.

In summary, we show here that eosinophils are recruited to the lung after *A. fumigatus* exposure and play a necessary role in the elimination of this pathogenic mold from the lungs. Our data further suggest that the eosinophil antimicrobial factors eosinophil peroxidase and major basic protein putatively mediate lung clearance. Moreover, we show that eosinophils possess potent activity against *A. fumigatus* *in vitro* and serve as a source of numerous cytokines and chemokines. Future studies targeted at understanding which PRRs play a role in eosinophil-mediated recognition of *A. fumigatus* as well as specific mechanisms of antifungal activity will further define the importance of eosinophils in host defense against the spectrum of diseases caused by exposure to *A. fumigatus*.

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