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The β -Glucan Receptor Dectin-1 Promotes Lung Immunopathology during Fungal Allergy via IL-22

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Sensitization to fungi, such as the mold *Aspergillus fumigatus*, is increasingly becoming linked with asthma severity. We have previously shown that lung responses generated via the β -glucan receptor Dectin-1 are required for lung defense during acute, invasive *A. fumigatus* infection. Unexpectedly, in an allergic model of chronic lung exposure to live *A. fumigatus* conidia, β -glucan recognition via Dectin-1 led to the induction of multiple proallergic (*Muc5ac*, *C1ca3*, CCL17, CCL22, and IL-33) and proinflammatory (IL-1 β and CXCL1) mediators that compromised lung function. Attenuated proallergic and proinflammatory responses in the absence of Dectin-1 were not associated with changes in *Ido* (IDO), *Il12p35/Ebi3* (IL-35), IL-10, or TGF- β levels. Assessment of Th responses demonstrated that purified lung CD4⁺ T cells produced IL-4, IL-13, IFN- γ , and IL-17A, but not IL-22, in a Dectin-1-dependent manner. In contrast, we observed robust, Dectin-1-dependent IL-22 production by unfractionated lung digest cells. Intriguingly, the absence of IL-22 alone mimicked the attenuated proallergic and proinflammatory responses observed in the absence of Dectin-1, suggesting that Dectin-1-mediated IL-22 production potentiated responses that led to decrements in lung function. To this end, neutralization of IL-22 improved lung function in normal mice. Collectively, these results indicate that the β -glucan receptor Dectin-1 contributes to lung inflammation and immunopathology associated with persistent fungal exposure via the production of IL-22. *The Journal of Immunology*, 2012, 189: 000–000.

Many asthmatics are able to keep their symptoms relatively under control with current therapies; however, a subset of asthmatics has multiple exacerbations annually that often require hospitalization. It has long been known that two-thirds of asthmatics are atopic to multiple allergens, and the severity often correlates with the degree of atopy (1). Although these allergens are common to many environments, fungi/molds are likely the most ubiquitous. Consequently, reports indicate that among severe asthmatics, sensitivity to fungi ranges from 25% to >70% (reviewed in Ref. 2) and correlates with hospital/intensive care unit admissions compared with asthmatics who do not require hospitalization (3). Although acknowledged to be associated with one of the severest forms of asthma, allergic bronchopulmonary aspergillosis (ABPA), hypersensitivity to *Aspergillus* alone, in the absence of a clinical ABPA diagnosis, is associated with asthma exacerbations (3). *Aspergillus*-sensitized asthmatics have lower lung function, more bronchiectasis, higher sputum neutrophil numbers, and higher steroid usage compared with asthmatics who are not sensitized (4, 5). Intriguingly, antifungal treatment of fungal-sensitized asthmatics (66% of whom demonstrated sensitivity to *Aspergillus*) resulted in better pulmonary function, lower serum IgE, and an improvement in Asthma Quality of Life Questionnaire score (6).

Severe forms of asthma are often associated with neutrophilic, rather than eosinophilic, infiltration (7, 8). IL-17A is a proinflammatory cytokine that upregulates a number of cytokines and chemokines leading to the recruitment of neutrophils to sites of inflammation (9). IL-17A gained prominence when it was discovered to be produced by CD4 T cells, a lineage now termed Th IL-17 or Th17 cells (10). With respect to human asthma, elevated IL-17A mRNA and protein expression has been observed in the lungs of asthmatics (11, 12). Early insight into a role for IL-17A in asthma-like changes came from experimental lung-overexpression studies, which demonstrated hypertrophic bronchial epithelium, mucus production, neutrophilic and eosinophilic infiltration, and asthma-associated chemokine induction (13). Moreover, stimulation of murine lung epithelial cells with IL-17A leads to the induction of multiple genes that may contribute to asthmatic phenotypes (14). Experimental models of asthma, including OVA challenge (15), house dust mite (16), respiratory syncytial virus (17), and cockroach allergen (18), have each demonstrated the induction of Th17 responses. In the widely used OVA model, some studies report beneficial effects when IL-17A is neutralized (15), although some studies argue for a protective role for IL-17A (19). However, in a striking finding, steroid-resistant allergic airway inflammation was found to be driven by Th17, but not Th2, responses (20). The explosion in IL-17A research over the last 15 y has also identified IL-22 as a Th17-associated cytokine (21). IL-22 is an intriguing cytokine in that it can act in both pro- and anti-inflammatory responses (22–23). Moreover, the limited cellular expression of the IL-22R, predominantly epithelial cells to date (24), makes it an attractive mediator to investigate in asthma pathogenesis. Like IL-17A, IL-22 is also reported to be elevated in asthmatics (25, 26). Experimental investigation in an OVA model has shown that IL-22 is required for the onset of allergic asthma; however, neutralization of IL-22 during Ag challenge enhanced allergic lung inflammation (25). In turn, administration of IL-22 has been shown to dampen allergic lung inflammation in mice (25, 27). We have recently shown a comprehensive role for the Dectin-1/IL-17A/IL-

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Abbreviations used in this article: ABPA, allergic bronchopulmonary aspergillosis; GMS, Gomori methenamine silver; PAS, periodic acid-Schiff; WT, wild-type.

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22 axis in protection against acute, invasive infection with *Aspergillus fumigatus* (28–30). In the current study, we investigated this axis during chronic *A. fumigatus* exposure, specifically in a live *A. fumigatus* conidia repetitive challenge model of fungal allergy. Despite being required for the elimination of *A. fumigatus* from the lungs during acute exposure, our data in this study support an immunopathogenic role for Dectin-1 and IL-22 during chronic fungal allergy.

Materials and Methods

Mice

C57BL/6NTac mice, 6–8 wk of age, were purchased from Taconic Farms (Germantown, NY). Dectin-1-deficient mice were generated on the 129/SvEv background, as previously described (31), backcrossed 10 generations to the C57BL/6 background, and bred at Taconic. IL-22-deficient mice (32) were provided by W. Ouyang (Genentech) and bred at the University of Alabama at Birmingham. Mice were maintained in a specific pathogen-free environment in microisolator cages within an American Association for Laboratory Animal Science-certified animal facility in the Lyons Harrison Research Building at the University of Alabama at Birmingham. Animal studies were reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

A. fumigatus chronic exposure model

A. fumigatus isolate 13073 (American Type Culture Collection, Manassas, VA) was maintained on potato dextrose agar for 5–7 d at 37°C. Conidia were harvested by washing the culture flask with 50 ml sterile 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. The repeated *A. fumigatus* exposure model was employed, as previously described (33). Briefly, mice were lightly anesthetized with isoflurane and administered 1×10^7 live *A. fumigatus* conidia in a volume of 50 μ l PBS intratracheally. Starting at day 7, mice were challenged intratracheally with 1×10^6 conidia live *A. fumigatus* in 50 μ l PBS daily for 5 d, rested for 2 d, and challenged daily for another 3 d. At 24 h after the final challenge, immune measures were assessed, as described below.

Muc5ac, *Clca3*, *Saa3*, *Ido1*, and *Il12p35/Ebi3* analysis

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 *Muc5ac* (Mm01276735_m1; Applied Biosystems), *Clca3* (Mm00489959_m1; Applied Biosystems), *Saa3* (Mm00441203_m1; Applied Biosystems), *Ido1* (Mm00492586_m1; Applied Biosystems), and *Il12p35/Ebi3* (Mm00434165_m1/Mm00469294_m1; Applied Biosystems) and was performed (iQ Supermix; Bio-Rad). mRNA levels were normalized to *Gapdh* mRNA levels (primers/probe from Applied Biosystems) using the $2^{-\Delta\Delta C_t}$ method (29, 30).

Whole lung cytokine and chemokine analysis, lung cell isolation, and culture

The left lung was homogenized in PBS supplemented with Complete 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 Bio-Plex multiplex suspension cytokine array (Bio-Rad Laboratories), according to the manufacturer's instructions. The data were analyzed using Bio-Plex Manager software (Bio-Rad Laboratories). IL-23, IL-33, CCL17, and CCL22 levels were quantified by ELISA (R&D Systems). For lung cell isolation, the lungs were collected and minced in IMDM media (Sigma-Aldrich, St. Louis, MO) supplemented with 1% penicillin-streptomycin-glutamine (Mediatech, Herndon, VA), 10% heat-inactivated FBS (Invitrogen, Carlsbad, CA), and 0.4 mg/ml polymyxin B (Thermo Fisher), followed by incubation for 60 min with tissue culture-grade type IV collagenase (1 mg/ml; Sigma-Aldrich) in a 37°C orbital shaker at 100 rpm. The cell suspension was filtered through sterile 70- μ m and 40- μ m nylon filters and RBCs lysed with ACK buffer (Lonza, Walkersville, MD) to create single-cell preparations. One million cells in a volume of 200 μ l were cultured overnight, followed by assessment of Th cytokine production by ELISA or Bio-Plex. For CD4 T cell purification, lung cells were stained for CD4, followed by FACS sorting. Purified CD4 T cells were stimulated with 5 μ g/ml anti-CD3 (clone 145-11)

and 2.5 μ g/ml anti-CD28 for 24 h. ELISA or Bio-Plex was employed to assess Th cytokine levels in supernatants.

Lung cell surface marker flow cytometry

Lung cells were isolated via bronchoalveolar lavage, as previously described (34). Cells were washed and Fc receptors were 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: eosinophils as CD11b⁺ Siglec F⁺ Ly-6G and Ly-6Clo/neg, neutrophils as CD11b⁺ Ly6G⁺ (1A8), dendritic cells as CD11b⁺ CD11c⁺, NK cells as CD11b⁺ DX5⁺, and T cells as CD3⁺ CD4⁺ (all Abs purchased from eBiosciences and BD Biosciences). Samples were acquired using a four-laser, 20-parameter analytic BD LSR II, and data were analyzed using FlowJo software (Tree Star, Ashland, OR). Unstained cells served as a control for background fluorescence and gating. Samples were acquired using BD LSR II cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

Pulmonary function assessment

A tracheostomy was performed on individual anesthetized *A. fumigatus*-exposed mice. Each animal was then attached to a computer-controlled volume ventilator (flexiVent; SCIREQ Montreal, PQ, Canada). 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 H₂O and measured during each breath stroke. Respiratory input impedance (Zrs) was measured using the forced oscillation technique controlled by the flexiVent system. The single-compartment model was used to describe dynamic lung resistance. All measurements were collected at baseline and after a linear dose response with methacholine challenge (10–40 mg/ml), as previously described (28).

IL-22 neutralization

For in vivo IL-22 neutralization, C57BL/6 mice were subjected to the repeated *A. fumigatus* exposure model, as described, and on days 7, 10, 13, and 16 administered 250 μ g anti-murine IL-22, clone 8E11, provided by W. Ouyang (Genentech) (32), or isotype control Ab i.p. Twenty-four hours after the last challenge, pulmonary function was assessed, as described.

Statistics

Data were analyzed using GraphPad Prism Version 5.0 statistical software. Comparisons between groups when data were normally distributed were made with the Student *t* test. Significance was accepted at a value of $p < 0.05$.

Results

The absence of Dectin-1 results in improved lung function after chronic fungal exposure

Although mice can be manipulated to develop allergic/Th2-type immune responses, this usually requires highly artificial commercial allergen sensitization procedures and the use of adjuvants. Whereas these experimental models clearly reproduce some aspects of human asthma and anti-inflammatory therapies have demonstrated efficacy in these models, many of these have failed to alter asthmatic/allergic responses in humans (reviewed in Refs. 35, 36). To alleviate this concern, recent animal models, particularly with fungi, have been developed in which repetitive exposure to a live pathogen induces an allergic response (33, 37). These models reproduce some critical features of allergic disease observed in persistently exposed individuals that are not present after a single exposure. A diagram of the chronic *A. fumigatus* exposure model is illustrated in Fig. 1A. Employing this model, we analyzed lung function 24 h after the last challenge. Initial analysis indicated that serum total IgE levels, which are undetectable in naive mice, reach nearly 300 ng/ml in both wild-type (WT) and Dectin-1-deficient mice after chronic fungal exposure (Fig. 1B) (33). Upon methacholine challenge, Dectin-1-deficient mice demonstrated significantly lower dynamic lung resistance (Fig. 1C) compared with WT mice. Thus, Dectin-1 signaling promoted responses in the lung that were detrimental to lung function.

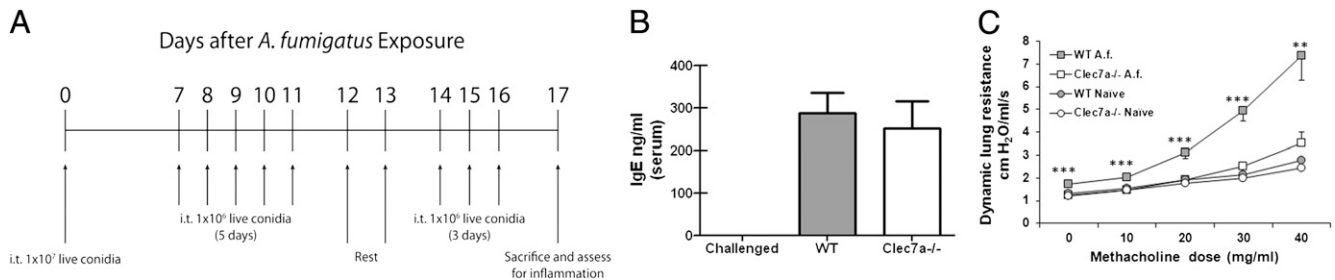


FIGURE 1. The absence of Dectin-1 results in improved lung function after chronic fungal exposure. **(A)** Diagram of the chronic *A. fumigatus* exposure model. **(B)** Total IgE levels in the serum of C57BL/6 (WT) and Dectin-1-deficient (Clec7a^{-/-}) mice after chronic fungal exposure. Challenged represents mice receiving a single challenge with *A. fumigatus*. **(C)** C57BL/6 (WT) and Dectin-1-deficient (Clec7a^{-/-}) mice were exposed to *A. fumigatus* conidia, as in (A). Twenty-four hours after the last challenge, dynamic lung resistance was analyzed via mechanical ventilation using the flexiVent system. This figure illustrates cumulative data from two independent studies. For all graphs, $^{**}p < 0.01$, $^{***}p < 0.001$, and $n = 4$ –5 mice/group for each study.

The absence of Dectin-1 results in attenuated proallergic and proinflammatory responses after chronic fungal exposure

We next determined the impact of Dectin-1 deficiency on proallergic and proinflammatory responses after repetitive *A. fumigatus* challenge. Better lung function in the absence of Dectin-1 was accompanied by significantly lower lung *Muc5ac* and *Clca3* (Gob5) mRNA expression (Fig. 2A), CCL17 and CCL22 levels (Fig. 2B), and IL-1 β and CXCL1 levels (Fig. 2C). In agreement with previous reports (33), C57BL/6 mice chronically exposed to *A. fumigatus* had higher eosinophil numbers compared with neutrophils, although neutrophils in lung lavage fluid were the lone population observed to be significantly lower in Dectin-1-deficient mice (Fig. 2D). Assessment of epithelial-derived mediators known to promote proallergic responses (38) revealed no detectable induction of thymic stromal lymphopoietin and similar levels of IL-25 in lung homogenates between WT and Dectin-1-deficient mice (data not shown). In contrast, we observed a significant reduction in the levels of the novel IL-1 family cytokine IL-33 in Dectin-1-deficient mice (Fig. 2E). Histological assessment of lung tissue sections revealed higher inflammatory cell recruitment to the lungs of WT mice (Fig. 2F) in the presence of enhanced goblet cell hyperplasia and mucus production (Fig. 2G). Gomori methenamine silver (GMS) staining of lung tissue sections showed similar levels of *A. fumigatus* organisms in the lungs of WT and Dectin-1-deficient mice (Fig. 2H). Collectively, these data indicate that Dectin-1 signaling contributes to lung proallergic and proinflammatory responses and immunopathogenesis during fungal allergy.

Dectin-1 deficiency is associated with attenuated lung cell IL-17A and IL-22 production during chronic fungal exposure

As the results in Fig. 1 indicated that CCL17, CCL22, and IL-33 production were attenuated in Dectin-1-deficient mice chronically exposed to *A. fumigatus*, we next assessed whether Th2 responses were modulated by Dectin-1 deficiency. Stimulation of lung cells isolated via enzymatic digestion with *A. fumigatus* for 24 h in vitro, which allows for quantification of the total amount of cytokine produced irrespective of the cell source (29, 30), revealed low production of IL-4 (Fig. 3A), IL-13 (Fig. 3B), and IFN- γ (Fig. 3C) by unfractionated WT lung cells. In contrast, we observed robust production of IL-17A (Fig. 3D) and IL-22 (Fig. 3E). Although produced at very low levels, IL-4 (Fig. 3A) and IFN- γ (Fig. 3C), but not IL-13 (Fig. 3B), production were lower in the absence of Dectin-1. Intriguingly, attenuated lung inflammation and better lung function during chronic *A. fumigatus* exposure in the absence of Dectin-1 correlated with significant reductions in unfractionated lung cell IL-17A (Fig. 3D) and IL-22 (Fig. 3E) pro-

duction. Anti-CD3/CD28 stimulation of CD4 T cells sorted by flow cytometry demonstrated Dectin-1-dependent production of IL-4 (Fig. 3A), IL-13 (Fig. 3B), IFN- γ (Fig. 3C), and IL-17A (Fig. 3D), but not IL-22 (Fig. 3E). Overall, although modulations in Th1 and Th2 responses were observed, the most apparent effect of Dectin-1 deficiency during chronic *A. fumigatus* exposure were attenuated IL-17A and IL-22 responses. Therefore, whereas our previous work indicated that acute exposure to *A. fumigatus* requires Dectin-1-mediated IL-17A and IL-22 for protection against infection, our data in this study suggest that Dectin-1-mediated immunopathology during fungal allergy may be due to IL-17A- and/or IL-22-mediated responses.

Attenuated *Saa3* expression and IL-23 production in the absence of Dectin-1 during chronic fungal exposure

To determine whether Dectin-1 deficiency was associated with a compensatory upregulation in regulatory responses, we assessed the expression of IDO (39) and IL-35 (40), both of which can negatively regulate IL-17A responses. However, neither *Ido* (Fig. 4A) nor *Il27p35/Ebi3* (IL-35) (Fig. 4B) mRNA expression was augmented in the lungs of Dectin-1-deficient mice, nor were the lung levels of IL-10 (Fig. 4C) and TGF- β (Fig. 4D). Therefore, lower lung IL-17A and IL-22 production in the absence of Dectin-1 was not a result of enhanced expression/function of immunoregulatory factors. In contrast, *Saa3* (serum amyloid A 3) mRNA levels, which can promote IL-17A responses, possibly via IL-23 induction (41), were significantly lower in the lungs of Dectin-1-deficient mice (Fig. 4E), as were IL-23 levels (Fig. 4F) in lung homogenates. These results suggest that lower IL-23 levels, possibly as a result of impaired *Saa3* induction, contribute to lower IL-17A and IL-22 production.

Lung inflammation requires IL-22 during chronic fungal exposure

During acute *A. fumigatus* exposure, we have shown that IL-22 is produced rapidly in the lungs at levels that are much higher than, and more Dectin-1 dependent than, IL-17A (29, 30). In addition, IL-22 was produced by unfractionated lung cells in higher amounts than IL-17A in chronically exposed and once again demonstrated more Dectin-1 dependency than IL-17A (Fig. 2). We therefore questioned the role of IL-22 in lung proallergic and inflammatory responses after repetitive *A. fumigatus* challenge. C57BL/6 and IL-22-deficient mice were repetitively exposed to *A. fumigatus* as in Fig. 1A, and parameters of lung inflammation were assessed. Results show that deficiency in IL-22 resulted in significant reductions in *Muc5ac* and *Clca3* (Gob5) mRNA expression (Fig. 5A), lung CCL17 and CCL22 levels (Fig. 5B), lung IL-33 levels (Fig. 5C), and IL-1 β and CXCL1 levels (Fig. 5D).

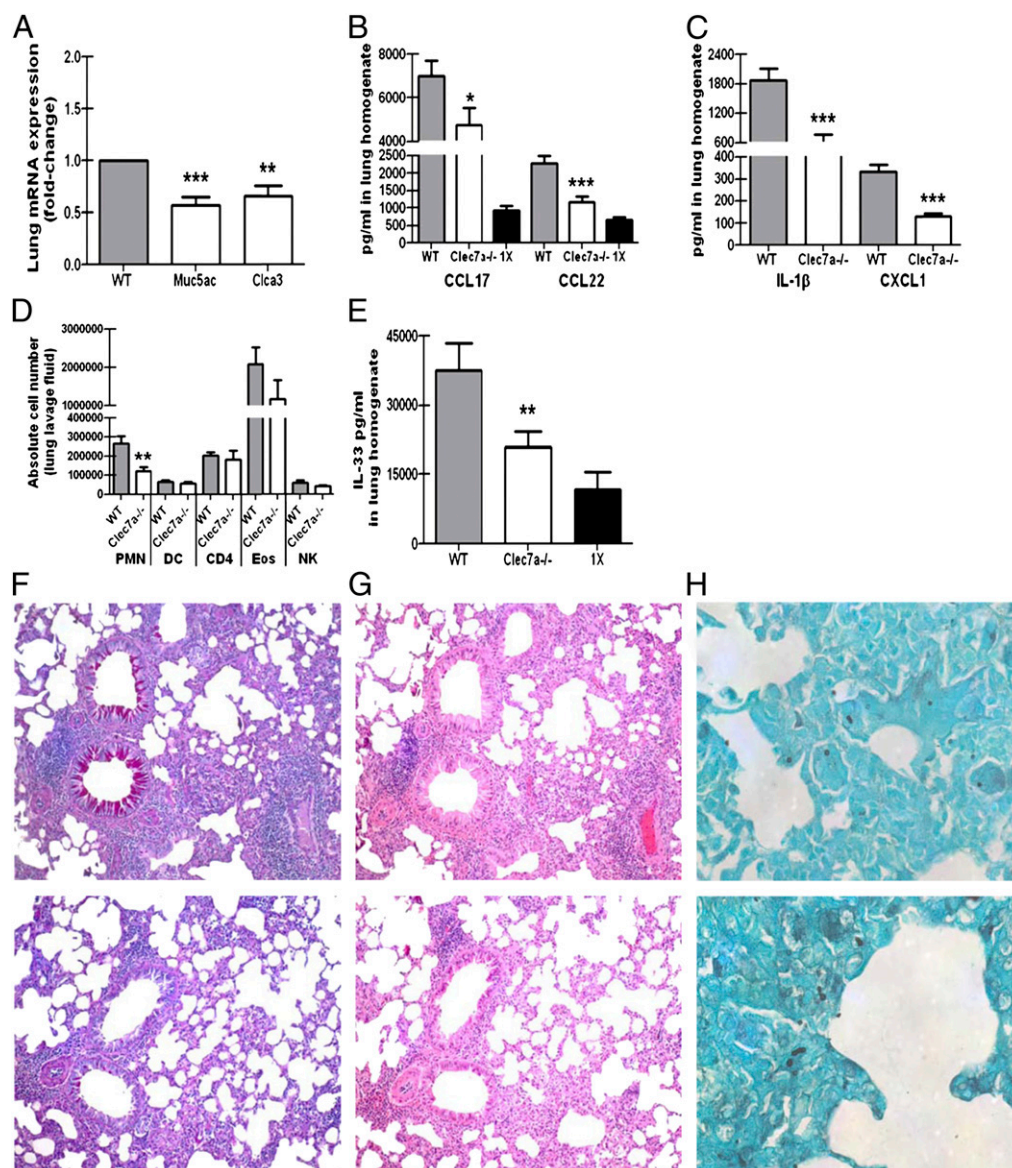


FIGURE 2. The absence of Dectin-1 results in attenuated proallergic and proinflammatory responses after chronic fungal exposure. **(A)** C57BL/6 WT and Dectin-1-deficient (Clec7a^{-/-}) mice were exposed to *A. fumigatus*, as described. Twenty-four hours after the last challenge, the left lungs were collected and *Muc5ac* and *Clca3* gene expression was quantified by real-time PCR and normalized to *Gapdh*. Fold changes between WT (set at 1) and Clec7a^{-/-} mice were determined using the $2^{-\Delta\Delta C_t}$ method. This figure illustrates cumulative data from three independent studies. **(B and C)** The right lungs were collected and homogenized, and CCL17 and CCL22 (**B**) and IL-1β and CXCL1 (**C**) levels were quantified in clarified lung homogenates by ELISA or Bio-Plex. This figure illustrates cumulative data from three independent studies. The 1X represents WT mice that received a single challenge of 1×10^6 *A. fumigatus* conidia for 24 h. **(D)** Lung cells were isolated by bronchoalveolar lavage, enumerated, Fc blocked, stained with a live/dead staining kit, followed by staining with fluorochrome-conjugated Abs corresponding to the various cell populations. This figure illustrates cumulative data from three independent studies. **(E)** IL-33 levels were quantified in clarified lung homogenates by ELISA. **(F–H)** Representative H&E-stained (**F**), PAS-stained (**G**), and GMS-stained (**H**) lung sections from WT (upper image) and Clec7a^{-/-} (lower image) mice. Original magnification $\times 200$ (H&E, PAS) and $\times 400$ (GMS). For all graphs, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and $n = 4$ –5 mice/group for each study.

Therefore, during chronic *A. fumigatus* exposure, Dectin-1-mediated IL-22 may be a central contributor to lung inflammation during fungal allergy.

Neutralization of IL-22 improves lung function after chronic fungal exposure

Cytokine modulation is currently undergoing clinical trials for efficacy in improving asthma control (35). Our data to date implicate Dectin-1-mediated responses, particularly the induction of IL-22, as a contributor to lung inflammation leading to decrements in lung function during chronic *A. fumigatus* exposure. To this end, we assessed the therapeutic efficacy of blocking IL-22 on improving

lung function during fungal allergy. For this, mice were exposed to *A. fumigatus* as in Fig. 1A and randomized to receive IL-22 neutralizing Abs or rat IgG isotype control Abs on days 7, 10, 13, and 16. Results show that, following methacholine challenge, mice receiving anti-IL-22 neutralizing Abs maintained lower dynamic lung resistance (Fig. 6), indicating that IL-22 drives lung inflammatory responses that have a negative impact on lung function during fungal allergy.

Discussion

We have previously reported that mice deficient in Dectin-1 acutely exposed to *A. fumigatus* had significantly lower levels of IL-17A in the lungs (28). In an extension of this work, we have recently re-

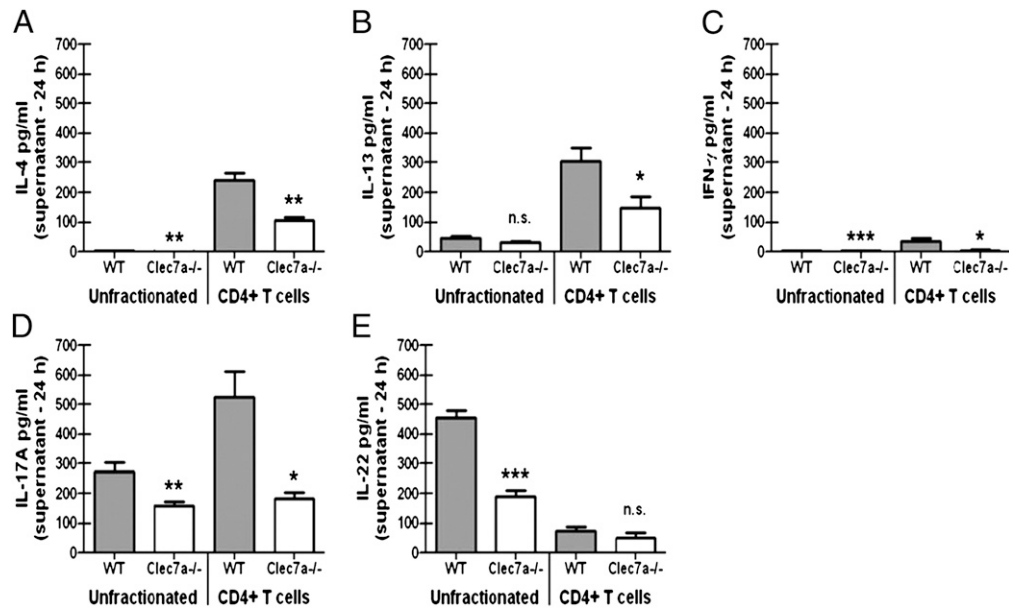


FIGURE 3. Dectin-1 deficiency is associated with attenuated lung cell IL-17A and IL-22 production during chronic fungal exposure. (A–E) C57BL/6 WT and Dectin-1-deficient (Clec7a^{-/-}) mice were exposed to *A. fumigatus*, as described. Twenty-four hours after the last challenge, the right lungs were collected and enzymatically digested, and unfractionated lung cells were cultured for 24 h in the presence of *A. fumigatus* conidia (1:3 cell to conidia ratio). In additional studies, CD4⁺ T cells were purified using flow cytometry and cultured for 24 h in the presence of anti-CD3/anti-CD28 Abs. IL-4 (A), IL-13 (B), IFN-γ (C), IL-17A (D), and IL-22 (E) levels were quantified in clarified coculture supernatants by ELISA or Bio-Plex. This figure illustrates cumulative data from three independent studies. For all graphs, **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

ported that during acute *A. fumigatus* exposure, neutrophils produce IL-17A in a Dectin-1- and IL-23-dependent manner (29). We have now extended the antifungal contribution of Dectin-1 to the induction of IL-22, as Dectin-1 deficiency resulted in a near total loss of lung IL-22 production after acute *A. fumigatus* exposure (30).

Importantly, neutralization of either IL-17A (28) or IL-22 (30) resulted in impaired clearance of *A. fumigatus*, illustrating a critical role for IL-17A and IL-22 in pathogen elimination during acute infection. In the current study, we examined the contribution of the Dectin-1/IL-17A/IL-22 axis to the development of fungal

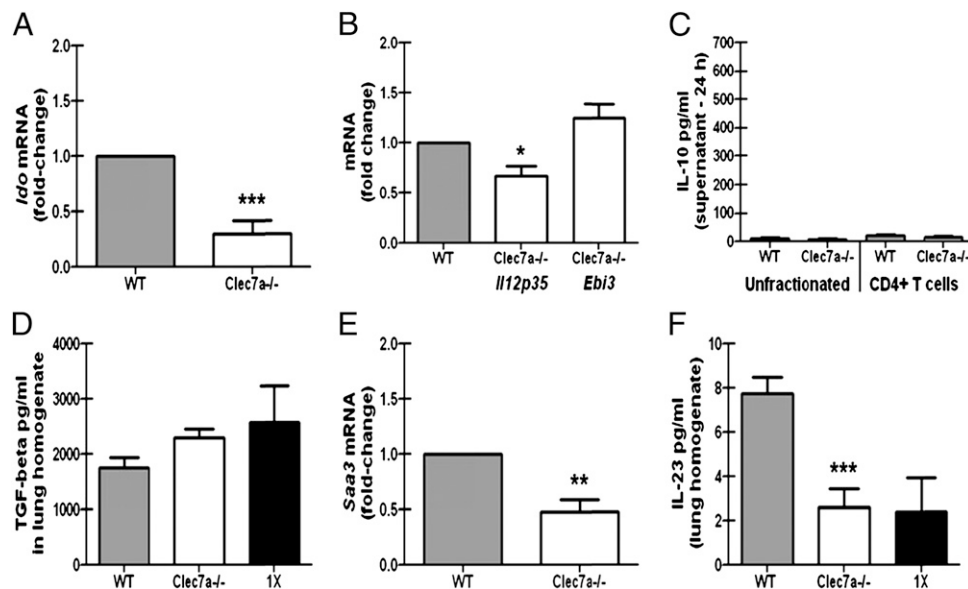


FIGURE 4. Attenuated *Saa3* expression and IL-23 production in the absence of Dectin-1 during chronic fungal exposure. (A and B) C57BL/6 WT and Dectin-1-deficient (Clec7a^{-/-}) mice were exposed to *A. fumigatus*, as described. Twenty-four hours after the last challenge, the left lungs were collected and *Ido* (A) and *Il12p35* and *Ebi3* (B) gene expression was quantified by real-time PCR and normalized to *Gapdh*. Fold changes between WT (set at 1) and Clec7a^{-/-} mice were determined using the 2^{-ΔΔC_t} method. This figure illustrates cumulative data from two independent studies. (C) Unfractionated lung cells and CD4⁺ T cells were isolated and cultured, as described, and IL-10 levels were quantified in coculture supernatants by Bio-Plex. This figure illustrates cumulative data from three independent studies. TGF-β (D) and IL-23 (F) were quantified in clarified lung homogenates by Bio-Plex and ELISA, respectively. This figure illustrates cumulative data from three independent studies. The 1X represents WT mice that received a single challenge of 1 × 10⁶ *A. fumigatus* conidia for 24 h. (E) *Saa3* gene expression was quantified by real-time PCR and normalized to *Gapdh*. Fold changes between WT (set at 1) and Clec7a^{-/-} mice were determined using the 2^{-ΔΔC_t} method. This figure illustrates cumulative data from two independent studies. For all graphs, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *n* = 4–5 mice/group for each study.

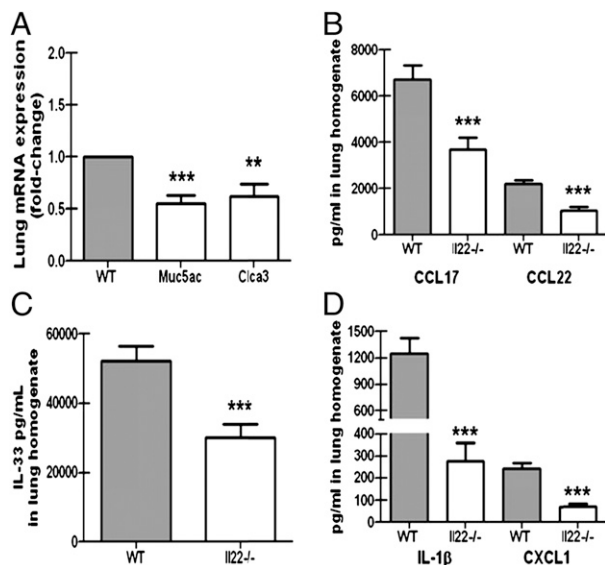


FIGURE 5. Lung inflammation requires IL-22 during chronic fungal exposure. (A) C57BL/6 WT and IL-22-deficient (IL-22^{-/-}) mice were exposed to *A. fumigatus*, as described. *Muc5ac* and *Clca3* gene expression was quantified by real-time PCR and normalized to *Gapdh*. Fold changes between WT (set at 1) and IL-22^{-/-} mice were determined using the $2^{-\Delta\Delta C_t}$ method. This figure illustrates cumulative data from three independent studies. (B–D) CCL17 and CCL22, (C) IL-33, and (D) IL-1 β and CXCL1 were quantified in clarified lung homogenates by ELISA or BioPlex. This figure illustrates cumulative data from four independent studies. For all graphs, ** $p < 0.01$, *** $p < 0.001$, and $n = 4$ –5 mice/group for each study.

allergy. Although required for host defense in an invasive infection model, our data show that Dectin-1, most likely via IL-22 production, contributes to immunopathogenesis in a model of fungal allergy.

Employing a repetitive live *A. fumigatus* challenge model (33), our initial data revealed that mice deficient in Dectin-1 had significantly better pulmonary function than control mice. To better understand these findings, we characterized the expression levels of mediators associated with the development of allergic responses. Analysis of *Muc5ac* and *Clca3*, two mucus-associated genes, demonstrated attenuated expression in the lungs of Dectin-1-deficient mice. This was supported by periodic acid-Schiff (PAS) staining of lung tissue showing that airway mucus was lower in mice deficient in Dectin-1. In addition, protein levels of the proallergic chemokines CCL17 and CCL22 were also attenuated in the absence of Dectin-1. Lower CCL17 levels are of particular

interest, as CCL17 (and CCL22 to a lesser extent) is considered a diagnostic and potentially prognostic marker during ABPA in individuals with cystic fibrosis (42, 43). We further identified IL-1 β and CXCL1 as proinflammatory mediators lowered by the absence of Dectin-1, which correlated with lower absolute neutrophil levels in lung lavage fluid and lower histological evidence of inflammation. As transgenic overexpression of CXCL1 in mice can enhance airway neutrophilia and hyperresponsiveness during fungal allergy (44) and CXCL1 levels in lavage fluid have recently been reported to differentiate severe versus moderate asthma in children (45), our data point to a possible immunopathogenic role for Dectin-1-mediated CXCL1 production.

Our data indicate that mice deficient in Dectin-1 exposed to repetitive *A. fumigatus* conidia have improved lung function that correlated with reductions in multiple proallergic and inflammatory mediators. Improved lung function in the absence of Dectin-1 also correlated with changes in CD4 T cell responses, specifically reductions in Th1, Th2, and Th17 responses. However, it is worth noting that in contrast to IL-4, IL-13, and IFN- γ , which were primarily produced by CD4⁺ T cells, IL-17A was produced by both unfractionated lung digest cells as well as purified CD4⁺ T cells. Moreover, we observed the most robust production of IL-22 in unfractionated lung digest cell cultures rather than purified CD4⁺ T cells, indicating that multiple cell types were contributing to the levels of IL-17A and IL-22 during chronic fungal exposure. Indeed, we have recently reported that neutrophils produce Dectin-1-dependent IL-17A during acute *A. fumigatus* exposure (29). Ongoing studies seek to identify the source of both IL-17A and IL-22 over the development of fungal allergy. The role of IL-17A in chronic fungal exposure has been recently investigated (33). In a similar repetitive *A. fumigatus* exposure model, although the effects of IL-17A on proallergic and proinflammatory responses as well as lung function were not investigated, neutralization of IL-17A or deficiency in IL-17RA resulted in attenuated neutrophil recruitment (33), as would be expected. A more recent study has shown that weekly *A. fumigatus* challenges in IL-17A-deficient mice over 4 wk resulted in attenuated lung inflammation, particularly in eosinophil numbers (46). In this report, it was hypothesized that, during fungal allergy, IL-17A may function to promote/mimic what is often associated with or attributed to Th2-mediated responses. How IL-17A may drive Th2-like inflammation is not currently known and the subject of future studies.

We posit in this work that IL-22 may also be involved in Th2-associated inflammation during fungal allergy. Our data indicate that Dectin-1 deficiency resulted in attenuated production of IL-22 in the presence of lower mucus gene expression, proallergic chemokine levels, and improved lung function. In turn, IL-22 deficiency also resulted in lower mucus gene expression and proallergic chemokine levels, and neutralization of IL-22 improved lung function. Common between Dectin-1 and IL-22 deficiency was attenuated production of IL-33. Therefore, one explanation for IL-17A/IL-22 mimicking Th2-like inflammation during fungal allergy may be due to an association with IL-33. Whether IL-17A or IL-22 has the ability to induce IL-33 directly is the subject of current studies. IL-33 is a potent inducer of Th2-associated mediators from multiple cell types (reviewed in Ref. 47). In a fungal allergy model employing i.p./s.c. sensitization with a commercial *Aspergillus* Ag in IFA, followed by three weekly intranasal Ag administrations and then a final challenge with live *A. fumigatus* conidia, ST2 (IL-33R) blockade ameliorated many pathological features of fungal asthma (48). However, this study focused solely on the Th2 aspects of fungal allergy. As ST2 forms a receptor complex with the commonly used subunit IL-1R accessory protein (49), IL-33 may also induce a number of proinflammatory mediators, such as TNF-

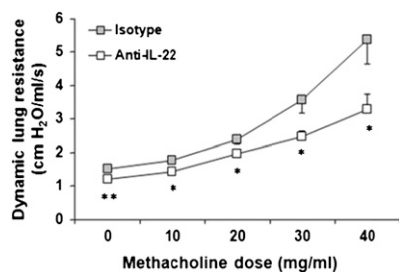


FIGURE 6. Neutralization of IL-22 improves lung function after chronic fungal exposure. C57BL/6 mice were exposed to *A. fumigatus*, as described. On days 7, 10, 13, and 16, mice received either 250 μ g anti-IL-22 Ab or isotype control Ab i.p. Twenty-four hours after the last challenge, dynamic lung resistance was analyzed via mechanical ventilation using the flexiVent system. This figure illustrates cumulative data from two independent studies. For all graphs, * $p < 0.05$, ** $p < 0.01$, and $n = 4$ –5 mice/group for each study.

α , IL-6, and IL-8 (reviewed in Ref. 47). Furthermore, neutrophil-derived elastase and cathepsin G can cleave IL-33 into a more active form, resulting in higher elicitation of inflammatory mediators such as IL-6 (50). Murine models of collagen-induced arthritis have shown that ST2 deficiency (51) and ST2 blockade (52) lead to reduced IL-17A levels, implicating a surprising role for IL-33 in IL-17A generation. IL-33 has also been shown to be overexpressed in clinical ulcerative colitis and in experimental enteritis, where IL-33 induced IL-17A production from mesenteric lymph node cultures (53). Finally, a new study employing an OVA allergic airway model has shown that eosinophil-deficient mice have augmented Th17 development and mast cells stimulated with IL-33 enhance Th17 development (54). Moreover, mast cells stimulated with IL-33 may themselves produce IL-17A (reviewed in Ref. 47). Collectively, the role of IL-33 is enigmatic and may function in any number of responses during fungal allergy, including promotion of Th2-associated inflammation (*Muc5ac*, CCL17, etc.), proinflammatory responses (IL-1 β , IL-6), or even IL-17A/IL-22 production in a feed-forward mechanism that promotes some of the inflammatory aspects of severe fungal asthma/allergy. Future studies will address the specific contribution of IL-33 in each of these responses.

In the commercial *Aspergillus* Ag/IFA fungal allergy model mentioned previously, mice deficient in TLR9 demonstrated higher serum IgE, but lower lung resistance (55). In this study, IL-17A levels trended lower, but were not significantly reduced, although Dectin-1 mRNA expression was significantly lower in the lung (55). In a subsequent report from the same group, IL-23 levels, Dectin-1 expression, and Th17 development were found to be significantly lower in TLR6-deficient mice, yet these mice demonstrated higher lung resistance and elevated expression of allergy biomarkers (56). These observations contradict the observations in TLR9-deficient mice, despite the fact that both studies reported lower Dectin-1 expression and IL-17A levels. IL-23 administration to TLR6-deficient mice augmented IL-17A and IL-22 levels and decreased lung resistance (56). In WT mice, neutralization of IL-17A increased *Muc5ac* and *Cla3* expression while paradoxically decreasing IL-33 levels, whereas neutralization of IL-22 decreased *Muc5ac* and *Cla3* expression while paradoxically increasing IL-33 levels (56). Therefore, although the TLR9 study implicates a role for IL-17A and Dectin-1 contributing to the development of fungal allergy, studies with TLR6-deficient mice suggest a protective role for IL-17A and IL-22 in fungal allergy. However, IL-17A and IL-22 neutralizations in the latter study complicate interpretations and indicate a complex role for both IL-17A and IL-22. IL-22 is elevated in asthmatics (25), and experimental investigation in an OVA model has shown that IL-22 contributes to the development of allergic responses (25). However, neutralization of IL-22 during Ag challenge enhanced allergic lung inflammation (25). These results are in contrast to our data in the fungal allergy model, which suggests that neutralization of IL-22 during challenge is effective at improving lung function. In the commercial *Aspergillus* Ag/IFA fungal allergy model, neutralization of IL-22 increased histological evidence of lung inflammation and lung IL-33 levels while paradoxically decreasing *Muc5ac* and *Cla3* expression (56). However, no significant effect on pulmonary function was observed after IL-22 neutralization (56). As both of these reports used adjuvant-associated sensitization models, they may not replicate allergen sensitization in humans. Nevertheless, these collective results suggest that IL-22 may function in different capacities over the development of allergic responses.

In summary, airborne fungi are well-recognized contributors to severe asthma. The model employed in the current study is solely

based on chronic exposure to a live, naturally occurring mold that is ubiquitous in the environment and has a known association with asthma (2, 3). Our data suggest that a mechanism behind the severity of fungal asthma is the elicitation of Dectin-1-dependent IL-22-mediated responses. Moreover, as *Aspergillus*-sensitized asthmatics have higher steroid usage (4, 5) and Th17/IL-17A and Th22/IL-22 responses are resistant to modulation by steroids (20), we can speculate that the mechanism connecting higher steroid usage during fungal allergy is the immunopathogenic, Dectin-1-dependent induction of IL-17A- and IL-22-mediated responses. These responses not only contribute to lung inflammation, but may also mimic Th2-mediated responses via the induction of IL-33. Thus, our data provide insight into immunopathogenesis during asthmatic/allergic responses in a model of chronic fungal exposure.

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Disclosures

The authors have no financial conflicts of interest.

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