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Dectin-1 and IL-17A Suppress Murine Asthma Induced by *Aspergillus versicolor* but Not *Cladosporium cladosporioides* Due to Differences in β -Glucan Surface Exposure

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There is considerable evidence supporting a role for mold exposure in the pathogenesis and expression of childhood asthma. *Aspergillus versicolor* and *Cladosporium cladosporioides* are common molds that have been implicated in asthma. In a model of mold-induced asthma, mice were repeatedly exposed to either *A. versicolor* or *C. cladosporioides* spores. The two molds induced distinct phenotypes, and this effect was observed in both BALB/c and C57BL/6 strains. *C. cladosporioides* induced robust airway hyperresponsiveness (AHR), eosinophilia, and a predominately Th2 response, whereas *A. versicolor* induced a strong Th17 response and neutrophilic inflammation, but very mild AHR. Neutralization of IL-17A resulted in strong AHR and eosinophilic inflammation following *A. versicolor* exposure. In Dectin-1-deficient mice, *A. versicolor* exposure resulted in markedly attenuated IL-17A and robust AHR compared with wild-type mice. In contrast, *C. cladosporioides* induced AHR and eosinophilic inflammation independent of IL-17A and Dectin-1. *A. versicolor*, but not *C. cladosporioides*, spores had increased exposure of β -glucans on their surface and were able to bind Dectin-1. Thus, the host response to *C. cladosporioides* was IL-17A- and Dectin-1-independent, whereas Dectin-1- and IL-17A-dependent pathways were protective against the development of asthma after exposure to *A. versicolor*. *The Journal of Immunology*, 2012, 189: 000–000.

Asthma is a major public health problem affecting nearly 23 million people in the United States, including 7 million children (1, 2). It is a complex disease with both genetic and environmental factors contributing to disease pathogenesis, and mold exposure has been implicated in the development and prevalence of asthma. In 2007, nearly half of the weekly requests received by the National Institute for Occupational Safety and Health concerned work-related asthma and mold exposure (3). In the Cincinnati Childhood Allergy and Air Pollution Study longitudinal birth cohort, mold exposure was associated with increased incidence of wheeze in infants (4), increased risk of developing asthma at age 3 y (5), and was a predictor of asthma development at age 7 y (6). The identification of mold as an important component of the environmental contribution to the asthma phenotype leads to questions about

possible interventions to prevent and/or attenuate mold-related health effects.

Two molds commonly identified in the homes of children enrolled in the Cincinnati Childhood Allergy and Air Pollution Study birth cohort were *Aspergillus versicolor* and *Cladosporium cladosporioides* (7). However, studies that examine the effects of these two molds on asthma are limited and largely descriptive. A study in Finland and another in New York City suggested that patients exposed to *C. cladosporioides* were more likely to have elevated IgE titers (8) and develop asthma symptoms compared with patients exposed to *A. versicolor* (8, 9). Additionally, murine studies indicated that *A. versicolor* protein extract had weak Th2 adjuvant activity compared with protein extract from *Alternaria alternata* (10). However, to our knowledge there are no murine studies with *C. cladosporioides*. Taken together, these studies indicate an important association between mold exposure and development of allergic disease, and they suggest that different molds can produce distinct immune responses.

IL-17 is a proinflammatory cytokine that has been implicated in several diseases, including asthma, and in response to molds. The role of IL-17 in asthma remains controversial. Some studies demonstrate that IL-17A contributes to the pathogenesis of asthma and can synergize with IL-13 to induce airway hyperresponsiveness (AHR) (11, 12), but IL-17 has also been shown to be a negative regulator of established murine asthma (13). Alternatively, it is well accepted that IL-17 has an important proactive role against infections with molds. Patients with deficiencies in IL-17 signaling, either due to inborn errors in IL-17 signaling (14) or autoantibodies against IL-17 cytokines, develop chronic mucocutaneous candidiasis (15, 16). Furthermore, IL-17 is downstream of Dectin-1, the receptor for β -glucans (17–22). Accordingly, deficiency in Dectin-1 has been associated with chronic mucocutaneous candidiasis (23), as well as invasive pulmonary aspergillosis in hematopoietic transplant patients (24). Mice de-

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Abbreviations used in this article: AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; i.t., intratracheal(ly).

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ficient in Dectin-1 develop fatal invasive pulmonary aspergillosis and do not clear *Candida* as efficiently as do wild-type mice (25–27). Furthermore, the response to *Aspergillus* in Dectin-1^{-/-} mice is characterized by decreased production of IL-17A (27, 28). Thus, IL-17 and Dectin-1 are important mediators in protection from infections with molds, but it is not known whether IL-17 and Dectin-1 have a role in mold-induced asthma.

In this study we hypothesized that *A. versicolor* and *C. cladosporioides* induce distinct pulmonary inflammation by differentially activating innate and adaptive immune pathways. To test our hypothesis we developed a murine model of exposure to mold spores and directly evaluated the contributions of *A. versicolor* versus *C. cladosporioides* to asthma, as well as the roles of IL-17A and Dectin-1. Exposure to *C. cladosporioides* spores induced a robust asthma phenotype, including AHR, eosinophilic inflammation, and the Th2 cytokines IL-4 and IL-13. In contrast, exposure to *A. versicolor* spores induced predominantly neutrophilic inflammation, IL-17A, and very mild AHR. IL-17A and Dectin-1 protected against AHR and eosinophilic inflammation following *A. versicolor*, but not *C. cladosporioides*, exposure.

Materials and Methods

Mice

Dectin-1^{-/-} mice have been previously described and were provided by Dr. Gordon Brown (25). Age- and sex-matched wild-type BALB/c and C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN). All mice were housed in a specific pathogen-free environment in the animal facility at Cincinnati Children's Hospital Medical Center. All procedures were performed in accordance with the ethical guidelines in the *Guide for the Care and Use of Laboratory Animals* of the Institutional Animal Care and Use Committee approved by Veterinary Services Department of Cincinnati Children's Hospital Medical Center.

Preparation of mold spores and spore challenge model

A. versicolor isolate 52173 and *C. cladosporioides* isolate 6721 (American Type Culture Collection, Manassas, VA) were grown on malt extract agar for 4–6 wk at 25°C. The spores were collected by agitating the culture plates with glass beads and rinsing the beads with saline supplemented with 0.05% Tween 80. The spores were stored at –80°C until use, and upon thawing were counted and resuspended in saline at a concentration of 2×10^7 spores/ml. Mice were lightly anesthetized with isoflurane and exposed to saline, 10^6 *A. versicolor* spores, or 10^6 *C. cladosporioides* spores in 50 μ l of saline intratracheally (i.t.) three times a week for 3 wk, and then were assessed 2 d after the last exposure. The mold spore dose was chosen based on the concentrations of these molds found in homes of children in the Cincinnati Childhood Allergy and Air Pollution Study birth cohort (29).

To test the role of IL-17A in our model we used rat anti-mouse IL-17A mAb (M210) and a control mouse IgG1 Ab were provided by Amgen (Seattle, WA). The rat M210 Ab was chimerized by fusing the V region domain of the rat IgG to mouse IgG1 constant domains. Abs were administered i.p. 3 d a week, 250 μ g/mouse, starting the day of the first spore administration. The dosing was determined from a previous publication using these Abs (12).

To quantify mold burden, mice were exposed i.t. to 5×10^6 of either *A. versicolor* or *C. cladosporioides* and sacrificed 24 h later, and the left lobe of the lung was fixed in paraformaldehyde, dehydrated, paraffin embedded, and cut into 5- μ m sections. Sections were stained with Grocott's methanamine silver stain. Total mold burden in the lungs was determined by averaging the number of spores in 40 high-power fields of the left lobe from each animal. Images were acquired at $\times 400$ using a Nikon 90i fully automated upright microscope system with a Nikon DS-Ri1 12-megapixel color camera. The software used to acquire the images was Nikon Elements quantitative analysis software. Images were color enhanced using Adobe Photoshop CS4.

Assessment of asthma phenotype

AHR to methacholine (acetyl- β -methylcholinechloride; Sigma-Aldrich, St. Louis, MO) was assessed in mice using flexiVent, a mechanical ventilator system (Scireq, Montreal, QC, Canada). Mice were anesthetized with ketamine, xylazine, and acepromazine and cannulated with a 20-gauge

blunt needle. Ventilation was set at 150 breaths/min, with volume and pressure controlled by the flexiVent system based on individual animal weights. Positive end expiratory pressure was set at 3.0 cm water. Two total lung capacity perturbations were performed for airway recruitment before baseline measurement and subsequent methacholine challenge at 0, 25, 50, and 100 mg/ml for BALB/c mice and 0, 75, 150, and 300 mg/ml for C57BL/6 mice. Measurements were made using a 1.25-s, 2.5-Hz volume-driven oscillation applied to the airways by the flexiVent system (SnapShot perturbation). Twelve SnapShot/ventilation cycle measurements were made. Dynamic resistance (*R*) and compliance (*C*) were determined by fitting the data to a single compartment model of airway mechanics where $P_{tr} = RV + EV + P_o$, and P_{tr} indicates tracheal pressure, *V*, volume, *E*, elastance, *P_o* is a constant, and $C = 1/E$. The maximum *R* value and minimum *C* value with a coefficient of determination of ≥ 0.9 (as determined by the flexiVent software) was used to determine the dose–response curve.

Bronchoalveolar lavage fluid collection and analysis

Bronchoalveolar lavage fluid (BALF) was collected and analyzed as previously described (30). Briefly, the lungs were lavaged with 1 ml PBS plus 2 mM EDTA. The total cell numbers were determined, and cells were spun onto slides and stained with the HEMA3 stain set (Fisher Scientific, Kalamazoo, MI). A minimum of 200 cells was counted and the total number of each cell type was calculated.

For detection of total serum IgE, plasma was diluted at 1:100 and the ELISA was performed as previously reported (30).

Isolation of lung cells and flow cytometry

Lungs were removed and the upper right lobe was minced and incubated at 37°C for 25–30 min in 2 ml RPMI 1640 containing Liberase DL (0.5 mg/ml; Roche Diagnostics, Indianapolis, IN) and DNase I (0.5 mg/ml; Sigma-Aldrich). Lung cells were passed through a 70- μ m cell strainer with a syringe rubber and the strainer washed with 5 ml RPMI 1640 plus DNase I media. Cells were centrifuged and resuspended in 2 ml RPMI 1640 before counting. Cell viability was confirmed by trypan blue exclusion.

Approximately 10^6 lung cells were transferred in to a V-bottom 96-well plate on ice, centrifuged, and resuspended in PBS containing Fc block (2.4G2 mAb; BioLegend, San Diego, CA) after stimulation with PMA and ionomycin in the presence of brefeldin A (eBioscience, San Diego, CA) and monensin (eBioscience). Cells were labeled with Live/Dead fixable aqua dead cell stain kit according to the manufacturer's instructions (Invitrogen/Life Technologies, Carlsbad, CA). T cells were stained with CD3e-FITC and CD4-Pacific Blue (BioLegend). Intracellular staining for IL-13-PE (eBioscience) and IL-17A-AF647 (BioLegend) was done using reagents from eBioscience. All flow cytometric data were acquired using the FACSCanto III (Becton Dickinson, Mountain View, CA), maintained by the Research Flow Cytometry Core in the Division of Rheumatology at Cincinnati Children's Hospital Medical Center. Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

RT-PCR

RNA was extracted from the lungs using TRIzol reagent (Life Technologies, Grand Island, NY), treated with DNase (Qiagen, Valencia, CA), and purified with an RNeasy MinElute kit (Qiagen). Reverse transcription was performed using iScript reverse transcription supermix (Bio-Rad, Hercules, CA). Real-time PCR was done using the SYBR Green Master kit and a LightCycler 480 instrument (Roche Diagnostics). Murine hypoxanthine phosphoribosyltransferase was used to normalize expression and was specifically amplified with forward primer 5'-TGC CGA GGA TTT GGA AAA AG-3' and reverse primer 5'-CCC CCC TTG AGC ACA CAG-3'. cDNA for murine IL-4 was specifically amplified using forward primer 5'-CTG TAG GGC TTC CAA GGT GCT TCG-3' and reverse primer 5'-CCA TTT GCA TGA TGC TCT TTA GGC-3'. cDNA for murine IL-17A was specifically amplified using forward primer 5'-ACT ACC TCA ACC GTT CCA CG-3' and reverse primer 5'-AGA ATT CAT GTG GTG GTC CA-3'. cDNA for murine IL-5 was specifically amplified using forward primer 5'-GAG GTT ACA GAC ATG CAC CAT T-3' and reverse primer 5'-CAG TTG GTA ACA TGC ACA AAG-3'. cDNA for murine eotaxin was specifically amplified using forward primer 5'-ATG AAA GGA GAT GTG GGA TTA TT-3' and reverse primer 5'-TTA TCC TCA GTT ACT CCT AAC TCG-3'.

β -glucan staining and Dectin-1 pulldown

Binding to recombinant murine Dectin-1 (R&D Systems, Minneapolis, MN) was determined by a pulldown assay. Mold spores were labeled

with Alexa Fluor 488 dye (Life Technologies), and 400,000 spores were incubated with recombinant murine Dectin-1 at a concentration of 1 nM. The recombinant murine Dectin-1 was pulled down with magnetic nickel beads (Life Technologies). The amount of spores pulled down with Dectin-1 was determined by reading the fluorescence on a Synergy H1 hybrid reader (BioTek, Winooski, VT) and normalizing to a standard curve.

To determine the amount and localization of exposed β -glucan on the spores, 10^6 spores were incubated with murine anti- β -glucan Ab, primary Ab (Biosupplies, Bundoora, VIC, Australia) at a concentration of 1 μ g/ml in 1% goat serum in 0.01% PBS Tween 20, and the secondary Ab was goat anti-mouse DyLight 594 (BioLegend). Images were acquired at $\times 1000$ using a Nikon 90i fully automated upright microscope system with a Nikon DSQIMc camera, with Z-sequence taken every 0.5 μ m. Nikon Elements quantitative analysis software was used to acquire the images.

Statistical analysis

All statistical analyses were done using Prism software (GraphPad Software, La Jolla, CA). Statistical significance was assessed using one-way ANOVA followed by a Newman–Keuls posttest or a two-way ANOVA followed by a Bonferroni posttest for dose–response data.

Results

A. versicolor and *C. cladosporioides* induce distinct inflammatory phenotypes in the lungs of mice

To determine the contribution of *A. versicolor* and *C. cladosporioides* to the development of murine asthma, we developed a murine model of mold-induced asthma by exposing mice to live mold spores (Fig. 1). *C. cladosporioides* induced robust AHR (Fig. 1A), whereas *A. versicolor* induced only mild AHR (Fig. 1A), suggesting that these two molds have distinct effects on lung physiology. Although both molds induced a significant increase in the total number of inflammatory cells found in the airways, there was a difference in the types of cells recruited (Fig. 1B). Inflammatory cell recruitment to the BALF in *A. versicolor*-exposed mice was characterized by predominantly neutrophils, whereas *C. cladosporioides* induced recruitment of both eosinophils and neutrophils (Fig. 1B). Furthermore, only mice exposed to *C. cladosporioides* had increased levels of total IgE (Fig. 1C). To determine whether the observed differences were dose-dependent, mice were exposed to 10^4 versus 10^6 spores. Mice exposed to 10^4 spores had less airway inflammation compared with mice exposed to 10^6 spores, but the phenotype at the lower exposure level was unchanged. Exposure to *C. cladosporioides* at the lower dose still induced eosinophilia, whereas *A. versicolor* did not induce eosinophilia at the low or high dose. *C. cladosporioides* exposure also induced a significant increase in serum IgE, even at the lower exposure level (Supplemental Fig. 1). Thus, our data indicate that *C. cladosporioides* induces allergic inflammation even at a lower exposure level.

No difference in distribution of mold spores in the lungs

To determine whether the observed differences in the inflammatory phenotype were due to a difference in the distribution of the mold spores in the lungs, mice were exposed to either *A. versicolor* or *C. cladosporioides* spores one time before harvesting tissues 24 h later. Both molds were uniformly distributed and showed similar distribution in the lungs. The spores were found mostly in the alveolar spaces with a few in the larger airways. Nearly all of the spores were taken up by APCs in the airways (Fig. 2A). We quantified the number of spores in the sections and did not observe any significant differences in mold burden between the two molds (Fig. 2B). Six weeks after exposure, there were no detectable spores in silver-stained lung sections from exposed mice (Supplemental Fig. 2), indicating that both molds were cleared from the lungs by 6 wk after a single

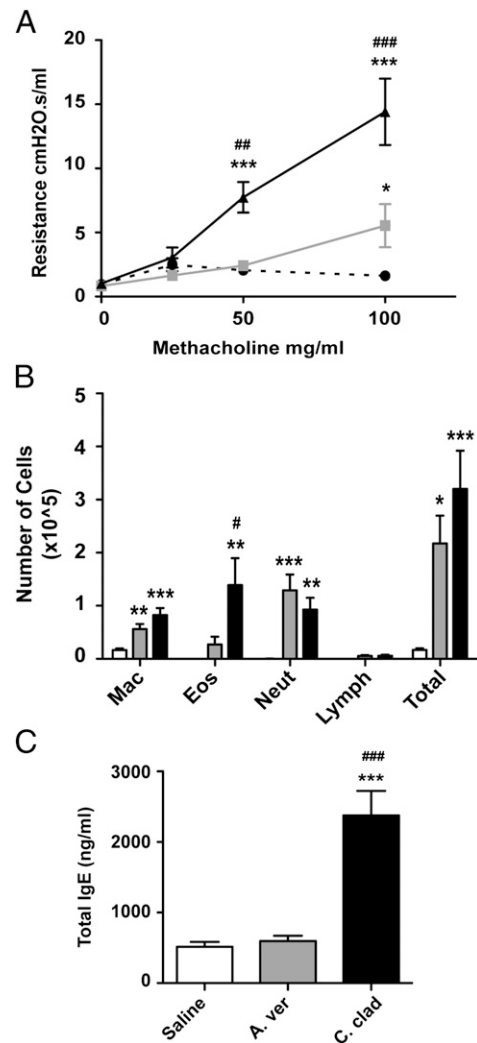


FIGURE 1. *A. versicolor* and *C. cladosporioides* induce distinct inflammatory phenotypes. (A) BALB/c mice were exposed i.t. to 10^6 *A. versicolor* or *C. cladosporioides* spores three times a week for 3 wk, and 48 h after the last challenge AHR was measured by flexiVent (dashed black line, saline; solid gray line, *A. ver*; solid black line, *C. clad*); (B) BALF was analyzed for total and differential counts (white bars, saline; gray bars, *A. ver*; black bars, *C. clad*); (C) total serum IgE. Data are representative of three independent experiments and are expressed as means $\pm$ SEM ($n = 6$ –8 mice/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with saline. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared with *A. versicolor*. *A. ver*, *A. versicolor*; *C. clad*, *C. cladosporioides*.

exposure. Thus, the molds did not actively grow and establish an infection in the lungs. These data indicate that the observed differences in inflammatory phenotypes induced by *A. versicolor* and *C. cladosporioides* are not due to differences in distribution, mold burden in the lungs of mice, or establishment of infection in the lungs.

Analysis of T cell responses to *A. versicolor* and *C. cladosporioides*

We next determined the types of effector T cell subsets present in the lungs after mold exposure. IL-17A production is required for optimal protection against fungal infections (14–16, 27, 28, 31). The frequency of IL-13–producing cells (Th2) and IL-17A (Th17) cells in the whole lung were determined by intracellular cytokine staining. *A. versicolor* induced predominantly Th17 cells whereas

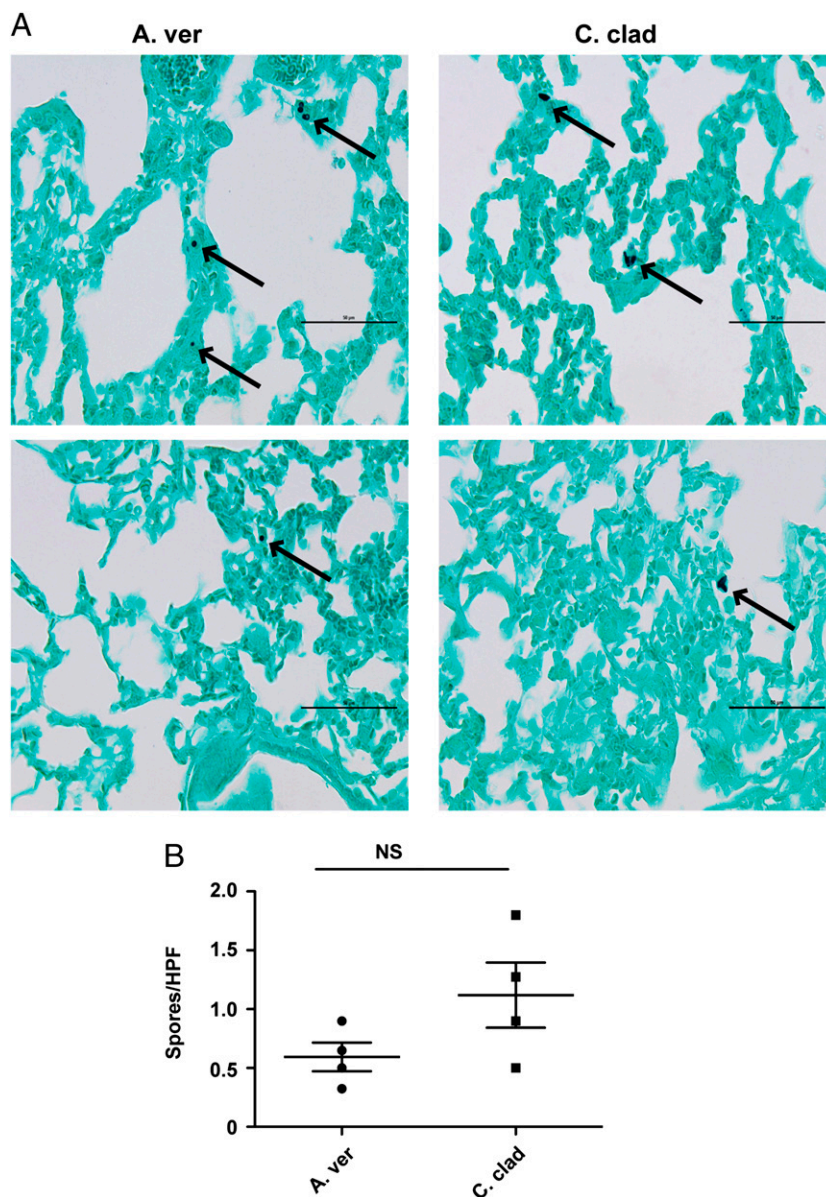


FIGURE 2. Similar distribution of *A. versicolor* and *C. cladosporioides* in the lungs of exposed mice. **(A)** Silver-stained sections from mice 24 h following a single exposure to 10^6 spores at an original magnification of $\times 400$. Arrows indicate pulmonary APCs containing mold spores. Scale bars, 50 μ m. **(B)** Quantification of spores per high power field from silver-stained sections. Data are representative of two independent experiments and are expressed as means $\pm$ SEM ($n = 3$ –4 mice/group). A. ver, *A. versicolor*; C. clad, *C. cladosporioides*.

C. cladosporioides induced predominantly Th2 cells (Fig. 3A, 3B). We also performed quantitative PCR on total lung RNA to determine cytokine expression (Fig. 3C). *C. cladosporioides* induced expression of both IL-4 and IL-17A, whereas *A. versicolor* induced expression of only IL-17. Molds have been reported to induce Th1 responses; however, we did not observe significant production of IFN- γ (Supplemental Fig. 3). Even though we observed fewer Th17 cells in the lungs of *C. cladosporioides*-exposed mice than in *A. versicolor*-exposed mice, we did observe similar mRNA expression of IL-17A in whole lungs between *A. versicolor* and *C. cladosporioides*, suggesting that there is another source of IL-17A in the lungs in addition to T cells. These data indicate that the T cell response to *A. versicolor* is predominantly a Th17 response, but the T cell response to *C. cladosporioides* is predominantly a Th2 cell response.

A. versicolor induces allergic airway disease when IL-17A is blocked

Given the central role of IL-17A in immunity to fungal infections, and our observation that IL-17A is differentially induced by

A. versicolor versus *C. cladosporioides*, we investigated the role of IL-17A in our model using a neutralizing Ab against IL-17A. Mice were exposed to *A. versicolor* or *C. cladosporioides* as before and also administered 250 μ g isotype control or anti-IL-17A Ab i.p. over the course of the experiment. Surprisingly, blockade of IL-17A in *A. versicolor*-exposed mice resulted in development of robust AHR compared with animals receiving isotype Ab (Fig. 4A). In fact, the *A. versicolor*-exposed mice treated with anti-IL-17A developed increased AHR at the highest dose of methacholine similar to that seen in *C. cladosporioides*-exposed mice (Fig. 4B). Furthermore, blockade of IL-17A in *A. versicolor*-exposed mice resulted in increased recruitment of eosinophils to the airways, a decrease in recruitment of neutrophils (Fig. 4C), and an increase in IL-5 expression (Fig. 4D). IL-17A blockade did not result in an increase in the frequency of Th2 cells in the lungs, in IL-13 in the BALF, or in expression of IL-4 mRNA (Supplemental Fig. 4). Despite a report that IL-17A downregulates eotaxin and goblet cell hyperplasia (13), we did not observe any differences in eotaxin expression (Supplemental Fig. 4C) or goblet cell hyperplasia (data not shown) in the lungs of isotype versus anti-IL-17A-

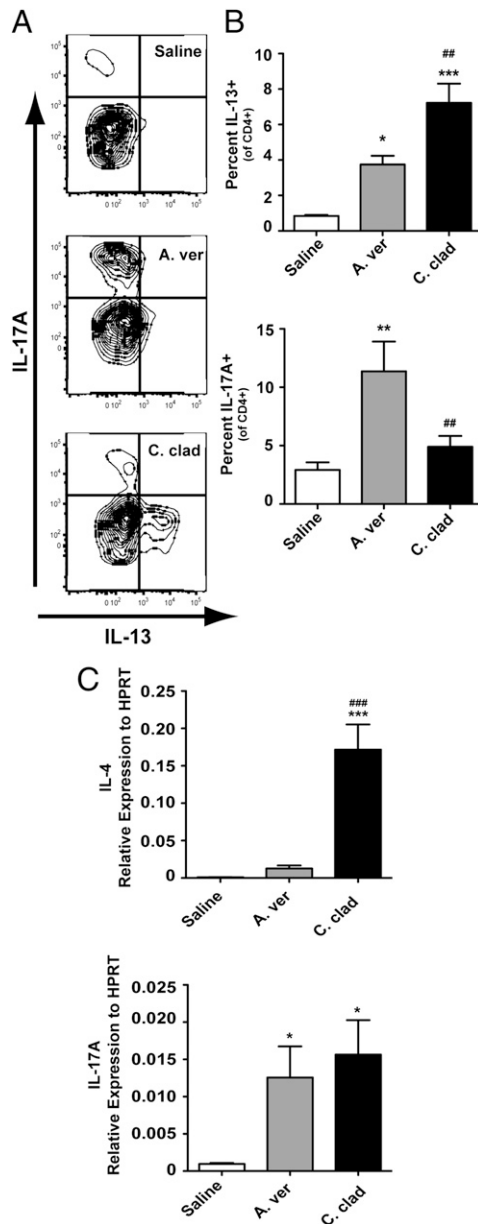


FIGURE 3. *A. versicolor* induces Th17 cells whereas *C. cladosporioides* induces Th2 cells. (A) Contour plots and (B) quantification of intracellular cytokine staining for IL-13 or IL-17A in CD4⁺ lung cells. (C) Relative levels of mRNA for IL-17A and IL-4 in whole lung. Data are representative of three independent experiments and are expressed as means $\pm$ SEM ($n = 6$ –8 mice/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with saline. ## $p < 0.01$, ### $p < 0.001$ compared with *A. versicolor*. A. ver, *A. versicolor*; C. clad, *C. cladosporioides*.

treated mice following mold exposure. These data indicate that IL-17A has a negative regulatory effect on development of allergic inflammation in response to *A. versicolor*, likely due to a negative effect on IL-5 and eosinophils.

Alternatively, blockade of IL-17A in *C. cladosporioides*-exposed mice resulted in development of higher AHR (Fig. 4A, 4B). This was not matched by an increase in recruitment of inflammatory cells to the airways (Fig. 4C). In fact, there was a decrease in the total number of cells recruited to the airways when IL-17A was neutralized in *C. cladosporioides*-exposed mice, likely due to a decrease in the number of neutrophils recruited to the airways.

IL-17A has a minimal role in the development of allergic inflammation after exposure to *C. cladosporioides*.

IL-17A is reduced in Dectin-1^{-/-} mice

Dectin-1 is critical in the recognition of different mold species (25–27), and signaling through Dectin-1 is important for production of IL-17 and Th17 responses (19, 22, 27). Wild-type C57BL/6 or Dectin-1^{-/-} mice were exposed to *A. versicolor* spores, and 2 d after the last exposure we determined the types of T effector cells in the lungs by intracellular cytokine staining. As expected, Dectin-1^{-/-} mice had fewer Th17 cells in the lungs compared with wild-type mice following *A. versicolor* exposure (Fig. 5A, 5B). In contrast, Th2 cells were increased in the Dectin-1^{-/-} mice compared with wild-type mice. In *C. cladosporioides*-exposed Dectin-1^{-/-} mice, there was a slight decrease in Th17 cells in the lungs, but no difference in Th2 cells between wild-type and Dectin-1^{-/-} mice (Fig. 5A, 5C). Our data support a role for Dectin-1 in development of Th17 responses to mold, and they suggests that pathways downstream of Dectin-1 can inhibit the formation of Th2 responses.

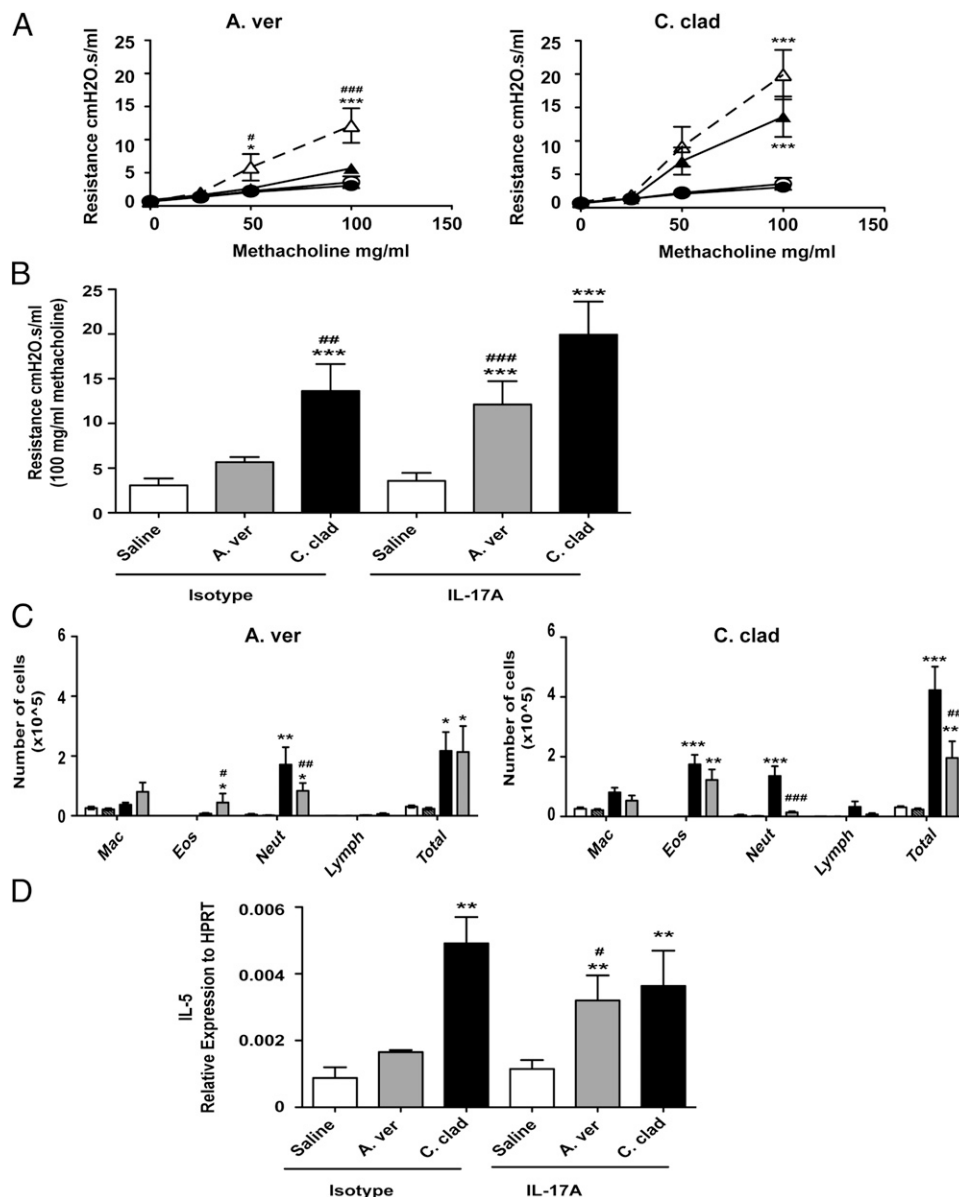
Dectin-1 inhibits AHR and allergic inflammation following A. versicolor exposure

Because in vivo blockade of IL-17A in *A. versicolor*-exposed mice resulted in increased AHR and airway eosinophils, and Dectin-1^{-/-} mice had decreased Th17 cells and increased Th2 cells in the lungs following *A. versicolor* exposure, we next determined the role of Dectin-1 in development of AHR and recruitment of airway eosinophils in *A. versicolor*- or *C. cladosporioides*-exposed mice. Wild-type C57BL/6 or Dectin-1^{-/-} mice exposed to mold spores were assessed for AHR and airway eosinophils 48 h after the last exposure. Following *A. versicolor* exposure, wild-type mice developed mild AHR, similar to our observations in BALB/c wild-type mice (Figs. 1A, 6A), indicating that our phenotype is not specific to one strain. However, in the absence of Dectin-1, *A. versicolor* exposure induced robust AHR and increased airway eosinophilia (Fig. 6B). Dectin-1^{-/-} and wild-type mice exposed to *C. cladosporioides* were similar in terms of AHR and BALF eosinophilia (Fig. 6C, 6D). These data demonstrate that Dectin-1 has an inhibitory effect on the development of allergic inflammation in response to exposure to *A. versicolor*. Furthermore, *C. cladosporioides* induces AHR and eosinophilia in a Dectin-independent manner.

A. versicolor has greater exposure of β -glucans on the spore surface and binding to Dectin-1 when compared with C. cladosporioides

Our data support that Dectin-1 inhibits AHR and allergic inflammation following *A. versicolor* exposure whereas *C. cladosporioides* acts in a Dectin-independent manner. This suggested that *A. versicolor*, but not *C. cladosporioides*, spores bind to Dectin-1. However, a previous report from our group revealed that *C. cladosporioides* spores have a greater β -glucan content than do *A. versicolor* spores (32). Therefore, we wanted to determine whether there is a difference in the exposure and availability of β -glucans on the surface of these spores. Staining for β -glucans on *C. cladosporioides* spores indicated that the β -glucans are only minimally exposed at the ends of the spores where they were connected in a growing chain before harvesting the spores (Fig. 7A). Alternatively, *A. versicolor* spores displayed strong staining for β -glucans throughout the spore surface (Fig. 7A), indicating that the β -glucans are more exposed in *A. versicolor* than in *C. cladosporioides*.

FIGURE 4. *A. versicolor* induces allergic airway disease when IL-17A is blocked. **(A)** BALB/c mice were exposed to *A. versicolor* or *C. cladosporioides* spores and treated with either isotype control Ab or anti-IL-17A. 48 h after the last challenge, AHR was measured by flexivent (solid circle/gray line, saline isotype; open circle/gray line, saline anti-IL17A; solid triangle/black line, *A. ver* or *C. clad* isotype; open triangle/dashed line, *A. ver* or *C. clad* anti-IL17A). **(B)** Comparison of AHR data at 100 mg/ml methacholine. **(C)** BALF total and differential counts (white bars, saline isotype; diagonal pattern bars, saline anti-IL17A; black bars, *A. ver* or *C. clad* isotype; gray bars, *A. ver* or *C. clad* anti-IL17A). **(D)** Relative levels of mRNA for IL-5 in whole lung. Data are representative of two independent experiments and expressed as means $\pm$ SEM ($n = 5$ –6 mice/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with saline. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared with *A. versicolor* or *C. cladosporioides* isotype Ab-treated mice. *A. ver*, *A. versicolor*; *C. clad*, *C. cladosporioides*.



We next determined the binding of each spore to Dectin-1 using recombinant Dectin-1 in a pulldown assay. We observed strong binding of *A. versicolor* spores to Dectin-1, but could not detect binding of *C. cladosporioides* spores (Fig. 7B). Thus, *A. versicolor* binds to Dectin-1 whereas *C. cladosporioides* does not, and this is likely due to the differences in surface exposure of β -glucans.

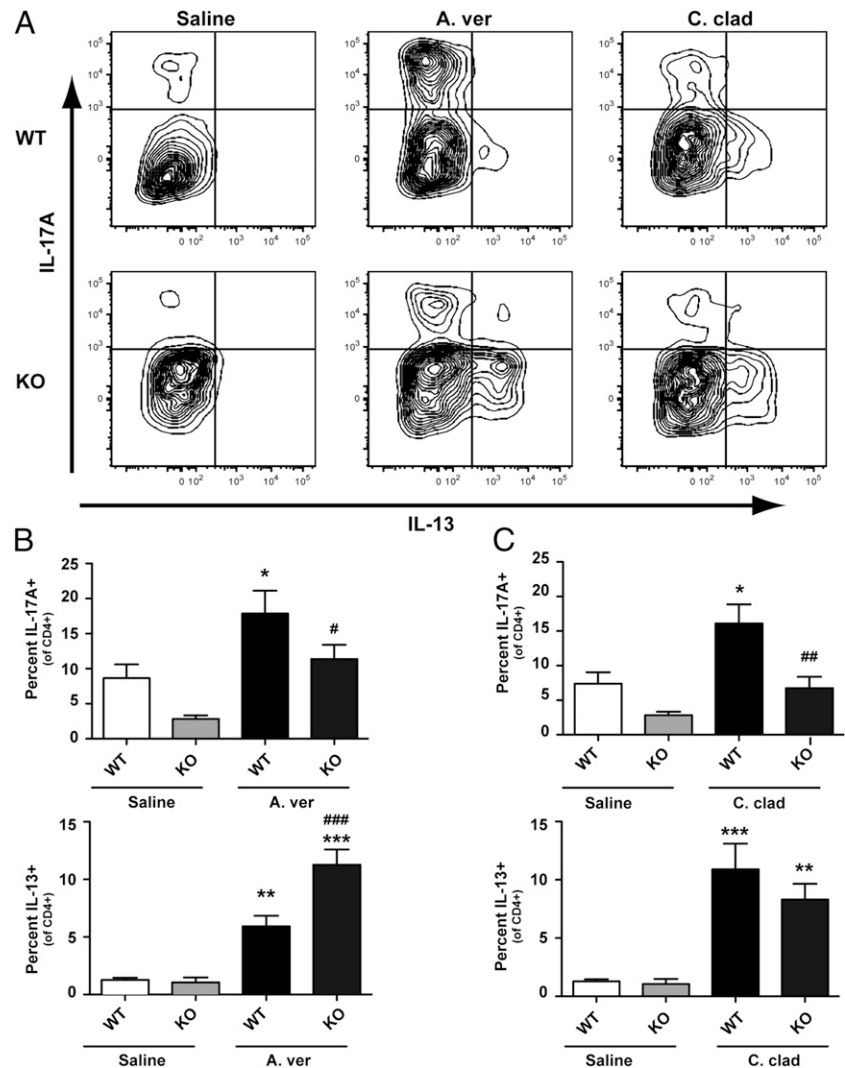
Discussion

The identification of mold as an important component of the environmental contribution to the asthma phenotype leads to questions about possible interventions to prevent and/or attenuate mold-related health effects. However, effective interventions to reduce mold exposure cannot presently be designed because they may or may not successfully target the relevant mold species. In this study, we characterized the responses to two common mold species that have been implicated in asthma. Our data demonstrate that different mold species are capable of inducing very distinct inflammatory phenotypes in the lungs, and they are in agreement with epidemiologic studies suggesting that exposure to *Cladosporium* species

is more likely to induce asthma symptoms in patients than exposure to *Aspergillus* species (8, 9).

We developed a model of mold exposure using *A. versicolor* or *C. cladosporioides* mold spores. This model has several advantages, including 1) utilization of whole spores rather than mold extracts, 2) continuous i.t. exposure during a period of 3 wk without use of adjuvants or prior sensitization, and 3) the dose of 10^6 spores was based on air sampling data from the Cincinnati Childhood Allergy and Air Pollution Study birth cohort (29). In this model, exposure to *C. cladosporioides* induced robust AHR, mixed eosinophilic/neutrophilic airway inflammation, Th2 cells, and elevated levels of serum IgE, an inflammatory phenotype characteristic of asthma. In contrast, exposure to *A. versicolor* induced predominantly neutrophilic airway inflammation and Th17 cells. There were minimal changes in lung physiology or serum levels of IgE after exposure to *A. versicolor*. There was no difference in mold burden, distribution of mold spores, or establishment of an infection by either mold species, indicating that our observations are independent of these factors. Furthermore, it is unlikely that the observed phenotypes are due to differences in spore size (32) because we observed only quantitative, but not

FIGURE 5. Increased Th2 cells and decreased Th17 cells in Dectin-1^{-/-} mice exposed to *A. versicolor*. **(A)** Contour plots and quantification of intracellular cytokine staining for IL-17A or IL-13 in CD4⁺ cells isolated from the lungs of *A. versicolor*-exposed mice **(B)** or *C. cladosporioides*-exposed mice **(C)**. Data are representative of three independent experiments and expressed as means $\pm$ SEM ($n = 4$ –6 mice/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with saline. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared with *A. versicolor* wild-type mice. A. ver, *A. versicolor*; C. clad, *C. cladosporioides*.



qualitative, differences in the phenotype with a lower exposure level (10^4 spores) compared with a higher exposure level (10^6 spores). Finally, the asthma phenotype was not strain-dependent, as we observed similar findings in both BALB/c and C57BL/6 mice.

IL-17A has been implicated in the pathogenesis of asthma and can synergize with IL-13 to induce AHR (11, 12), but it has also been shown to be a negative regulator of established murine asthma (13). Although the role of IL-17 is controversial in asthma, it is well established that signaling through Dectin-1 induces Th17 cells (19, 21, 22, 33), and that both Dectin-1 and IL-17A are necessary for optimal protection from fungal infections (14–16, 27, 28, 31). In a recent study, TLR6 protected from development of asthma through induction of IL-17, possibly by regulating Dectin-1 expression (34). Using a mold-induced asthma model, another study found that TNF- α positively regulates IL-17 and negatively regulates IL-5 levels, and that dendritic cells deficient in Dectin-1 produce significantly less TNF- α than do wild-type cells (35). Both of these studies suggest a possible role for Dectin-1 in mold-induced asthma. In the present study, we provide direct support for this and demonstrate that the ability of a mold to bind Dectin-1 is an important determinant of the nature of the immune response that ensues following exposure to a given mold. Our data reveal IL-17A- and Dectin-1-dependent pathways suppress asthma development in mice exposed to *A. versicolor*

spores; blockade of IL-17A or Dectin-1 deficiency in mice exposed to *A. versicolor* spores mice resulted in development of robust AHR and eosinophilia. Our data are supported by previous reports suggesting that IL-17 prevents eosinophil recruitment in asthma (13, 34, 35). Additionally, a recent study by Werner et al. (28) demonstrated decreased IL-17A and increased airway eosinophils in Dectin-1^{-/-} mice exposed to *Aspergillus fumigatus*. Data from our study now provide direct evidence that Dectin-1 and downstream IL-17 can inhibit eosinophil recruitment and asthma development following exposure to another *Aspergillus* species. Importantly, our work uses a model of chronic pulmonary exposure to mold spores, rather than utilizing a model in which mice are systemically sensitized before exposure to mold spores.

In contrast, *C. cladosporioides*-induced asthma was independent of Dectin-1 and IL-17A. These data suggest that the Dectin-1 and downstream IL-17A pathway inhibit allergic inflammation in response to *A. versicolor* but not in response to *C. cladosporioides*. The mechanism underlying the distinct inflammatory phenotypes induced by *A. versicolor* and *C. cladosporioides* is likely due to the differences in the ability of the mold spores to bind to Dectin-1. The ability to bind to Dectin-1 is directly correlated with the surface exposure of the β -glucans in *A. versicolor* and *C. cladosporioides*, with *A. versicolor* having greater β -glucan exposure and binding to Dectin-1 than does *C. cladosporioides*.

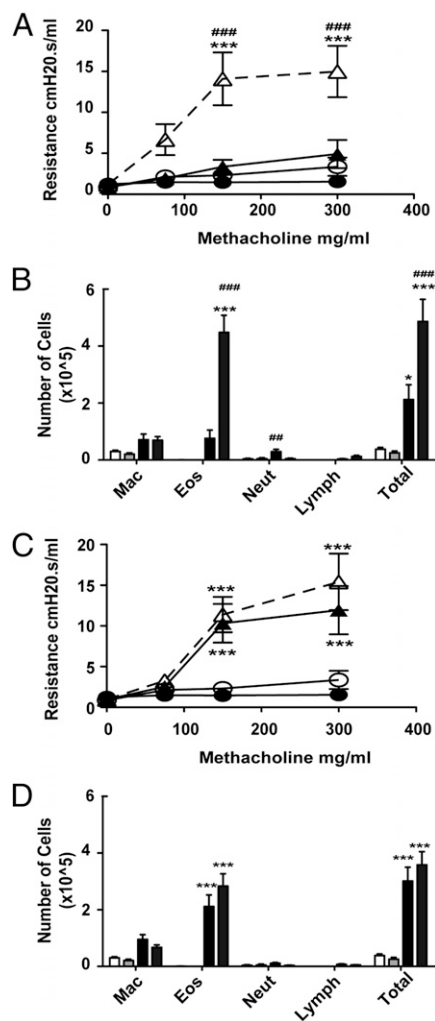


FIGURE 6. Dectin-1 protects against allergic inflammation induced by *A. versicolor*. **(A and C)** Wild-type C57BL/6 or Dectin-1^{-/-} mice were exposed to *A. versicolor* (A) or *C. cladosporioides* (C) spores and AHR was assessed 48 h later. Solid gray circles/gray line, saline WT; open circles/gray line, saline KO; solid triangle/black line, A. ver or C. clad WT; open triangle/dashed line, A. ver or C. clad KO. **(B and D)** Total and differential counts of BALF for *A. versicolor* (B) or *C. cladosporioides* (D) exposed mice. White bars, saline WT; light gray bars, saline KO; black bars, A. ver or C. clad WT; dark gray bars, A. ver or C. clad KO. Data are representative of three independent experiments and expressed as means $\pm$ SEM ($n = 4-6$ mice/group). * $p < 0.05$, *** $p < 0.001$ compared with saline. # $p < 0.05$, ### $p < 0.001$ compared with exposed Dectin-1^{-/-} mice. A. ver, *A. versicolor*; C. clad, *C. cladosporioides*.

oides. Our data now add to the present literature and demonstrate that Dectin-1 and IL-17A can protect against mold-induced asthma. Furthermore, this effect was dependent on the particular mold species and β -glucan surface availability; *C. cladosporioides* exposure induced asthma independent of both Dectin-1 and IL-17A.

In conclusion, Dectin-1 and IL-17A suppress murine asthma induced by *A. versicolor* but not *C. cladosporioides*. *C. cladosporioides*-induced asthma is independent of IL-17A and Dectin-1. We propose a model in which exposure of β -glucans on *A. versicolor* promote binding to and signaling through Dectin-1, induction of a Th17 response, and protection against the development of allergic disease. In the absence of Dectin-1, an alternate pathway is used, which promotes a Th2 response and the development of

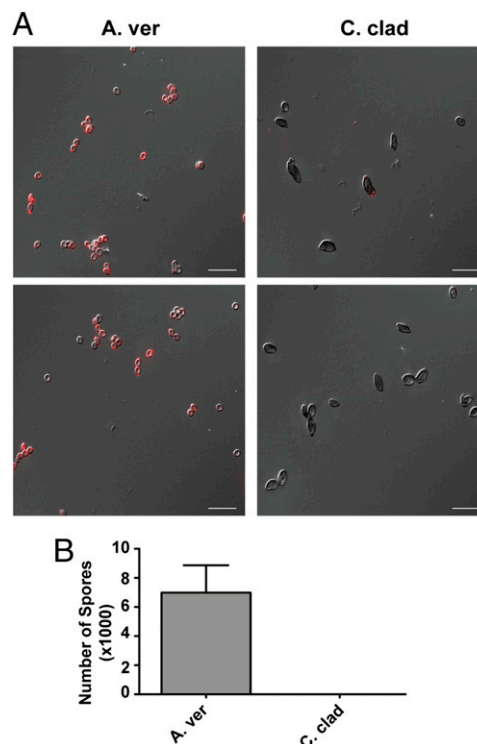


FIGURE 7. *A. versicolor* spores have increased β -glucan surface availability and Dectin-1 binding compared with *C. cladosporioides* spores. **(A)** Immunofluorescence images of β -glucan staining taken at original magnification of $\times 1000$ for *A. versicolor* and *C. cladosporioides* spores. Scale bars, 10 μ m. **(B)** Dectin-1 binding assay for *A. versicolor* and *C. cladosporioides* spores. Data are representative of two independent experiments and are expressed as means $\pm$ SEM ($n = 3$ samples/group). A. ver, *A. versicolor*; C. clad, *C. cladosporioides*.

an asthma phenotype (Fig. 8). In contrast, *C. cladosporioides* spores have minimal surface availability of β -glucans and do not bind to Dectin-1. As a result, *C. cladosporioides*-induced asthma is independent of IL-17A and Dectin-1. A recent study suggested that melanin in fungal spores blocks signaling through pattern recognition receptors (36). It is interesting to speculate that

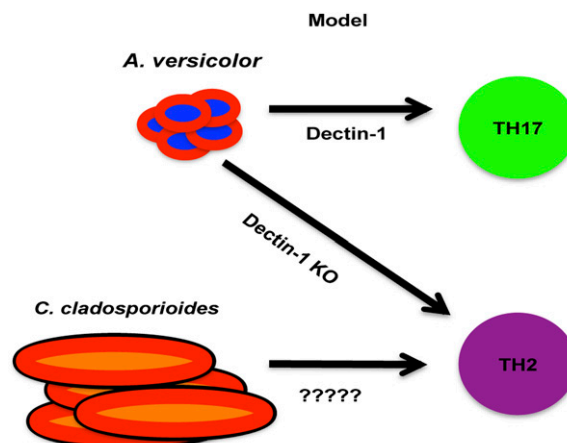


FIGURE 8. Working model. Exposure of β -glucans (red) on the surface of *A. versicolor* promote signaling through Dectin-1 to induce a Th17 response. In the absence of Dectin-1, *A. versicolor* signals through an alternate pathway to induce Th2 cells and asthma. β -glucans (red) in *C. cladosporioides* are not readily available on the surface (black), thus preventing signaling through a Dectin-1 and promoting the use of an alternate pathway to induce a Th2 response and asthma.

C. cladosporioides spores contain a substance that covers the β -glucans found in the cell wall, prevents signaling through Dectin-1, and, by default, *C. cladosporioides* spores signal through an alternate pathway to induce allergic disease.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental Material

Supplemental Figure 1: Recruitment of eosinophils to the airways and increased IgE in

mice exposed to varying doses of mold spores. (A) Total and differential BALF counts from mice exposed to 10^4 or 10^6 *A. versicolor* or *C. cladosporioides* spores 3 times a week for 3 weeks. (B) Measurement of total serum IgE by ELISA. Data are expressed as mean and SEM. N=4-6 mice per group.

Supplemental Figure 2: No growth of mold spores in the lungs six weeks after exposure.

Silver stained sections from lungs of mice sacrificed 6 weeks after a single exposure to 5×10^6 spores of either *A. versicolor* or *C. cladosporioides*. Pictures are 400x magnification and the scale bar is 50um. N=3-4 mice per group.

Supplemental Figure 3: No increase in Th1 cells after exposure to mold spores.

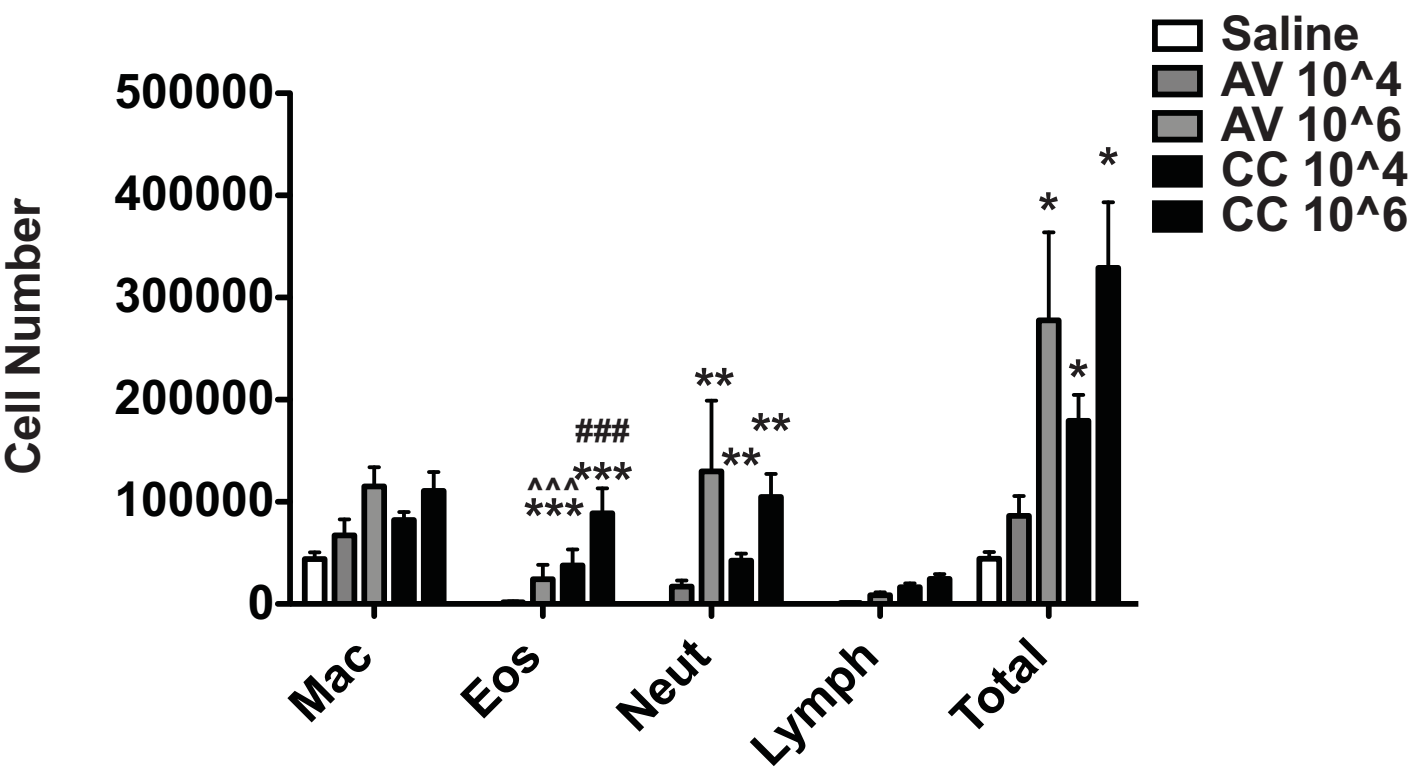
(A) Contour plots and (B) quantification of intracellular cytokine staining for IFN γ in lung cells. Data are representative of three independent experiments and expressed as mean and SEM. N=6-8 mice per group.

Supplemental Figure 4: No increase in Th2 response after exposure to *A. versicolor* in IL-

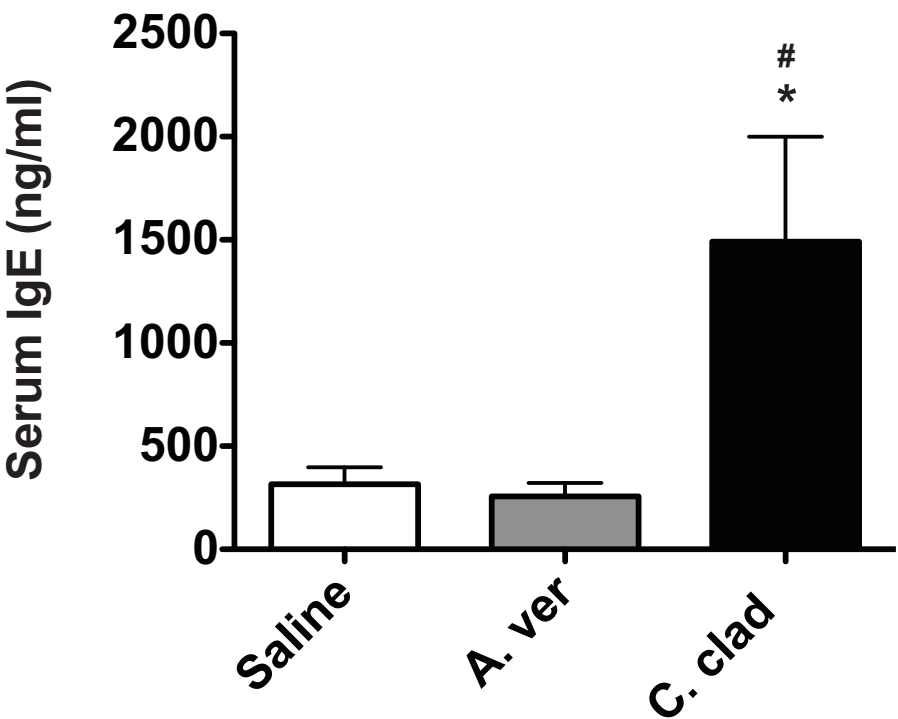
17A neutralized mice. (A) Contour plots and (B) quantification of intracellular cytokine staining for IL-13 in lung cells. (C) ELISA of IL-13 in BALF (D) Relative levels of IL-4 mRNA in whole lungs (E) Relative levels of Eotaxin mRNA in whole lungs Data are representative of two independent experiments and expressed as mean and SEM. N=4 -6 mice per group.

Supplemental Figure 1

A.

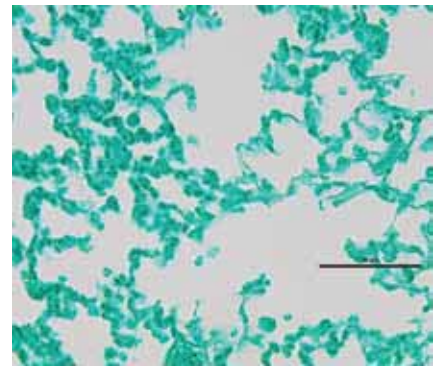
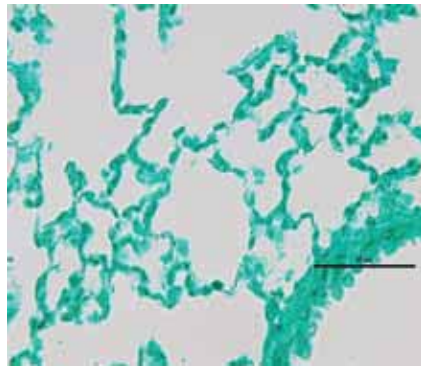
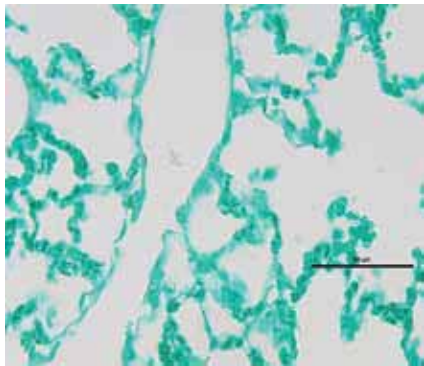


B.

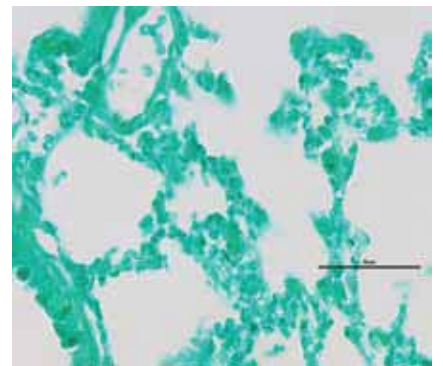
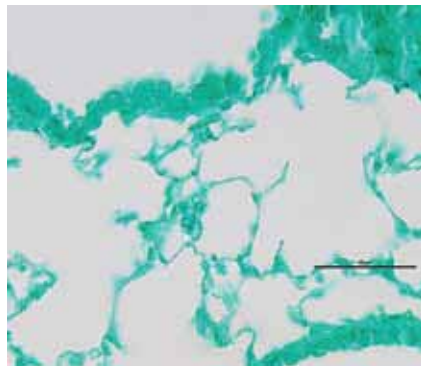
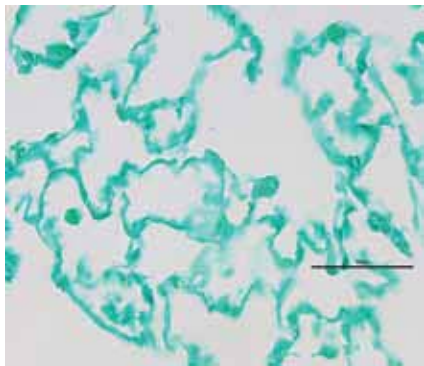


Supplemental Figure 2

A. ver

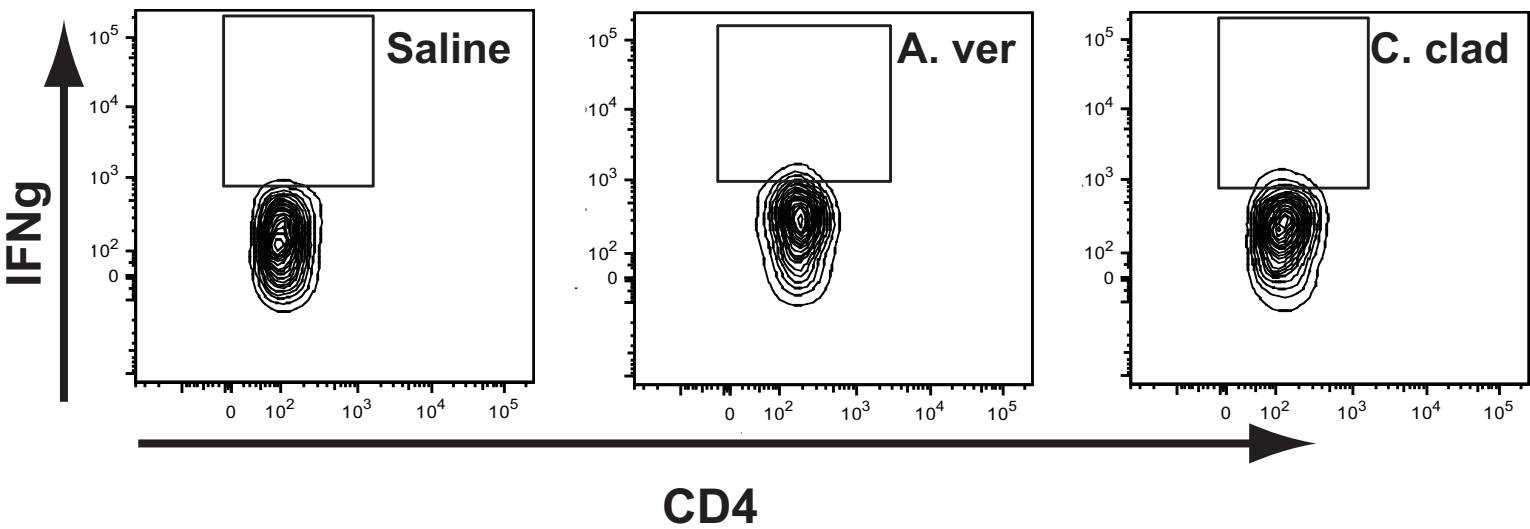


C. clad

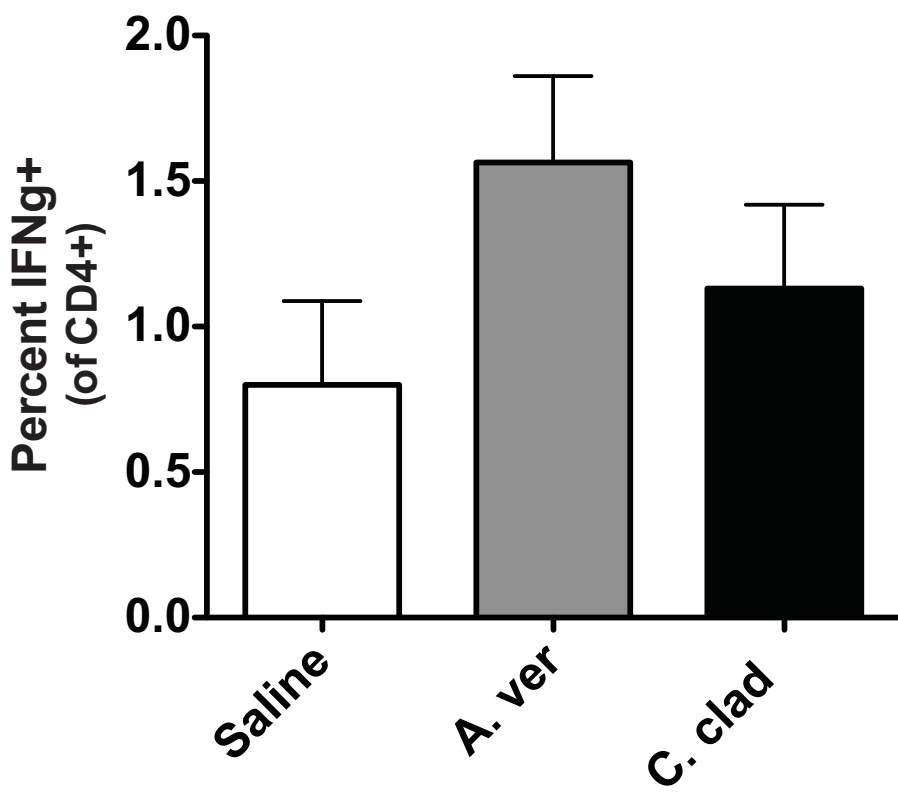


Supplemental Figure 3

A.

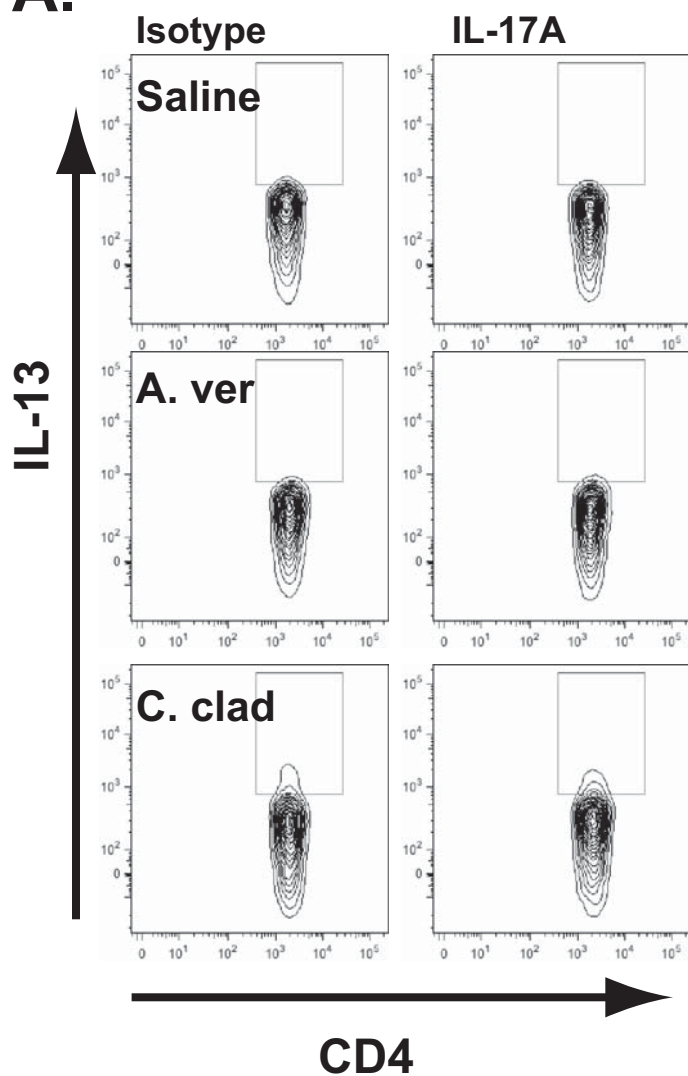


B.

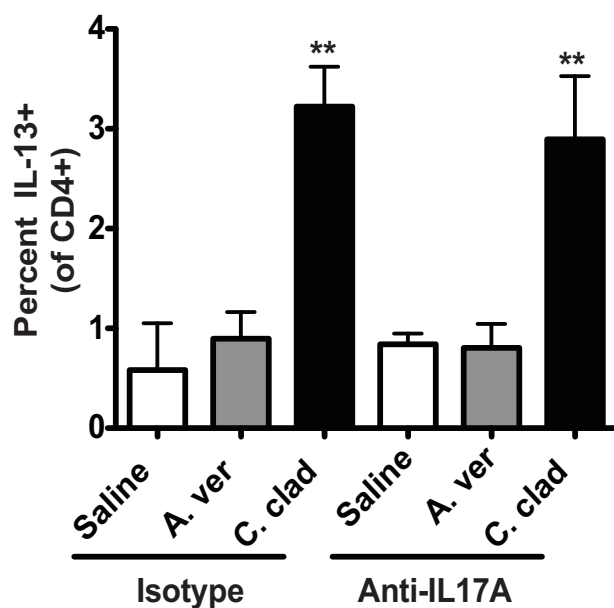


Supplemental Figure 4

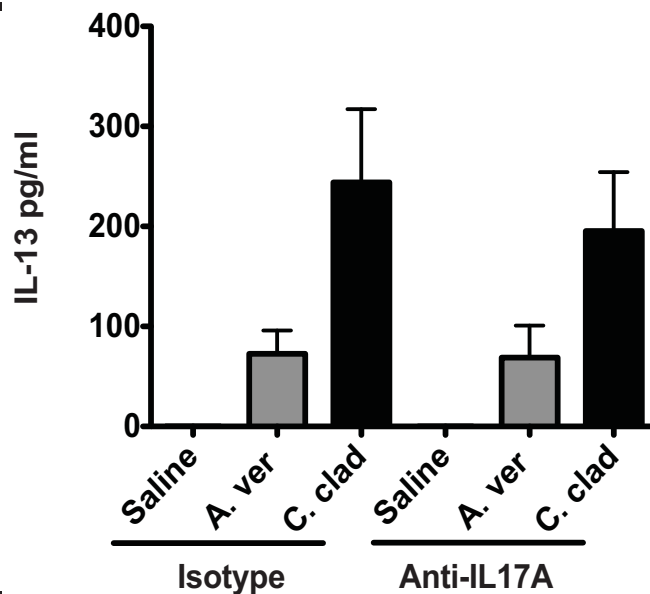
A.



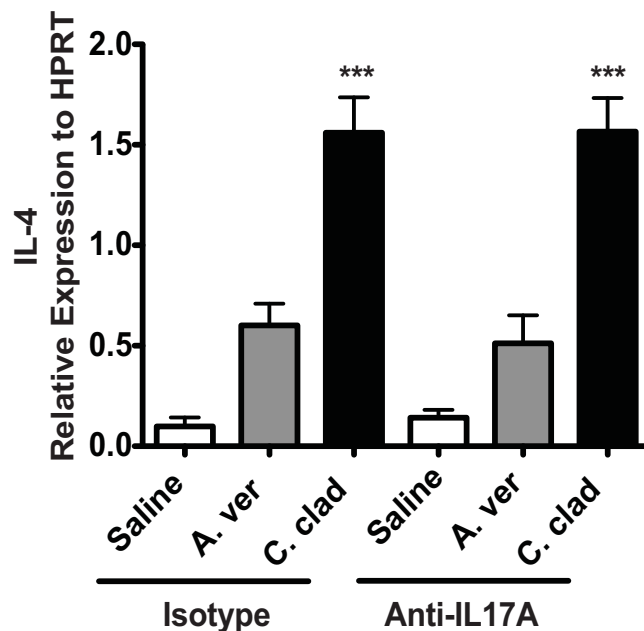
B.



C.



D.



E.

