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

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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.)

Key words: *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 infections[21-23] 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]

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

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-methylcholinechloride; 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 RPMI 1640 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-μm 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 μg/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× by using a Nikon 90i Fully Automated Upright Microscope System with a Nikon DSQiMc camera, with Z-sequence taken every 0.5 μm. Nikon