Lung eosinophil recruitment in response to *Aspergillus fumigatus* is correlated with fungal cell wall composition and requires γδ T cells

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Abstract

The differential recognition of fungal cell wall polysaccharides that program innate and adaptive immunity to the human opportunistic fungal pathogen *Aspergillus fumigatus* has been a focus of considerable interest. In a mouse model of fungal conidia aspiration, decreased relative levels of cell wall core carbohydrates β-1,3-glucan to chitin in *A. fumigatus* isolates and mutant strains were correlated with increased airway eosinophil recruitment. In addition, an increase in fungal surface chitin exposure induced by the β-1,3-glucan synthesis-targeting drug caspofungin was associated with increased murine airway eosinophil recruitment after a single challenge of conidia. The response to increased *A. fumigatus* chitin was associated with increased transcription of IL-17A after a single aspiration, although this cytokine was not required for eosinophil recruitment. Rather, both RAG1 and γδ T cells were required, suggesting that this subset of innate-like lymphocytes may be an important regulator of potentially detrimental type 2 immune responses to fungal inhalation and infection.

Key words: Aspergillus fumigatus, lung immune responses, eosinophils, gammadelta T cells, chitin, caspofungin

1. Introduction

Species of the filamentous fungus *Aspergillus* are associated with allergy and asthma in otherwise healthy individuals, while the respiratory tract of immune deficient individuals may be colonized and ultimately lead to a fatal disseminated infection [1]. However, the ability to colonize host tissue and the potential for invasive infection vary between and within species. *A. fumigatus*, widely considered the most pathogenic species, is believed to account for 90% of invasive aspergillosis cases [2, 3]. Genetic variability within *A. fumigatus* isolates is also considerable, and the resulting variation of phenotypic factors such as *in vitro* growth rate and metabolic adaptation appear to be correlated with *in vivo* virulence [4-6]. The virulence of *A. fumigatus* has also been attributed in part to the ability to germinate at physiological temperatures [7]. Germination of dormant *A. fumigatus* conidia exposes immunostimulatory β-glucan and chitin on the surface that would otherwise be masked from the host immune recognition [8, 9]. Thus, requirements for and changes to *A. fumigatus* conidia during germination determine both the ability of *A. fumigatus* to invade host tissues and the initial character of the host immune response.

Though germination has been shown to direct airway immune responses to *Aspergillus* conidia, the effect of specific fungal genes has not been well-characterized. Numerous fungal virulence factors have been identified that are likely to influence protective immunity to *A. fumigatus*. The yeast nucleolar ortholog CgrA was shown to mediate germination of *A. fumigatus* conidia at physiologic temperatures, thus enhancing virulence in a mouse model of invasive aspergillosis [10]. Although a mutant strain Δ*cgrA* exhibited markedly decreased virulence, the effect of delayed germination on the generation of anti-fungal immune responses remains unknown. In addition to regulation of germination, other virulence factors protect *A. fumigatus*

from environmental stress, such as the unfolded protein response (UPR) regulator HacA [11], the ER-stress sensor IreA [12], or the fungal pigment dihydroxynapthalene (DHN) melanin [13]. Disruption of the ER stress response genes *hacA* and *ireA* resulted in decreased cell wall β-glucan and secretion of proteases, including those necessary for nutrient acquisition and invasion of host tissues [11, 12]. Recently, the effect of pigment mutation on lung cytokine levels or airway leukocyte recruitment in response to *A. fumigatus* conidia was examined [14, 15]. In these studies, the relative levels of lung IL-17A, IFN-γ, and IL-10 were markedly different in UV-generated color mutants of the commonly used clinical isolate Af293 [15], and airway eosinophil recruitment was increased in response to conidia lacking the melanin-pathway genes *arp2* and *alb1* [14]. Interestingly, one of the melanin mutant strains that induced increased lung eosinophil accumulation (Δ*arp2*[14]) was also reported to display increased surface chitin, but not β-glucan exposure [16]. We have previously reported that increased expression of cell wall chitin in an isolate of *A. fumigatus* resulted in increased eosinophil recruitment in a murine model of repeated aspiration [17]. This study further examined the role of eosinophils in protection from invasive aspergillosis in neutropenic mice with type 2-skewed immunity, and our results suggested that eosinophils inhibit fungal clearance and increase disease severity in this setting. Cell wall chitin was also increased when *A. fumigatus* was cultured in the presence of the β-glucan synthesis-inhibiting antifungal drug caspofungin, suggesting that synthesis of β-glucan and chitin may be reciprocally regulated [18, 19]. Although it is accepted that immune responses to β-glucan and chitin are skewed towards Th1/17 and Th2 profiles, respectively, an increase in detrimental eosinophil recruitment or type 2 immunity in response to inhalation of caspofungin-modulated *A. fumigatus* has not been reported [20].

The immune mechanism responsible for chitin-mediated eosinophil recruitment and induction of type 2 immunity in response to *A. fumigatus* is not well-understood. To date, many studies have focused on immune responses to particulate chitin. Results of these studies indicated that the size and acetylation of chitin are important factors in determining the nature of the resultant immune response to exposure an inhalation [21]. Purified chitin induced TNFα, IL-10 and IL-17A production in macrophages in a size-dependent manner [22-24]. However, the role of these immune effectors in lung responses to viable *A. fumigatus* conidia remains unknown.

In this study, we observed that strains that were previously reported to exhibit a decreased ratio of cell wall β-glucan/chitin exhibited increased airway eosinophil recruitment in response to repeated aspiration of *A. fumigatus* conidia. Furthermore, fungal growth and germination of conidia in the presence of the β-glucan synthesis-inhibiting antifungal caspofungin resulted in increased chitin exposure and airway eosinophil recruitment in response to fungal aspiration. Although lung IL-17A transcription was increased in response to single aspiration of high-chitin expressing *A. fumigatus* conidia, the presence of IL-17A was not required for eosinophil recruitment. In contrast, expression of RAG1 and the presence of γδ T cells were required, suggesting that these innate-like lymphocytes are involved in lung eosinophil recruitment and subsequently promote the development of detrimental type 2 immune responses to *A. fumigatus*.

2. Materials and Methods

2.1. Growth and handling of fungi

A. *fumigatus* (Af293) was purchased from the Fungal Genetics Stock Center. Additional wild type (H237, KuA) and mutant strains (Δ*cgrA*, Δ*hacA*, Δ*ireA*) of *A. fumigatus* were provided by Dr. David Askew (University of Cincinnati) [10-12]. Fungi were cultured on malt extract agar (MEA) or MEA plates supplemented with vegetable extract at room temperature (RT). For experiments involving the Δ*ireA* strain, experimental and parent strains were grown on osmotic stress medium (OSM) agar plates at 30^0C [12]. In some instances, the Af293 isolate was cultured on MEA plates containing 16μg/ ml caspofungin diacetate (Sigma) and incubated at 37^0C for 4 days. Conidia were isolated from culture plates kept at RT for 14 days by applying and gently shaking 1g of glass beads (0.5 mm, Braun-Melsungen), then placed in suspension by pouring the beads into a tube with sterile phosphate buffered saline (PBS). For flow cytometric analysis of conidia, harvested conidia were swollen in RPMI for 4 hrs at 37^0C and subsequently fixed with 4% paraformaldehyde. In some experiments, conidia were swollen in the presence of 16μg/ml of caspofungin in RPMI. Fixed, swollen conidia were washed with ammonium chloride and DPBS and resuspended on DPBS for aspiration or surface staining and flow cytometric analysis. For surface stain, swollen conidia were stained with carbohydrate binding lecithin, Wheat Germ Agglutinin (conjugated with allophycocyanin (APC) for surface chitin detection and analyzed on flow cytometry for quantification. For mouse aspiration, conidia were harvested using glass beads, enumerated and resuspended in DPBS.

2.2. Mouse aspiration, sacrifice, histological staining, and collection of BALF

All animal procedures were approved by the Animal Care and Use Committee of Indiana State University, the host campus of IUSM-Terre Haute. BALB/c or C57BL6/J mice were obtained

from Envigo or Jackson Laboratory, IL-17A-/- mice were obtained from Dr. David Wilkes, TCRδ-/- mice aged 5 weeks were obtained from Jackson Laboratory. Mice were allowed to rest 1-4 weeks prior to experiments. A subset of mice were bred at the IUSM-Terre Haute animal facility with offspring used in subsequent experiments at 7-10 weeks of age. For repeated aspiration, suspensions of 2 x 10^6 conidia were delivered involuntarily as previously described [25]. After two weeks of twice weekly aspiration, mice were rested for two weeks, when a final aspiration was administered. Mice were euthanized 72 hrs after the final challenge with sodium pentobarbitol, and lungs were perfused with 10 ml phosphate buffered saline (PBS). Bronchoalveolar lavage fluid (BALF) was collected from the perfused lungs as previously described [26].

2.3. Flow cytometric analysis of bronchoalveolar lavage fluid and lung homogenates

BALF cell composition was determined by flow cytometric analysis of recovered lavage cells in suspension and stained with surface markers. In brief, BALF was centrifuged for 5 min at 1500 rpm, the supernatant removed, and the cell pellet resuspended and washed in 1 ml of FACS buffer (Phosphate Buffered Saline, 5% fetal bovine serum, 0.05% sodium azide). The washed pellet was resuspended and stained in a solution containing FACS buffer with 10% rat serum, Fc-receptor blocking antibody (clone 24G2) and the following antibodies: rat-anti-mouse Ly-6G-FITC, rat-anti-mouse Siglec-F-PE, pan-leukocyte rat-anti mouse CD45-PerCP, and rat-anti-mouse CD11c-APC. For γδ T cell staining the following antibodies were used; rat-anti-mouse CD3ε-PeCy7, CD4-APC and TCRδ-PE (BD Biosciences). After staining, cells were washed and fixed with BD Cytofix. Populations of cells were evaluated by flow cytometric analysis on a Guava EasyCyte 8HT (EMD Millipore).

2.4. Intracellular cytokine staining

T-cell cytokine production on a per-cell level was determined by fluorescent intracellular cytokine staining (ICS) as previously described [27]. Briefly, the BALF suspension was centrifuged for 5 min at 1500 rpm and washed in 1 ml complete RP10 medium. The supernatant was discarded and a solution of Leukocyte Activation Cocktail with GolgiPlug (BD Biosciences) was added to each sample for stimulation of cytokine production and simultaneous inhibition of cytokine secretion. Cells were incubated at 37°C for 4 hrs. After the incubation, cells were washed in FACS buffer and stained for flow cytometric analysis using the surface antibodies rat-anti-moue CD4 PerCP and rat-anti-mouse CD8 FITC on ice (eBioscience). After a 30 min incubation, cells were washed in FACS buffer and centrifuged, and cell pellets were resuspended in BD Cytofix/Cytoperm for 15 min to allow fixation and permeabilization for subsequent intracellular cytokine staining. Cells were washed and resuspended in Permwash (BD). Each sample was divided into two tubes and stained with rat-anti-mouse IFN-γ-APC (eBioscience) and rat-anti-mouse IL-17A-PE, or with control isotype antibodies (eBioscience).

2.5. Total RNA processing and gene expression analysis

Lungs were removed and flash frozen in liquid nitrogen for RNA extraction. Total RNA was extracted from whole lungs homogenized in Trizol reagent (Invitrogen). Following the aqueous upper phase separation further RNA purification was performed using Qiagen RNEasy column with on column DNAse treatment per manufacturer`s recommendations. 2μg of total RNA was transcribed using High-capacity cDNA synthesis kit (Life Technologies) according to manufacturer`s protocol. For qPCR, Power-Up Sybr Green PCR Master Mix (Applied Biosystems) was used with Mxp3500 Real-time PCR system (Agilent). Custom cytokine expression array plates were obtained from SABiosciences.

2.6. Data analysis methods

Analysis of mouse data was performed with FlowJo software (TreeStar). GraphPad Prism was used for generation of graphs and figures and for statistical analyses (GraphPad Software). Unpaired t-tests were used to measure statistical significance when two groups were compared, and one or two-way analysis of variance (ANOVA) tests were used along with Tukey's or Sidak's post-tests for multiple comparisons, respectively. Differences between experimental groups that resulted in a p-value of less than 0.05 were considered significant.

3. Results

3.1. Strain-dependent eosinophil recruitment and T cell cytokine secretion in response to repeated aspiration of A. fumigatus conidia.

We and others have observed phenotypic variation in isolates of *A. fumigatus* that could affect immune recognition and thus alter protective immunity [5, 28-31]. A recent study reported distinct patterns of lung cytokine production in response to different *A. fumigatus* isolates [15], although differences in airway recruitment of innate and adaptive immune cells were not examined. Using our previously described model of repeated aspiration of conidia (Fig. 1C and [17, 26]), we observed that recruitment of airway leukocytes and neutrophils was similar between all clinical and environmental isolates examined (Figs. 1A, 1D, and 1H). Although airway eosinophil recruitment was equivalent in response to the clinical isolates Af293, Af13073 and the environmental isolate Af164, it was markedly increased in response to the environmental isolate Af5517 (Figs. 1A and 1E). The frequency of CD4 T cells in the lymphocyte population was increased in response to Af5517 in comparison to repeated Af293 and Af164 aspiration, but not in comparison to Af13073 (Figs. 1B and 1F), however, the frequency of IFN-γ-secreting CD4+ T cells was decreased in response to Af5517 (Fig. 1G). No major differences in CD8 T cells with respect to frequency, total number, or cytokine production were observed (Fig. 1B and data not shown). Although these results are in agreement with our recent study that reported increased chitin-mediated eosinophil recruitment in response to Af5517 [17], airway responses to additional *A. fumigatus* isolates were otherwise equivalent to the clinical isolate Af293.

Our previous results suggested that the ability of *A. fumigatus* to germinate at physiological temperatures may be important for induction of adaptive lung immune responses [26]. Furthermore, another study has demonstrated that the cell wall architecture of *A. fumigatus*

196 is altered in response to ER stress [17], and this might result in differential immune recognition.
197 Therefore, we also compared the airway response to repeated aspiration of the parent wild-type
198 strains with that of the thermotolerance-defective Δ*cgrA* and the ER stress-response deficient
199 Δ*hacA* and Δ*ireA* strains [10-12]. Possibly due to the relative avirulence of Δ*ireA* conidia,
200 repeated aspiration resulted in decreased leukocyte infiltration in comparison with the parent
201 wild-type strain, while leukocyte infiltration remained essentially unchanged in response to
202 Δ*cgrA* and Δ*hacA* mutants (Fig. 2E). Furthermore, the total number of neutrophils, eosinophils,
203 and CD4+ T cells were similar between each parent and mutant strain (Figs. 2A, 2B (bottom
204 panel), and 2C), although the frequency of eosinophils among CD45hiLy6G- cells was increased
205 in response to Δ*hacA* and Δ*ireA* strains (Fig. 2B, top panel). The frequencies of IFN-γ and IL-
206 17A-secreting CD4+ and CD8+ T cells were increased in response to Δ*cgrA* and Δ*ireA* strains,
207 respectively, with a decrease in total number of IFN-γ-secreting cells in response to Δ*ireA* (Fig.
208 2D and data not shown). Thus, similar to the response to repeated aspiration of Af5517 conidia,
209 airway immunity to Δ*hacA* and Δ*ireA* strains resulted in increased frequency of airway
210 eosinophils and modulated profiles of T cell cytokine secretion.

211 *3.2. Growth in the presence of caspofungin increases surface chitin exposure in germinating A.*
212 *fumigatus conidia and promotes airway eosinophil recruitment.*

213 Numerous antifungal drugs specifically target the expression of cell wall components that are
214 critical for growth of pathogenic fungi within susceptible host tissue. As a result, *in situ* cell wall
215 remodeling may alter the pattern recognition of cell wall components by host defenses and thus
216 influence the character of susbsequent immune responses. Germination and growth of
217 *Aspergillus fumigatus* isolate Af293 conidia in the presence of caspofungin, which targets β-1,3-
218 glucan, resulted in increased chitin expression (Fig. 3A and [18, 19]). When mice aspirated

Af293 conidia that were cultured and germinated in the presence of caspofungin, airway eosinophil recruitment was significantly increased when compared to aspiration of untreated germinated conidia, while total cells and neutrophils remained unchanged (Figs. 3B and 3C and data not shown). Thus, growth and germination of *A. fumigatu*s in the presence of caspofungin promotes increased chitin expression and airway eosinophil recruitment in response to a single aspiration of conidia.

3.3. Lung transcription of IL-17A is increased in response to Af5517 conidia and is not required for chitin-mediated eosinophil recruitment.

We observed in a previous study that multiple aspirations of Af5517 conidia resulted in an increase in lung expression of Th2 chemokines and a decrease in IFN-γ when compared to repeated aspiration of Af293 [17]. However, the early signals in response to increased chitin expression in *A. fumigatus* remain unclear. In contrast to our chronic exposure model, a single aspiration of Af5517 resulted in an increased in IL-17A transcription (Fig. 4A). To determine if IL-17A was important for chitin-mediated eosinophil recruitment, we compared leukocyte recruitment in wild-type and IL-17A-deficient mice that had received single or multiple aspirations of Af5517 conidia. After a single aspiration of Af5517 conidia, no significant difference in BALF leukocytes, neutrophils, or eosinophils was observed between wild-type and IL-17A-deficient mice (Figs. 4B-D). Furthermore, IL-17A was required for airway neutrophil recruitment after multiple aspirations of conidia (Fig. 4F). However, lack of IL-17A expression did not significantly affect total airway leukocytes or eosinophil recruitment (Figs. 4E and 4G). These results suggest that although IL-17A transcription is increased in response to Af5517, this increase does not play a role in chitin-mediated eosinophil recruitment.

3.4. Airway eosinophil recruitment in response to high chitin-expressing Af5517 is not increased in RAG1-deficient mice.

Previously, innate eosinophil recruitment was reported in response to pure chitin particles, a phenotype that was also observed in recombinase activating gene (RAG)-deficient mice that lack adaptive immunity [32]. Therefore, we aimed to determine if adaptive immunity was necessary for the increased eosinophil recruitment observed in response to a single aspiration of swollen Af5517 conidia in comparison to Af293. We observed no significant differences in total leukocytes, neutrophils or eosinophils when airway responses to Af293 and Af5517 were compared in RAG1-/- mice (Figures 5A-C). Thus, in contrast to the previous reported response to chitin particles [32], RAG1 expression was required for the observed increase in eosinophil recruitment in response to Af5517 conidia.

3.5. Airway eosinophil recruitment in response to high chitin-expressing Af5517 is partly dependent on γδ T cells.

The lack of chitin-enhanced eosinophil recruitment in RAG1-deficient mice suggests that lymphocytes with antigen receptors generated by somatic recombination are required for this early response phenotype. Therefore, we examined the role of γδ T cells that are known to undergo rapid activation at sites of injury or infection and promote eosinophil recruitment in lung allergic inflammation [33-35]. CD3+γδTCR+ T cells were observed in the lungs of mice 48 hrs after a single aspiration of swollen Af293 or Af5517 conidia, with no significant increase observed in response to Af5517 (Figures 6A,B). However, in mice deficient in γδ T cells, airway eosinophil recruitment was markedly decreased in response to single aspiration of Af5517 in comparison to wild-type mice, while neutrophils and total leukocytes were unaffected (Figures 6C-E). The phenotype was maintained after multiple aspirations of Af5517 (Figures 6F-H), more

specifically in a decreased eosinophil frequency along with a concomitant increase in airway neutrophils in γδ T cell-deficient mice (Figures 6G,H). Thus, chitin-mediated eosinophil recruitment in response to *A. fumigatus* is partially dependent on the presence of γδ T cells.

4. Discussion

Our results and the results of others suggest that specific patterns of cell-wall modulation promote distinct immune responses. Of the strains we examined, the three that were reported to exhibit a decreased ratio of cell wall β-glucan/chitin (Af5517, Δ*hacA*, and Δ*ireA)* [5, 11, 12] also induced increased airway frequency and/or total number of eosinophils after repeated conidial aspiration in comparison to other wild-type and mutant strains. The relevance of this association is further enhanced by the results of previous studies that demonstrated increased surface chitin exposure or airway eosinophilia in response to repeated aspiration of melanin-deficient strains, in particular the melanin-deficient Δarp2 [14, 16]. However, these studies are not altogether definitive, and it will thus be necessary to characterize the immune responses to additional strains with similar cell wall phenotypes to more fully understand the extent of this host-pathogen relationship. Indeed, there are other strains and growth conditions that have been reported to exhibit similar cell wall phenotypes. For example, cell wall chitin was also increased in an *A. fumigatus* strain with a deficiency in glycoprotein synthesis caused by conditional inactivation of Stt3, a subunit of the N-glycosylating enzyme oligosaccharyltransferase [36]. Increased lung eosinophils observed in response to repeated aspiration of this strain would provide additional support of a correlation with cell wall β-glucan/chitin content or exposure.

In addition to natural or targeted mutation, conditions of environmental stress may also alter cell wall composition and therefore differentially prime host immunity. *A. fumigatus* grown under hypoxic conditions similar to those encountered in host tissue during invasive infection resulted in increased cell wall β-glucan and chitin capable of stimulating increased macrophage and neutrophil activation [37]. Antifungals are another potential source of environmental stress encountered in host tissue, as echinocandins and nikkomycins directly target the synthesis of cell

wall β-glucan and chitin, respectively [38]. Our results confirmed those of other studies that demonstrated inhibition of β-glucan synthesis in *A. fumigatus* by the echinocandin caspofungin resulted in increased cell wall chitin [18, 19]. In addition to increased surface chitin, we observed increased airway eosinophil recruitment in mice after aspiration of caspofungin-treated Af293. This suggests that caspofungin therapy might in some aspergillosis patients increase detrimental eosinophil activation, and the results of another study using a mouse model of invasive aspergillosis provide support for this hypothesis (Amarsaikhan et al., submitted). This is further supported by other reports of decreased caspofungin efficacy in mouse models of invasive aspergillosis [39, 40].

In the strains we examined, statistically significant decreases in cell wall β-glucan were not unifor mly accompanied with increases in chitin [11, 12]. Moreover, in the environmental isolate Af5517, we observed markedly increased cell wall chitin along with a modest increase in β-glucan composition compared to the other isolates examined in this study [5]. Of these two cell wall components, the immune response to β-glucan has been more extensively studied in the context of fungal infection, being implicated in neutrophil recruitment and macrophage activation via Dectin-1 recognition [9, 41, 42]. Although several innate immune receptors have been shown to mediate chitin-induced responses (TLR2, TLR9, NOD2, mannose receptor) [23, 24], a chitin-specific receptor has not been identified. Our lab demonstrated that increased surface chitin exposure promotes increased eosinophilia and Th2 immune responses to repeated aspiration of the isolate Af5517 [17]. Furthermore, eosinophils appeared to contribute to detrimental Th2 responses in a model of invasive aspergillosis. In addition to identifying host factors that predispose individuals to inappropriate immune responses to environmental and

pathogenic fungi, it will be equally important to understand how concurrent recognition of these core cell wall components program immunity to inhaled fungi.

The early innate immune responses that program subsequent immunity to fungal infections are not well understood. In this study, we observed that a single aspiration of high-chitin-expressing Af5517 conidia induced lung transcription of IL-17A, while Af293 conidia did not (Fig. 4A). However, expression of IL-17A was not required for lung eosinophil recruitment in response to single or multiple aspirations of Af5517 conidia (Fig. 4B-G). Rather, neutrophil recruitment after multiple aspirations of Af5517 was markedly decreased in IL-17A-deficient mice (Fig. 4F). This result is not surprising, as the role of IL-17A in neutrophil recruitment is well-established [43]. Interestingly, eosinophils were recently identified as a source of IL-17A in response to *A. fumigatus* inhalation [44]. It is therefore possible that the increase in IL-17A transcription that we observed in response to a single aspiration of Af5517 was due to increased eosinophil recruitment, rather than a contributing factor. Instead, our results with RAG1 and γδ T-cell-deficient mice suggest that this subset of innate-like T cells are required for maximal eosinophil recruitment in response to *A. fumigatus* (Figs 5 and 6). γδ T cells are also known to be a potent source of innate IL-17A in the lungs in response to infection [45]; however, we did not observe increased production of IL-17A in γδ T cells in response to Af5517 (data not shown). Although our results suggest that γδ T cells play a role in eosinophil recruitment in response to *A. fumigatus*, the effector mechanism responsible remains unknown. One likely possibility is γδ T cell secretion of IL-4, which was shown to mediate eosinophil recruitment in a mouse model of allergy [34], and we intend to examine the role of this cytokine in γδ T cells in future studies. A caveat of our results is that our initial studies were done in BALB/c mice, whereas our experiments with IL-17A and γδ T cell-deficient mice required the use of B6-background control

and knockout animals. However, we previously reported increased lung eosinophil recruitment in response to Af5517 in both BALB/c and B6 mice [17], suggesting that although the mechanisms of recruitment may not be identical, the phenotype of chitin-mediated eosinophil recruitment is not strain-specific. Our future studies will examine in more detail the role of γδ T cells in chitin-mediated eosinophil recruitment in response to *A. fumigatus* inhalation and infection in both strains of mice.

In this study, we have identified strains (natural or with virulence factor deletions) of *A. fumigatus* that concomitantly exhibit a decreased ratio of cell wall β-glucan/chitin content and increased airway eosinophil recruitment. However, *A. fumigatus* is a complex organism comprised of many structural components that are temporally expressed and thus recognized by the immune system during growth and germination. Future studies will need to account for the array of potential interactions between secreted and structural components of fungi and the diverse immune repertoire of susceptible individuals in order to develop the most effective therapies in the treatment of *Aspergillus*-related immune pathogenesis.

Conflict of Interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Comparison of airway leukocyte recruitment in response to different *A. fumigatus* isolates in a mouse model of repeated conidia aspiration. (A) Representative FACS plots of bronchoalveolar lavage fluid with neutrophil, eosinophil and alveolar macrophage gates and frequencies. (B) Representative FACS plots depict gating, CD4+ and CD8+ T cell frequencies, and frequencies of IFN-γ and IL-17A producing cells within each subset. (C) Schedule of BALB/c mouse aspiration. (D-F) Frequency (top panels) and total number (bottom panels) of the indicated BALF cell populations. (G) Frequency (top panels) and total numbers (bottom panels) of CD4+ T cells producing IFN-γ or IL-17A, as indicated. (H) Total number of BALF leukocytes. Data shown are a summary of 2-3 experiments with 5-15 mice in each group. *p<0.05. **p<0.01. ****p<0.0001.

Figure 2. Airway leukocyte recruitment in response to repeated aspiration of of wild-type and mutant strains of *A. fumigatus* conidia. Mice were subjected to repeated aspirations of parent wild-type or Δ*cgrA*, Δ*hacA*, or Δ*ireA* mutant strains with resulting airway leukocyte populations quantified by flow cytometric analysis as described for Figure 1. (A-C) Frequency (top panel) and total number (bottom) of the indicated BALF cell subsets. (D) Frequency (top panels) and total numbers (bottom panels) of IFN-γ or IL-17A-producing T cells within each subset. Data shown are a summary of 2 experiments with 5-12 mice in each group. (E) Total leukocytes. *p<0.05, **p<0.01.

Figure 3. Growth and germination in the presence of caspofungin promotes increased fungal chitin exposure and airway eosinophil recruitment in response to *A. fumigatus*. Af293 conidia

were cultured and germinated (4 hrs at 37°C) in the presence of caspofungin or control conditions, then fixed prior to WGA-APC staining (A,B) or aspiration of BALB/c mice (C,D). (A) Representative histogram overlay from 3 experiments. (B,C) Indicated BALF populations 48 hrs after aspiration as a summary of 3 experiments. *p<0.05, ****p<0.0001.

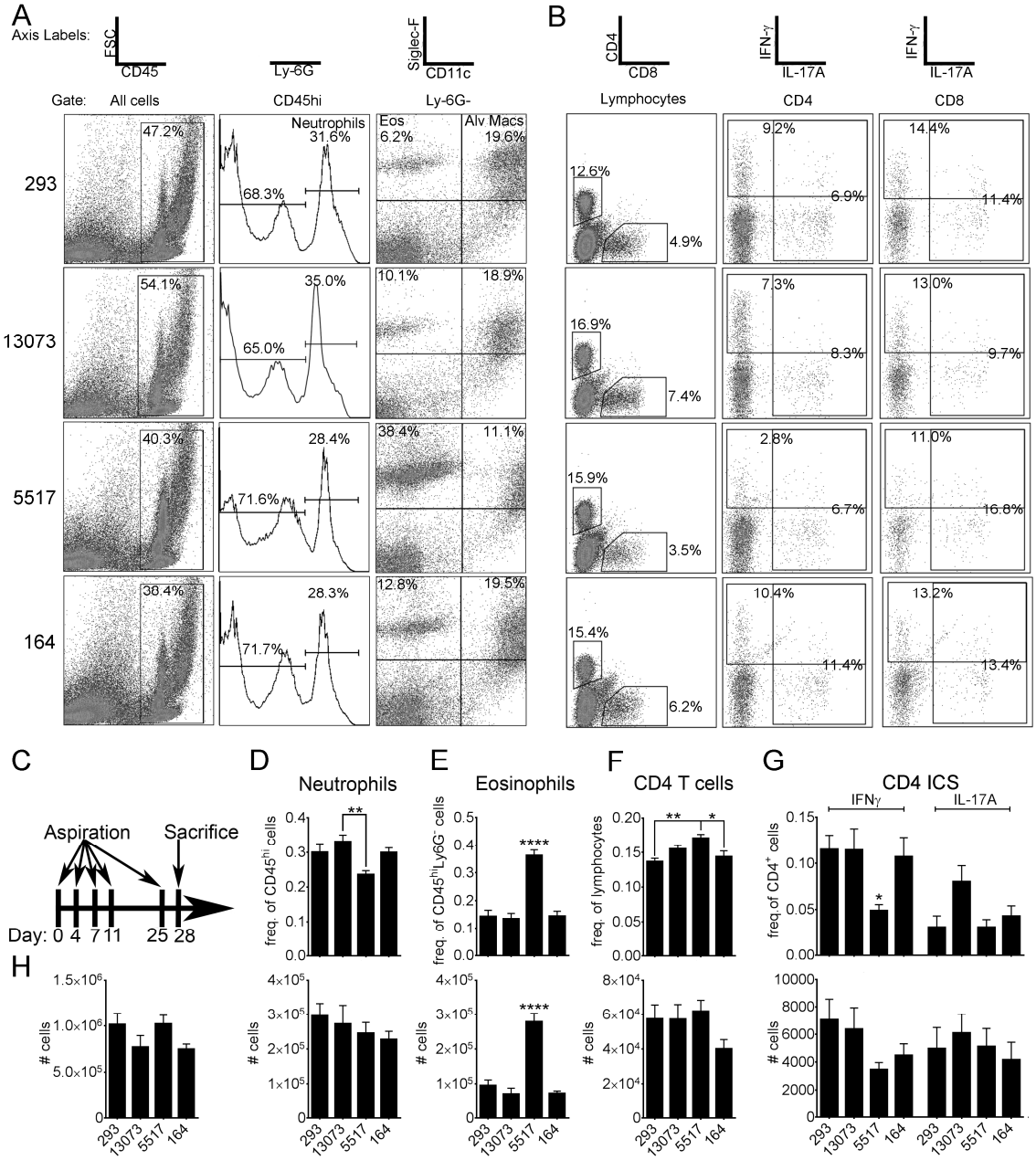
Figure 4. IL-17A is not required for airway eosinophil recruitment in response to Af5517 conidia.

(A) Lung homogenate mRNA of indicated cytokine/chemokine genes in BALB/c mice was compared with single aspirations of Af293 or Af5517 conidia. Gene expression was assessed by qRT-PCR (summary of 2 experiments with a total of 6 mice/group). (B-G) Flow cytometric quantification of indicated BALF cell subsets from wild-type (B6) or IL17A-deficient mice upon single (B-D, 48 hrs post-challenge) or multiple (E-G, 72 hrs post-challenge) aspiration(s) of Af5517 conidia (summary of 2 experiments). ***p<0.001. ****p<0.0001.

Figure 5. RAG1 activity is required for chitin-mediated airway eosinophil recruitment in response to fungal aspiration. (A-C) RAG1-deficient mice aspirated Af293 or Af5517 isolates, and were sacrificed 48 hrs later. BALF was recovered and the indicated cell populations were quantified. Data are shown as a summary of 3 independent experiments.

Figure 6. γδ T cells promote airway eosinophil recruitment in response to fungal inhalation. (A,B) Wild-type B6 mice were challenged with conidia from indicated isolates and lungs were harvested 48 hrs later, homogenized, with γδ T cells quantified by flow cytometry. (A) Frequency. (B) Total number of cells. (C-E) Indicated BALF cell populations 48 hrs after a

523 single aspiration of Af5517 conidia in wild-type B6 or γδ T cell deficient mice. (F-H) Indicated
524 BALF populations in response to repeated aspirations of Af5517 conidia at 72 hrs after the final
525 challenge. Data shown are a summary of 2 experiments. *$p<0.05$. **$p<0.01$.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