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Surface availability of beta-glucans is critical determinant of host immune response to *Cladosporium cladosporioides*

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Abstract

Background: It is well accepted that mold exposure is a major contributor to the development of asthma, and beta-glucans are often used as a surrogate for mold exposure in the environment. Beta-glucans are an important component of mold spores and are recognized by the immune system by their receptor, Dectin-1. *Cladosporium cladosporioides* spores have a high beta-glucan content, but the beta-glucans are not available on the surface of live spores.

Objective: We sought to determine whether altering the exposure of beta-glucans in *C cladosporioides* through heat killing could alter the immune response through binding to Dectin-1.

Methods: In a murine model of mold-induced asthma, mice were repeatedly exposed to either live or heat-killed *C cladosporioides* and the phenotype was determined by the measurement of airway hyperresponsiveness, airway inflammation, and cytokine production. Pro-inflammatory cytokines from dendritic cells were measured by using quantitative PCR and ELISA.

Results: Live *C cladosporioides* induced robust airway hyperresponsiveness, eosinophilia, and a predominately T_H2 response, while heat-killed *C cladosporioides* induced a strong T_H17 response and neutrophilic inflammation, but very mild airway hyperresponsiveness. Heat killing of *C cladosporioides* spores effectively exposed beta-glucans on the surface of the spores and increased binding to Dectin-1. In the absence of Dectin-1, heat-killed spores induced a predominantly T_H2 response analogous to live spores. Furthermore, the production of T_H17-skewing IL-6, IL-23, and TNF- α by dendritic cells in response to heat-killed *C cladosporioides* was dependent on Dectin-1.

Conclusions: The host immune response to *C cladosporioides* is dependent on the surface availability of beta-glucans rather than the total beta-glucan content. (*J Allergy Clin Immunol* 2013;132:159-69.)

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Keywords

Mold; *Cladosporium cladosporioides*; beta-glucans; asthma; Dectin-1

Fungal spores are ubiquitously distributed in both the indoor and outdoor environment and are often associated with respiratory disease.¹ Numerous studies have implicated mold exposure in the development and prevalence of asthma. Chronic mold exposure in a high-risk birth cohort was associated with persistent wheeze,² and persistent childhood asthma was associated with sensitivity to mold in another pediatric cohort.³ In adult asthma, allergic sensitization to molds was associated with more hospital and intensive care unit admissions due to asthma.^{4,5} These studies underscore the importance of mold exposure as a public health concern and the relevance of mold exposure to asthma.

A major component of fungal cell walls is (1-3)-beta-D-glucans, and these are commonly used as a marker of mold exposure in the environment.⁶ Beta-glucans account for up to 60% of the weight of the cell wall,⁷ and they are important for the recognition of molds by the immune system. Dectin-1, the receptor for beta-glucans,⁸ is expressed on macrophages, monocytes, neutrophils, and dendritic cells.^{9,10} Signaling through Dectin-1 promotes fungal immunity, specifically by inducing dendritic cells to polarize T cells toward T_H17 cells.¹¹⁻¹⁴ Accordingly, deficiency in IL-17A or Dectin-1 has been associated with an increased risk for fungal infections in humans.¹⁵⁻²⁰ Results from animal studies also demonstrate that the absence of Dectin-1 leads to increased susceptibility to fungal infections²¹⁻²³ and decreased production of IL-17A.^{23,24}

Several studies indicate that the beta-glucans in mold spores are important for the development of an immune response and type of immune response. Removal of rodA, either genetically or chemically, from *Aspergillus fumigatus* spores increases both the activation of dendritic cells and the binding of Dectin-1.²⁵ Heat-killed *Candida albicans* has greater binding to Dectin-1 than do live spores, and the heat-killed spores are better at activating antigen-presenting cells.^{26,27} Another study demonstrated that distinct immune responses develop in response to live and heat-killed mold spores,²⁸ suggesting that exposure of beta-glucans on the surface of spores may alter the immune response. Furthermore, recognition of different stages of mold growth is dependent on the accessibility of beta-glucan and binding to Dectin-1.²⁹ We recently reported that Dectin-1 and IL-17A prevent the development of asthma in mice after exposure to *A versicolor*, but not *C cladosporioides*, due to differences in the surface availability of beta-glucans between the 2 spores and, therefore, differences in binding to Dectin-1.³⁰ Collectively, these studies indicate that the surface exposure of beta-glucans alters the immune response to molds.

In this study, we hypothesized that exposure of beta-glucans on *C cladosporioides* spore surface by heat killing will prevent the development of asthma through a Dectin-1-dependent mechanism. Our data demonstrate that heat-killed *C cladosporioides* spores display more surface beta-glucans and Dectin-1 binding than do live spores. Furthermore, heat-killed *C cladosporioides* spores induced an attenuated asthma phenotype compared with live spores. The inflammatory phenotype induced by heat-killed *C cladosporioides* spores was

predominately a T_H17 response marked by neutrophilic inflammation in the airways, and this response was dependent on Dectin-1.

METHODS

Mice

Dectin-1^{-/-} mice have been previously described and were generously provided by Dr Gordon Brown.²¹ Age- and sex-matched wild-type BALB/c and C57Bl/6 mice were purchased from Harlan Laboratories (Indianapolis, Ind). 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 the Veterinary Services Department of the Cincinnati Children's Hospital Medical Center.

Preparation of mold spores and spore challenge model

C. cladosporioides isolate 6721 (American Type Culture Collection, Manassas, Va) was grown on malt extract agar for 4 to 6 weeks at 25°C. The spores were collected by agitating the surface of fungal cultures 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. Heat killing of spores was achieved by autoclaving the spores. Mice were lightly anesthetized with isoflurane and exposed to saline, 10^6 live *C. cladosporioides* or 10^6 heat-killed *C. cladosporioides* spores in 50 µL intratracheally 3 times a week for 3 weeks, and then were assessed 2 days after the last exposure. The mold spore dose used is the same as previously published.³⁰

Assessment of airway hyperresponsiveness

Airway hyperresponsiveness (AHR) to methacholine (acetyl-b-methyl-cholinechloride; Sigma, St Louis, Mo) was assessed in mice by using flexiVent (SCIREQ, Montreal, Quebec, Canada) as previously described.³⁰

Bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BMDCs) were generated as previously described.³¹ BMDCs were stimulated with either live or heat-killed *C. cladosporioides* spores at a 1:2 cell-to-spore ratio. After 24 hours, supernatants and RNA were collected.

Bronchoalveolar lavage fluid collection and analysis

Bronchoalveolar lavage fluid (BALF) was collected and analyzed as previously described.³² For detection of total serum IgE, plasma was diluted at 1:50 and the ELISA was performed as previously reported.³² Detection of IL-4 and IL-17A was performed according to manufacturer instructions (BioLegend, San Diego, Calif) in undiluted BALF. IL-6 and TNF-α were also detected according to manufacturer instructions (BioLegend) in supernatants from BMDCs diluted 1:5.

Isolation of lung cells and flow cytometry

Lungs were removed, and the upper right lobe was minced and incubated at 37°C for 25 to 30 minutes in 2 mL of RPMI1640 containing Liberase DL (0.5 mg/mL; Roche Diagnostics, Indianapolis, Ind) and DNase I (0.5 mg/mL; Sigma). Lung cells were passed through a 70- μ m cell strainer. Cells were centrifuged and resuspended in 2 mL of RPMI. Cell viability was confirmed by trypan blue exclusion.

Approximately 10^6 lung cells were transferred into a V-bottom 96-well plate on ice, centrifuged, and resuspended in PBS containing FcBlock (2.4G2 mAb, Biolegend) after stimulation with phorbol 12-myristate 13-acetate (50 ng/mL; Sigma) and ionomycin (500 ng/mL; Sigma) in the presence of Brefeldin A (eBioscience, San Diego, Calif) and monensin (eBioscience) diluted 1:1000. Cells were labeled with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit according to manufacturer's instructions (Invitrogen by Life Technologies, Carlsbad, Calif). T cells were stained with CD4-Pacific Blue (BioLegend). Intracellular staining for IL13-PE (eBioscience) and IL17A-AF647 (BioLegend) was done by using reagents from eBioscience. All flow cytometric data were acquired by using the LSR Fortessa (Becton Dickinson, Mountain View, Calif), maintained by the Research Flow Cytometry Core in the Division of Rheumatology at Cincinnati Children's Hospital Medical Center. Data were analyzed by using FlowJo software (Tree Star, Ashland, Ore).

RT-PCR

RNA was extracted from the lungs or from BMDCs by using TRIzol reagent (Life Technologies, Grand Island, NY). Reverse transcription was performed by using iScript Reverse Transcription Supermix (BioRad, Hercules, Calif). Real-time PCR was done by 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 by 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 by 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-6 was specifically amplified by using forward primer 5'-TGA TGC ACT TGC AGA AAA CA-3' and reverse primer 5'-ACC AGA GGA AAT TTT CAA TAG GC-3'. cDNA for murine TNF- α was specifically amplified by using forward primer 5'-TGT GCT CAG AGC TTT CAA CAA-3' and reverse primer 5'-CTT GAT GGT GGT GCA TGA GA-3'. cDNA for murine IL-23p19 was specifically amplified by using forward primer 5'-ACT CAG CCA ACT CCT CCA GCC AG-3' and reverse primer 5'-CTG CTC CGT GGG CAA AGA CCC-3'.

Beta-glucan staining and Dectin-1 pulldown

Binding to recombinant murine Dectin-1 (R&D Systems, Minneapolis, Minn) was determined as previously described.³⁰ Briefly, mold spores were labeled with Alexa Fluor 488 dye (Life Technologies), incubated with recombinant murine Dectin-1 (R&D Systems), and 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 beta-glucans on the spores, 10^6 spores were incubated with murine anti-beta-glucan antibody, primary antibody (Biosupplies, Bundoora, Australia), at a concentration of 1 $\mu\text{g}/\text{mL}$ in 1% goat serum in 0.01% PBS Tween-20, and the secondary antibody was goat antimouse DyLight 594 (BioLegend). Images were acquired at 1000 \times by using a Nikon 90i Fully Automated Upright Microscope System with a Nikon DSQiMc camera, with Z-sequence taken every 0.5 μm . Nikon Elements Quantitative Analysis software was used to acquire the images. For the measurement of mean fluorescence intensity, the fluorescence of each spore in 10 fields of view was measured by using Nikon Elements Quantitative Analysis software. The mean was calculated by adding the fluorescence of each individual spore and dividing by the total number of spores.

Statistical analysis

All statistical analyses were done by using PRISM software (GraphPad Software, Inc, La Jolla, Calif). Statistical significance was assessed by using 1-way ANOVA followed by a Neuman-Kohls posttest or a 2-way ANOVA followed by a Bonferroni posttest for dose-response data.

RESULTS

Heat-killed *C cladosporioides* spores induce neutrophilic inflammation in the lungs of mice

Our previous data indicate that Dectin-1 prevents the development of murine asthma in response to the environmental mold *A versicolor* but not *C cladosporioides*. This was due to differences in the surface exposure of beta-glucans on the 2 molds and thus the differing capabilities of the molds to bind to Dectin-1.³⁰ Previous studies reported that heat killing of *Candida albicans* results in exposure of beta-glucans.^{26,27} Therefore, we wanted to determine whether heat-killed *C cladosporioides* spores would induce an inflammatory phenotype in the lungs of mice similar to *A versicolor*, which express higher levels of surface beta-glucans.³⁰ Exposure of mice to live *C cladosporioides* spores induced robust AHR, a strong eosinophilic response, and an increase in total IgE (Fig 1), as we previously reported.³⁰ In contrast, exposure of mice to heat-killed *C cladosporioides* spores resulted in the development of minimal AHR, a predominately neutrophilic response, and no increase in serum IgE (Fig 1). Thus, heat-killed *C cladosporioides* spores induce a predominantly neutrophilic response in the lungs of mice similar to our published data with *A versicolor*.³⁰

Lack of a T_H2 cytokine response in the lungs of mice exposed to heat-killed *C cladosporioides*

We next investigated the nature of the immune responses that developed in the lungs of mice after exposure to live versus heat-killed *C cladosporioides* spores. While both live and heat-killed spores were able to induce T_H2 cells (Fig 2, B), only the heat-killed spores induced T_H17 cells (Fig 2, A). The gating strategy is demonstrated in Fig E1 in this article's Online Repository at www.jacionline.org. However, we found by analyzing IL-17A and IL-4 in the

BALF by ELISA (Fig 2, *C*) or in the lungs by quantitative PCR (Fig 2, *D*) that heat-killed spores induced significant amounts of IL-17A and very little IL-4 than did live spores (Fig 2, *C* and *D*). Thus, heat-killed spores induce a strong T_H17 response and a relatively weak T_H2 response than do live spores.

Heat killing of *C cladosporioides* exposes beta-glucans

To establish the impact of heat killing on the level of beta-glucans exposed on the surface of *C cladosporioides* spores, we performed immunofluorescence on the spores by using an anti-beta-glucan antibody. In both live and heat-killed spores, we observed staining predominately at the ends of spores at the bud scars (Fig 3, *A*). Importantly, the heat-killed spores were intact and the process of heat killing did not disrupt the spores (Fig 3, *A*). Furthermore, we did not observe any autofluorescence in the AF488 channel because only spores stained with an AF488 dye demonstrated any fluorescence (see Fig E2 in this article's Online Repository at www.jacionline.org). The staining intensity was stronger in the heat-killed spores, supporting that there are more beta-glucans exposed following heat killing (quantified in Fig 3, *B*). To confirm this, we directly examined the ability of the heat-killed spores to bind recombinant Dectin-1. As shown in Fig 3, *C*, heat-killed spores demonstrated increased binding to recombinant Dectin-1 than did live spores (Fig 3, *C*), consistent with the greater exposure of beta-glucans on heat-killed spores.

Dectin-1 prevents the induction of murine asthma in response to heat-killed spores

We investigated whether the phenotype induced by heat-killed *C cladosporioides* was dependent on Dectin-1. Wild-type mice exposed to heat-killed *C cladosporioides* spores developed a mixed neutrophilic/eosinophilic response but no AHR (Fig 4). Dectin-1^{-/-} mice exposed to heat-killed *C cladosporioides* spores developed robust AHR and eosinophilia. Furthermore, they did not develop any neutrophilic inflammation (Fig 4). In contrast, Dectin-1 had minimal impact on the development of AHR and eosinophilia in response to live spores (data not shown and previously published³⁰). Similar to our previously published data,³⁰ C57B1/6 mice developed more robust eosinophilia than did BALB/c mice. Thus, these data indicate that the inflammatory phenotype induced by heat-killed *C cladosporioides* spores is dependent on Dectin-1.

T_H17 response induced by heat-killed *C cladosporioides* spores is dependent on Dectin-1

We next determined whether the development of a T_H17 response following exposure to heat-killed *C cladosporioides* spores (Fig 2) was dependent on Dectin-1. We characterized the T cells in the lungs of wild-type and Dectin-1^{-/-} mice following repeated exposure to heat-killed *C cladosporioides* spores. As shown in Fig 5, there was a significant decrease in the frequency of T_H17 cells in Dectin-1^{-/-} mice than in wild-type mice (Fig 5, *A* and *B*). Similarly, IL-17A was decreased in the BALF and IL-17A expression was decreased in the lungs of Dectin-1^{-/-} mice, but not wild-type mice, exposed to heat-killed *C cladosporioides* spores (Fig 5, *C*). Furthermore, there was an increase in T_H2 cells in Dectin-1^{-/-} mice than in wild-type mice exposed to heat-killed *C cladosporioides* spores (Fig 5, *A* and *B*). While there was no difference in IL-4 BALF levels between wild-type and Dectin-1^{-/-} mice, IL-4 mRNA levels were increased in the lungs of Dectin-1^{-/-} versus wild-type mice following exposure to heat-killed *C cladosporioides* spores (Fig 5, *D*). Induction of T_H2 cytokines by

live *C cladosporioides* spores is independent of Dectin-1 (data not shown and previously published³⁰). Thus, the T_H17 response induced by heat-killed spores is dependent on Dectin-1. In the absence of Dectin-1, the frequency of T_H2 cells and IL-4 expression in the lungs were increased.

T_H17-skewing cytokines are decreased in the lungs of Dectin-1-deficient mice

Because the T_H17 response induced by heat-killed *C cladosporioides* spores is dependent on Dectin-1, we hypothesized that altered upstream expression of T_H17-skewing cytokines by heat-killed versus live spores may be the mechanistic basis for our observations. IL-6 is important for priming T_H17 cells in the mucosal tissues,³³ and TNF- α production by dendritic cells is important for regulating IL-17 levels in the lungs.³⁴ Therefore, we examined the expression of IL-6 and TNF- α in the lungs of mice exposed to live or heat-killed *C cladosporioides* spores in wild-type and Dectin-1^{-/-} mice. Mice exposed to live *C cladosporioides* spores had a significant increase in the expression of IL-6 than did saline-exposed mice, and this increase was independent of Dectin-1. There was no significant difference between wild-type mice and Dectin-1^{-/-} mice exposed to live *C cladosporioides* spores (Fig 6, A). There was only a slight, nonsignificant, induction of TNF- α by live *C cladosporioides* spores in both wild-type and Dectin-1^{-/-} mice (Fig 6, B). However, heat-killed *C cladosporioides* spores induced expression of both IL-6 and TNF- α in a Dectin-1-dependent manner (Fig 6, C and D). In the absence of Dectin-1, expression of IL-6 and TNF- α was significantly reduced (Fig 6, C and D). Thus, Dectin-1 promotes the induction of T_H17-skewing cytokines, but only in response to heat-killed spores that express surface beta-glucans.

Dendritic cell production of proinflammatory cytokines is dependent on Dectin-1

Because we observed altered expression of IL-6 and TNF- α in response to heat-killed spores in Dectin-1^{-/-} mice, we wanted to determine whether the expression of Dectin-1 in dendritic cells could contribute to altered production of T_H17-skewing cytokines. Signaling through Dectin-1 on dendritic cells has been shown to induce pro-T_H17 factors including TNF- α , IL-6, and IL-23.^{13,21,35,36} Therefore, we investigated the role of Dectin-1 in the response of dendritic cells to heat-killed versus live *C cladosporioides* spores. When BMDCs were exposed to live spores, the presence or absence of Dectin-1 did not impact the induction of IL-6, TNF- α , or IL-23 (Fig 7). In contrast, on exposure to heat-killed spores, BMDCs from Dectin-1^{-/-} mice showed significantly attenuated expression of IL-6, IL-23, and TNF- α compared with wild-type mice (Fig 7, A-C). Similarly, in the absence of Dectin-1, BMDCs secreted significantly less IL-6 and TNF- α (Fig 7, D and E). Thus, Dectin-1 promotes the induction of proinflammatory cytokines, but only in response to heat-killed spores that express surface beta-glucans, not in response to live spores.

DISCUSSION

These data demonstrate that the surface availability of beta-glucans is a critical determinant of the type of inflammatory response induced by specific mold species. Our previous work demonstrated that the distinct pulmonary inflammatory phenotypes are induced by *A versicolor* and *C cladosporioides*, common environmental molds. *C cladosporioides* induces

strong eosinophilic inflammation and AHR through a Dectin-1-independent mechanism, while *A versicolor* induces neutrophilic inflammation and mild AHR and this is dependent on Dectin-1. In the absence of Dectin-1, *A versicolor* induces a response similar to that of *C cladosporioides*, indicating that Dectin-1 inhibits eosinophilic inflammation and AHR in response to *A versicolor*.³⁰ In this study, we demonstrate that exposure of beta-glucans in *C cladosporioides* by heat killing induces a neutrophilic inflammatory phenotype characterized by T_H17 cytokines similar to that induced by *A versicolor*. In the absence of Dectin-1, heat-killed *C cladosporioides* induce strong AHR, eosinophilia, and T_H2 cytokines. Thus, the availability of beta-glucans on the surface of a mold, and not the viability of the mold spores, is an important determinant in the type of inflammatory response that ensues.

Furthermore, our data support a mechanistic role for dendritic cells in the observed differences in mold-induced inflammation. The presence or absence of Dectin-1 did not impact the induction of proinflammatory cytokines by live *C cladosporioides* spores, but induction of proinflammatory cytokines IL-6, IL-23, and TNF- α , all of which have been shown to be important in the development of T_H17 inflammatory responses,^{33,34,37,38} was significantly attenuated in the absence of Dectin-1 following exposure to heat-killed *C cladosporioides* spores. Despite the production of IL-6, IL-23, and TNF- α dendritic cells after exposure to live *C cladosporioides* spores, we did not observe a significant increase in T_H17 cells in mice exposed to live spores. It is likely that live *C cladosporioides* spores are inducing these cytokines through a non-Dectin-1^{-/-} pathway that does not contribute to the development of T_H17 responses.

Quantification of beta-glucans in the environment is often used as a surrogate for mold exposure.³⁹⁻⁴¹ Several studies have examined the direct relationship between beta-glucans and health effects (reviewed in Douwes⁷) with inconsistent results. One study found that low levels of beta-glucans were positively correlated with a positive asthma predictive index but high levels of beta-glucans were negatively correlated with a positive asthma predictive index.⁴² A study in Germany similarly found a negative association between beta-glucan levels in mattress dust and sensitization to aeroallergens.⁴¹ Another study also found a negative correlation because there was a trend toward increased IFN- γ in patients exposed to beta-glucans rather than T_H2 cytokines.⁴⁰ Studies examining airway function and inflammation also contradict each other.⁷ The inconsistencies in the literature may be explained by the fact that it is not the amount of beta-glucans present but rather the surface availability of beta-glucans on the mold spores in the samples taken. Notably, *C cladosporioides* has a higher beta-glucan content than does *A versicolor*⁴³; thus, one might predict that *C cladosporioides* would mediate its effects through Dectin-1 binding; however, *C cladosporioides* induces a strong asthma phenotype independent of Dectin-1.³⁰ It is the surface availability of beta-glucans that is important for determining the immune response, not the beta-glucan content of spores. This study demonstrates that in the same mold species, increasing the availability of beta-glucans on the surface of the mold spore (without changing the total beta-glucan content) alters the immune response.

Several studies support the concept that exposure of beta-glucans on spore surface modulates the immune response. Heat killing of *C albicans* exposes the beta-glucans and alters the immune response,^{26,27} and exposure of beta-glucans in *A fumigatus* by genetic or

chemical depletion alters the immunogenicity of mold spores.²⁵ Our data now add to the current literature and demonstrate that differences in the exposure of surface beta-glucans in the same species of mold can alter the immune response in a Dectin-1-dependent manner. In addition, our data suggest that when quantifying mold exposure, it is important to consider to develop methods that assess the availability of beta-glucans on the molds, in addition to the total content of beta-glucans present. It is intriguing to speculate that one may be able to modify the immune response to molds by altering the beta-glucan availability.

Extended Data

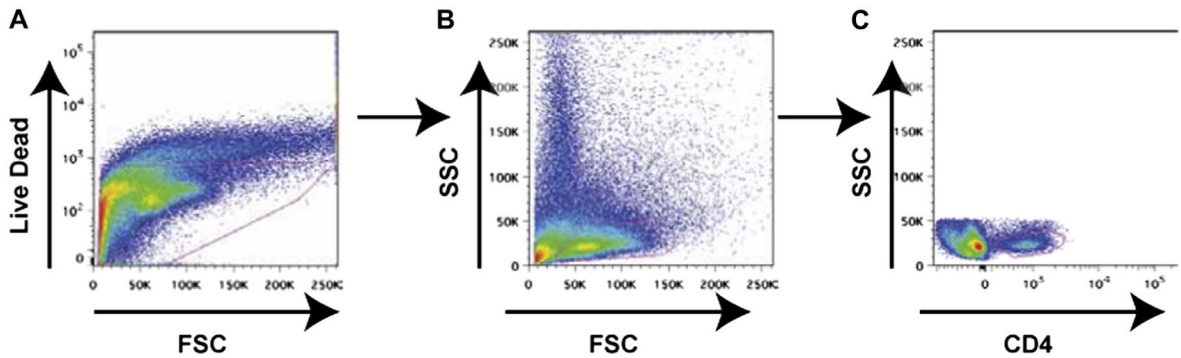
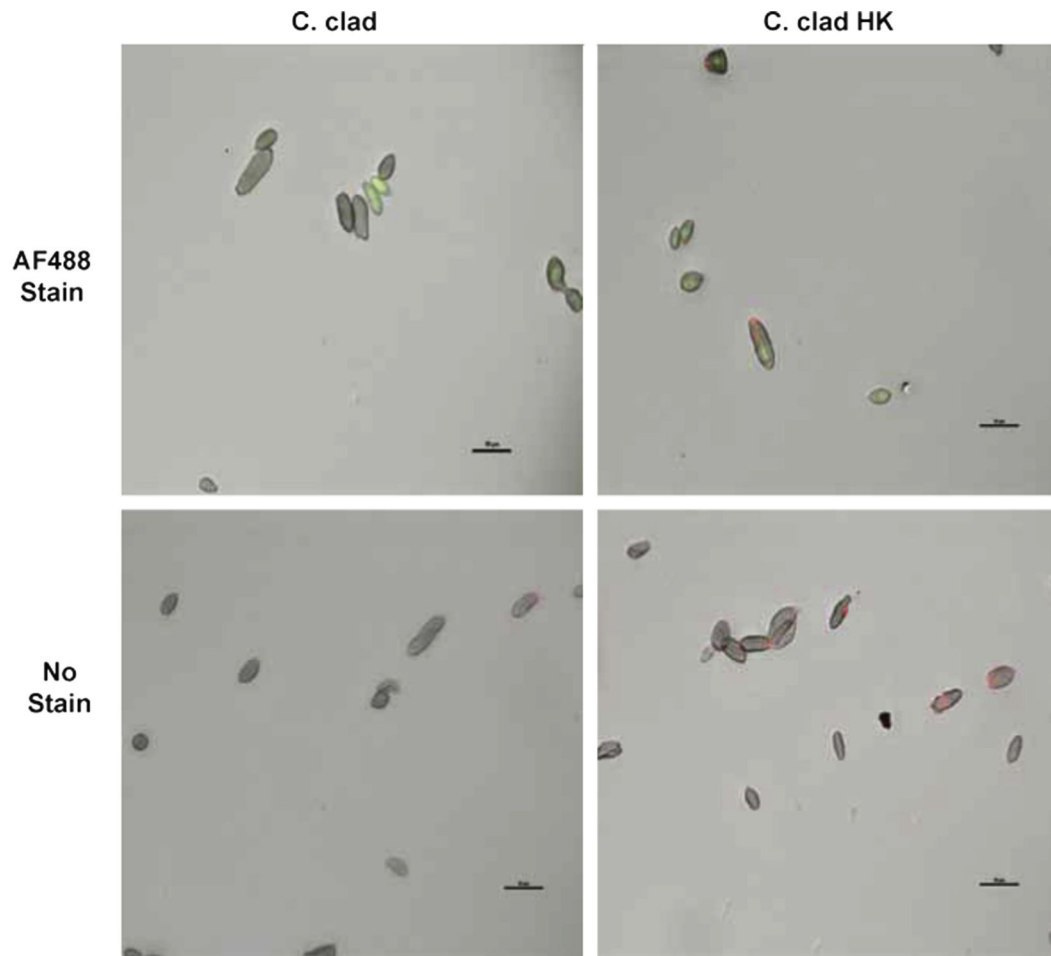


FIG E1.

A, Live cells gating with live dead stain (Invitrogen). **B**, Gating of lymphocytes in the live population based on forward scatter (*FSC*) and side scatter (*SSC*). **C**, CD4 gating from the lymphocyte population.

**FIG E2.**

C. clad or *C. clad* HK spores were stained with an AF488 dye (*green*) or not stained and then incubated with anti-beta glucan and a secondary antibody (*red*). *C. clad* HK, Heat-killed *C. cladosporioides*.

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Abbreviations used

AHR	Airway hyperresponsiveness
BALF	Bronchoalveolar lavage fluid
BMDCs	Bone marrow–derived dendritic cells

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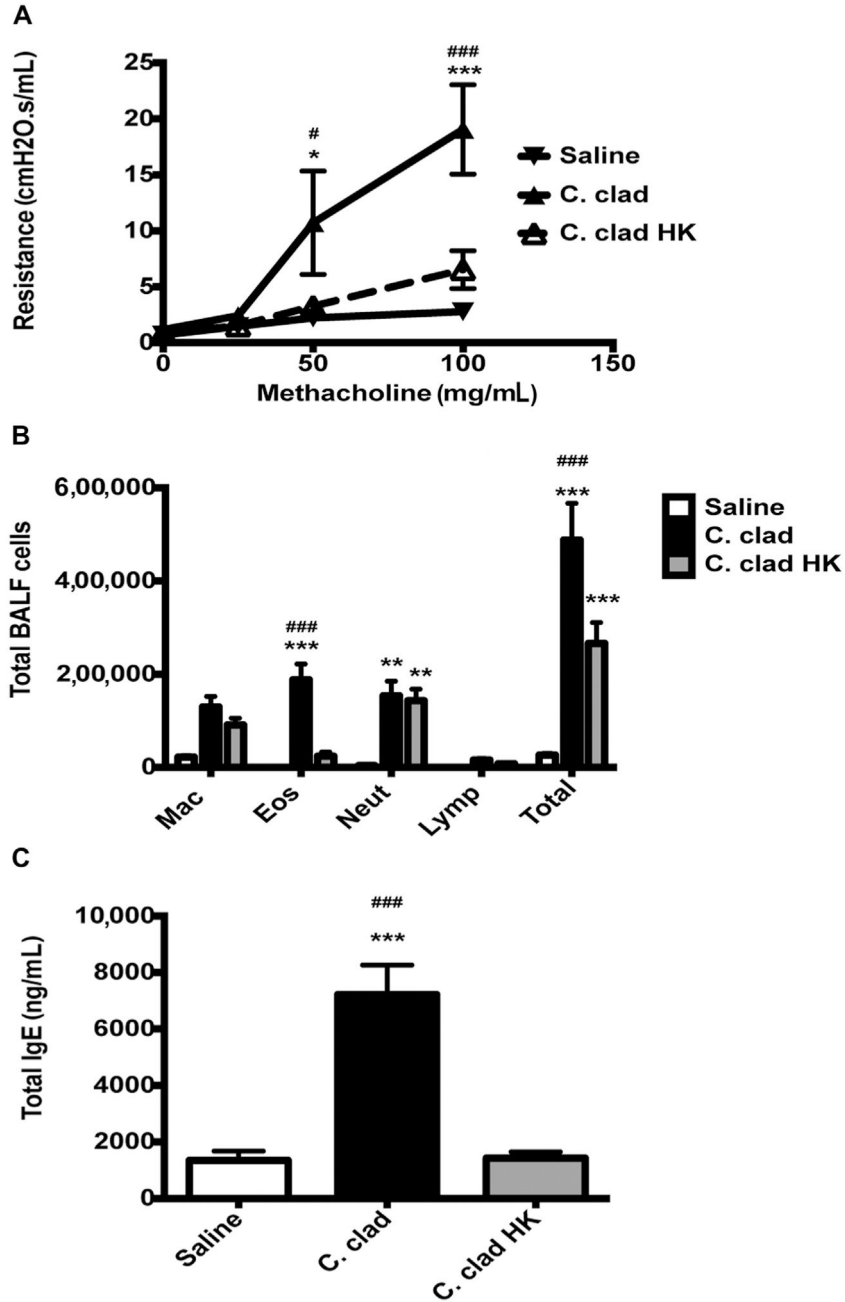
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Key messages

- Heat-killed *C cladosporioides* spores effectively expose beta-glucans on the surface of spores.
- Alteration of beta-glucan surface availability influences the host response to *C cladosporioides* through differential binding to Dectin-1.
- Beta-glucan exposure and signaling through Dectin-1 attenuates the induction of allergic disease, likely through the promotion of T_H17 responses.

**FIG 1.**

Heat killing of *C cladosporioides* prevents the development of murine asthma. **A**, BALB/c mice were exposed i.t. to 10^6 live or heat-killed *C cladosporioides* (C clad or C clad HK) spores 3 times a week for 3 weeks and 48 hours after the last challenge, AHR was measured by flexiVent. **B**, BALF was analyzed for total and differential counts. **C**, Total serum IgE. Data are representative of 3 independent experiments and are expressed as mean and SEM. $n = 4$ to 6 mice per group. *Eos*, Eosinophils; *HK*, heat killed; *i.t.*, intratracheally; *Lymp*, lymphocytes; *Mac*, macrophages; *Neut*, neutrophils. * $P < .05$, ** $P < .01$, and *** $P < .001$ as compared with saline. # $P < .05$ and ### $P < .001$ as compared with C clad HK.

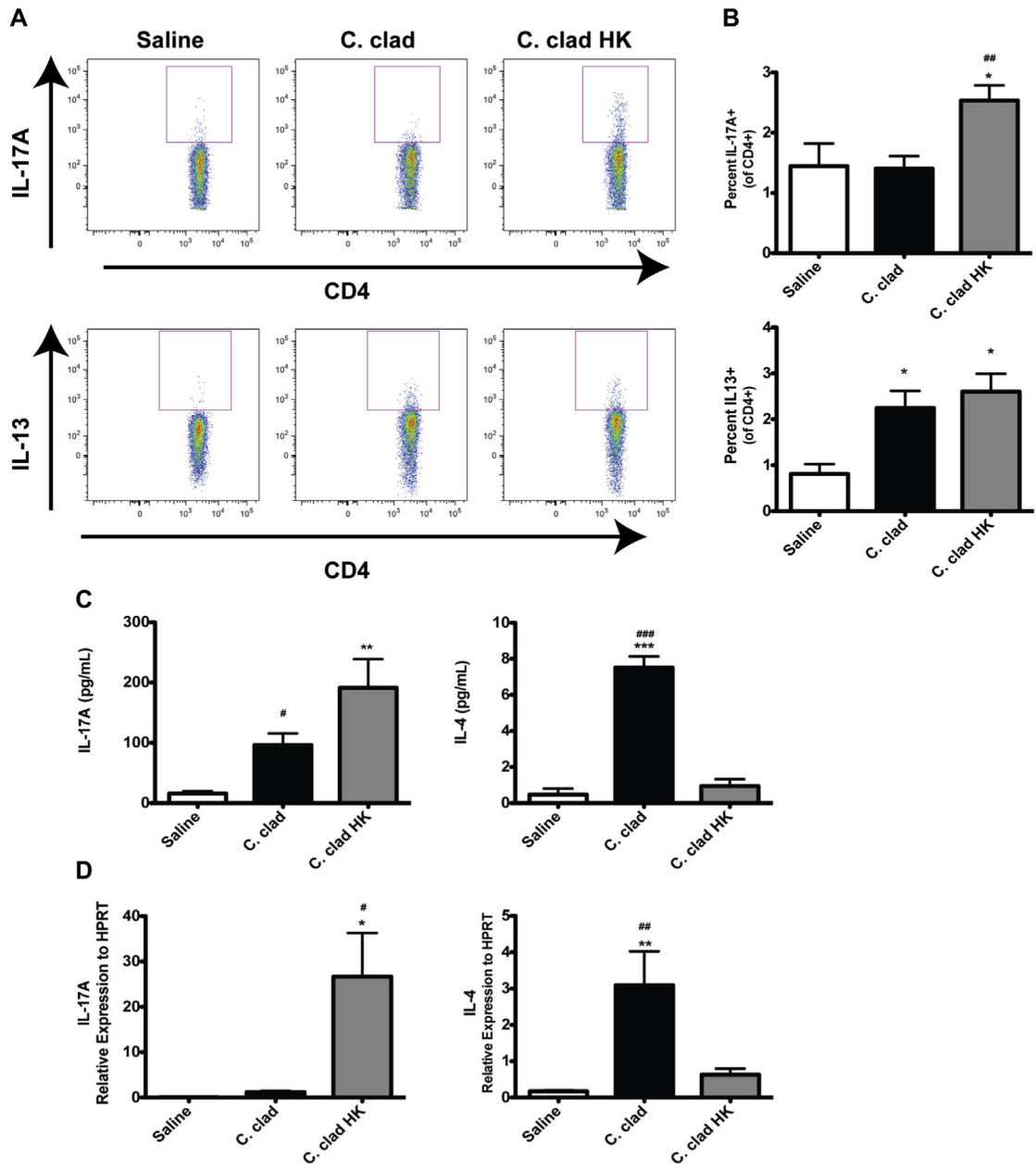


FIG 2. Induction of a T_H17 response by heat-killed *C. cladosporioides* (*C. clad HK*). Contour plots (A) and quantification of intracellular cytokine staining for IL-13 or IL-17A in CD4+ lung cells (B). C, Levels of IL-17A and IL-4 in BALF. D, Relative levels of mRNA for IL-17A and IL-4 in whole lung. Data are representative of 3 independent experiments and are expressed as mean and SEM. $n = 4$ to 6 mice per group. *HPRT*, Hypoxanthine phosphoribosyltransferase. * $P < .05$, ** $P < .01$, and *** $P < .001$ as compared with saline. # $P < .05$, ## $P < .01$, and ### $P < .001$ as compared with *C. clad HK*.

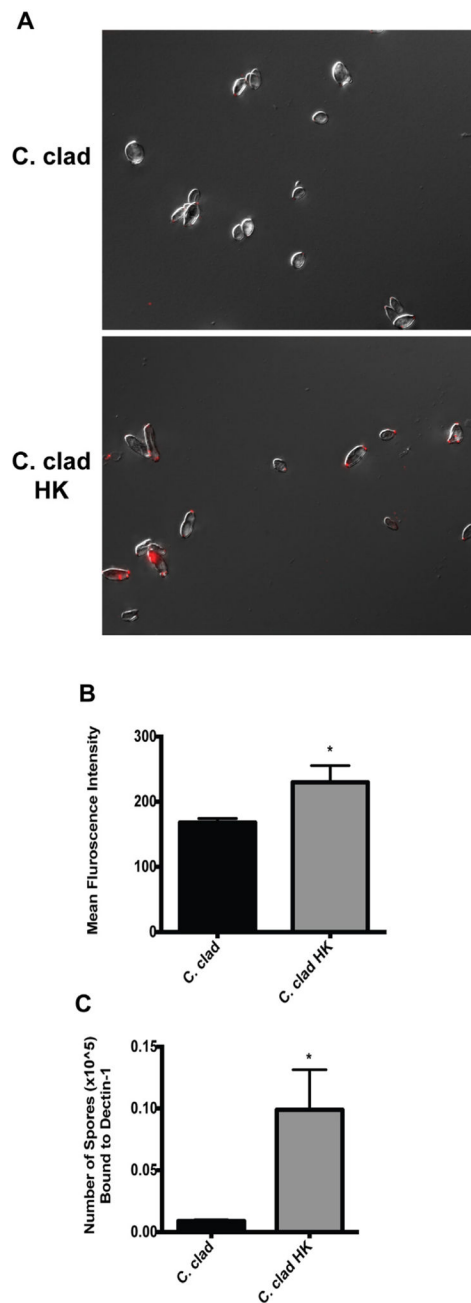


FIG 3. Heat-killed *C cladosporioides* have increased surface exposure of beta-glucans and binding to Dectin-1. **A**, Immunofluorescence images of beta-glucan staining taken at $\times 1000$ for live and heat-killed *C cladosporioides* spores. Scale bar is 10 μm . **B**, Quantification of beta-glucan fluorescence intensity per spore. **C**, Number of spores bound to recombinant Dectin-1. Data are representative of 2 independent experiments and are expressed as mean and SEM. $n = 3$ samples per group. *C clad HK*, Heat-killed *C cladosporioides*. * $P < .05$.

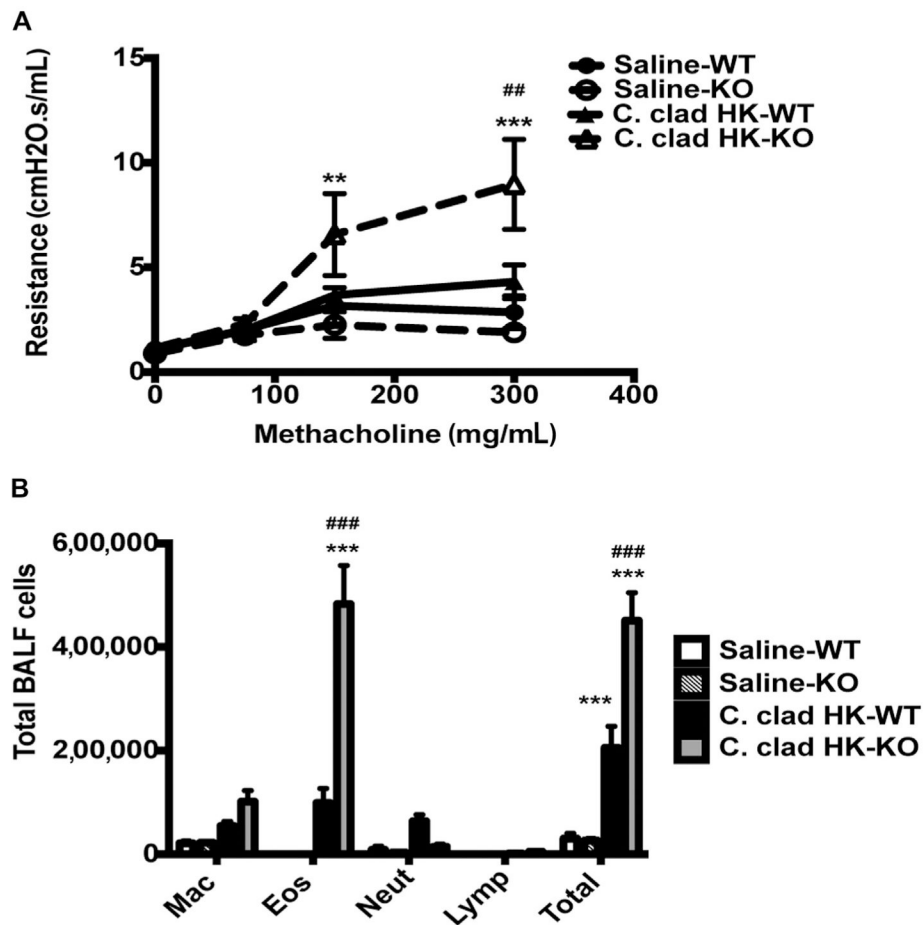
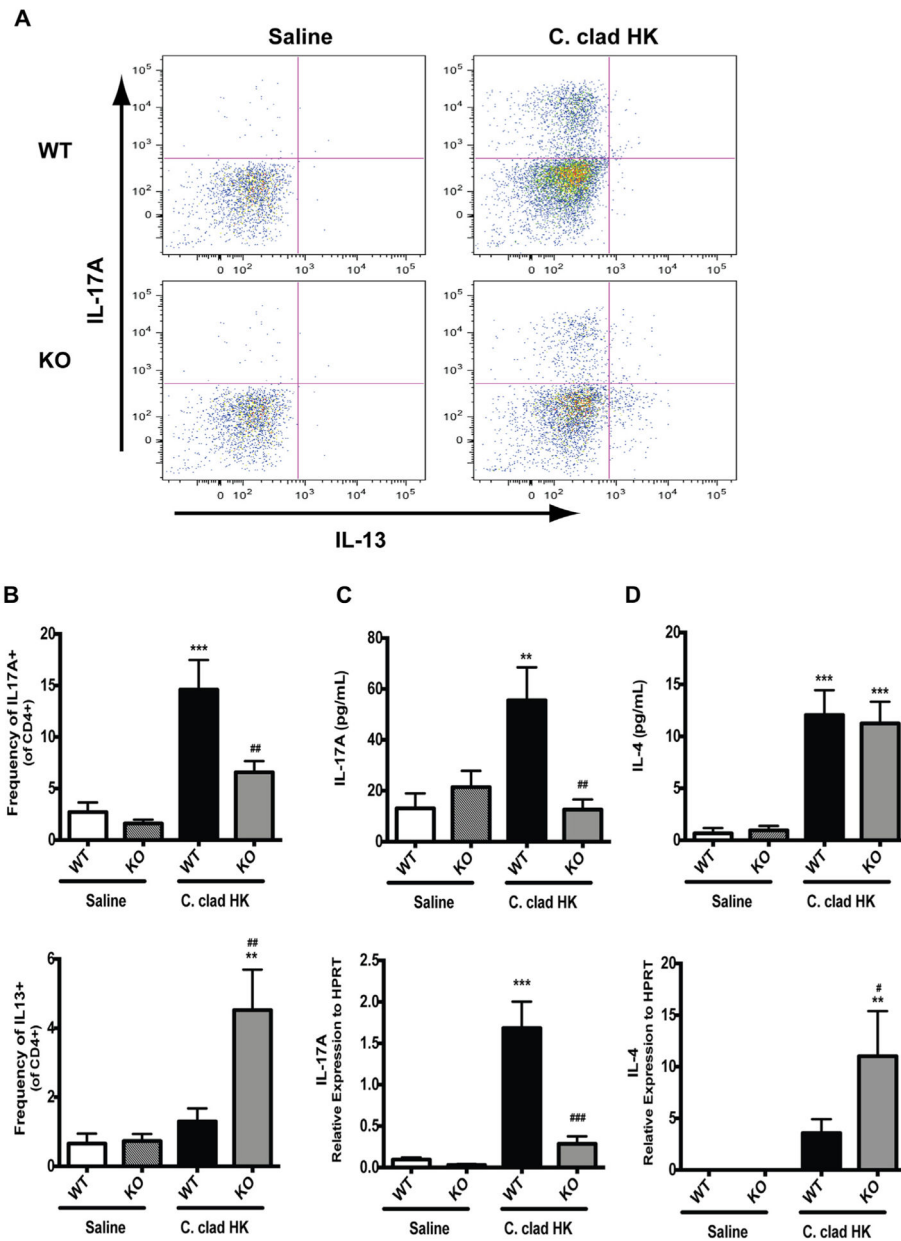


FIG 4. Heat-killed *C cladosporioides* prevents murine asthma in a Dectin-1–dependent manner. **A**, Wild-type C57BI/6 or Dectin-1^{-/-} mice were exposed to heat-killed *C cladosporioides* spores, and AHR was assessed 48 hours later. **B**, Total and differential counts of BALF. Data are representative from 2 independent experiments and are expressed as mean and SEM. n = 4 to 6 mice per group. *C clad HK*, Heat-killed *C cladosporioides*; *Eos*, eosinophils; *KO*, knockout; *Lymp*, lymphocytes; *Mac*, macrophages; *Neut*, neutrophils; *WT*, wild type. ***P* < .01 and ****P* < .001 as compared with saline. ##*P* < .01 and ###*P* < .001 as compared with wild-type mice exposed to *C cladosporioides*.

**FIG 5.**

In the absence of Dectin-1, heat-killed *C. cladosporioides* induces a T_H2 response. Contour plots (A) and quantification of intracellular cytokine staining for IL-13 or IL-17A in CD4+ lung cells (B). C, Levels of IL-17A in BALF and mRNA in whole lung. D, Levels of IL-4 in BALF and mRNA in whole lung. Data are representative from 2 independent experiments and are expressed as mean and SEM. n = 4 to 6 mice per group. *C. clad* HK, Heat-killed *C. cladosporioides*; KO, knockout; WT, wild type. ***P* < .01 and ****P* < .001 as compared with saline. #*P* < .05, ##*P* < .01, and ###*P* < .001 as compared with wild-type mice exposed to *C. cladosporioides*.

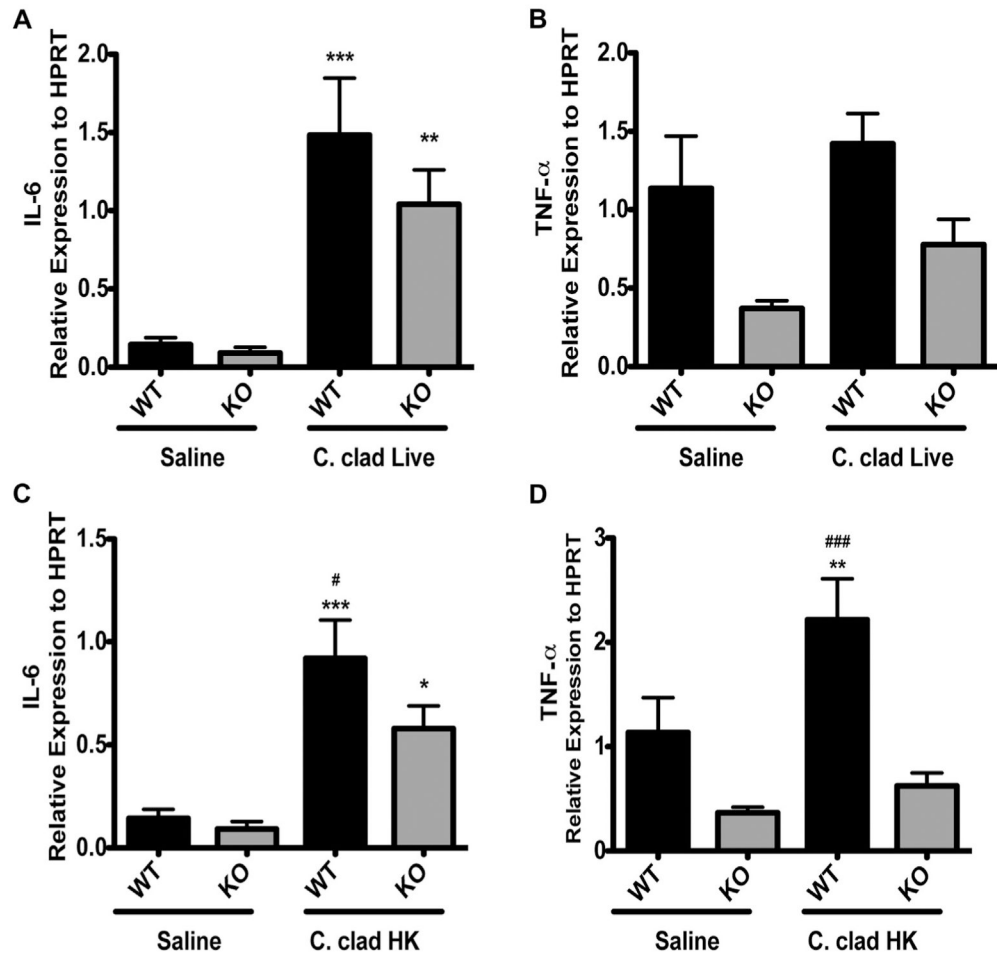
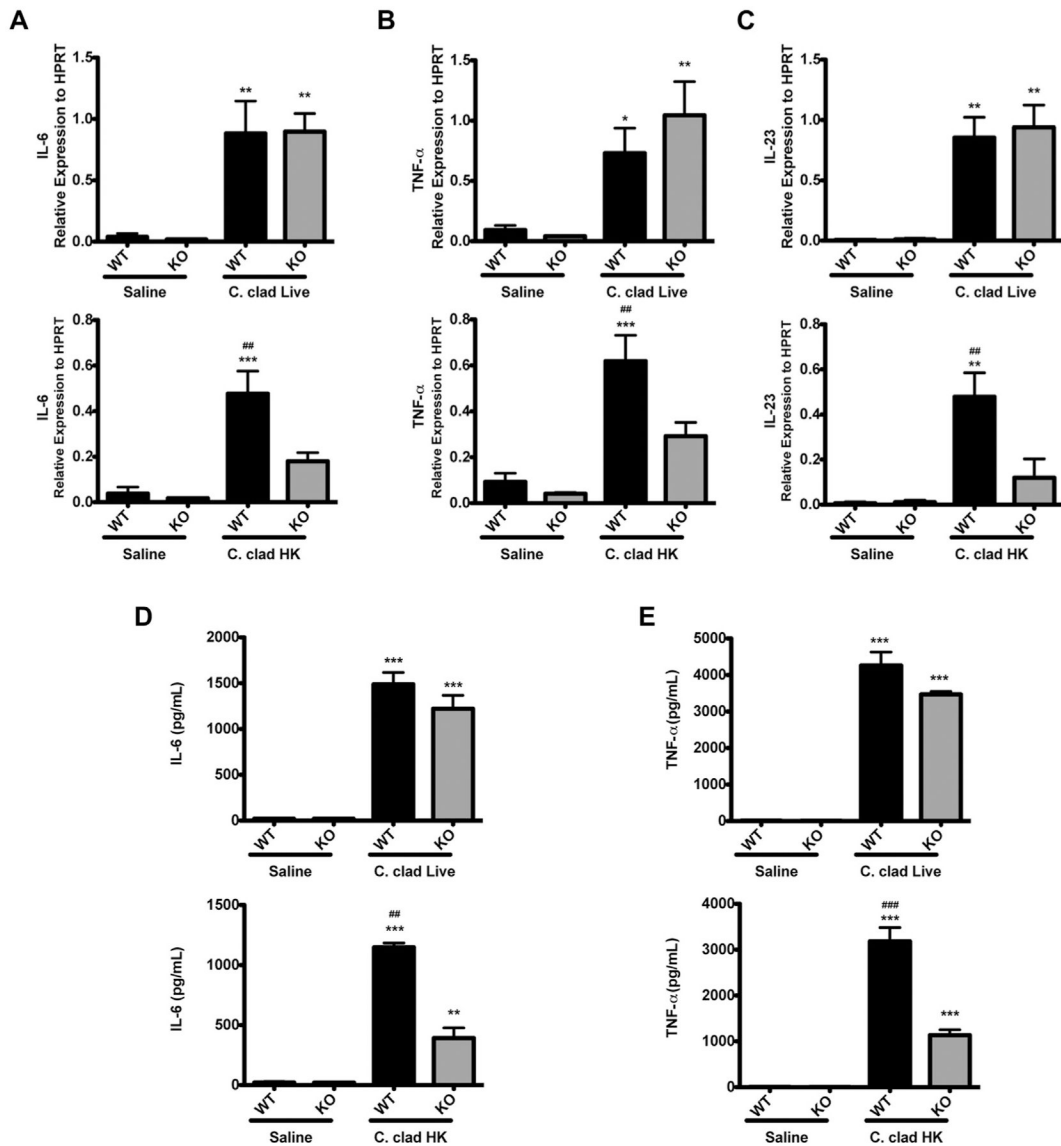


FIG 6.

Expression of T_H17 -skewing cytokines in the lungs is dependent on Dectin-1. **A** and **C**, Expression of IL-6 in the lungs of mice exposed to live (Fig 6, **A**) or heat-killed (Fig 6, **C**) *C. cladosporioides*. **B** and **D**, Expression of TNF- α in the lungs of mice exposed to live (Fig 6, **B**) or heat-killed (Fig 6, **D**) *C. cladosporioides*. Data are representative from 2 independent experiments and are expressed as mean and SEM. n = 4 to 6 mice per group. *C. clad HK*, Heat-killed *C. cladosporioides*; *HPRT*, hypoxanthine phosphoribosyltransferase; *KO*, knockout; *WT*, wild type. * $P < .05$, ** $P < .01$, and *** $P < .001$ as compared with saline. # $P < .05$ and ### $P < .001$ as compared with Dectin-1^{-/-} mice exposed to *C. cladosporioides*.

**FIG 7.**

Proinflammatory cytokine production in BMDCs is dependent on Dectin-1. Expression of IL-6 (A), TNF- α (B), and IL-23 (C) in BMDCs. Level of IL-6 (D) and TNF- α (E) in culture supernatants of BMDCs. Data are representative of 3 independent experiments and are expressed as mean and SEM. $n = 4$ samples per group. *C. clad HK*, Heat-killed *C. cladosporioides*; *HPRT*, hypoxanthine phosphoribosyltransferase; *KO*, knockout; *WT*, wild type. * $P < .05$, ** $P < .01$, and *** $P < .001$ as compared with saline. ## $P < 0.01$ and ### $P < .001$ as compared with Dectin-1^{-/-} BMDCs exposed to *C. cladosporioides*.