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Mold-Induced Asthma: Not all Molds are Created Equal

A dissertation submitted to the Graduate School of the University of Cincinnati in partial fulfillment of the requirements for the degree of

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by

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Abstract

There is considerable evidence supporting that mold exposure is a major contributor to the development of asthma. The identification of mold as an important component of the environmental contribution to the development and pathogenesis of asthma leads to questions about possible interventions to prevent and/or attenuate mold-related health effects. In epidemiologic studies it is difficult to determine the species of mold that are the causative agents since many subjects are exposed to more than one mold. This lead us to develop a murine model of chronic mold exposure to examine the inflammatory phenotypes induced by individual molds and the mechanisms underlying those phenotypes. The work in this dissertation uses two molds that are common in the Ohio River Valley, Aspergillus versicolor and Cladosporium cladosporioides. In our model 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, while 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, robust AHR, and eosinophilic inflammation compared to wild type mice. A similar phenotype was observed in mice deficient in MyD88. In the absence of MyD88, IL-17A was attenuated in response to A. versicolor, and there was an induction of robust AHR and eosinophilic inflammation. In contrast, C. cladosporioides induced AHR and eosinophilic inflammation independent of IL-17A, Dectin-1, and MyD88. A. versicolor, but not C. *cladosporioides*, spores had increased exposure of beta-glucans on their surface and were able to

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bind Dectin-1. Thus, the host response to C. cladosporioides was IL-17A- and Dectin1independent, while Dectin-1 and IL-17A-dependent pathways were protective against the development of asthma after exposure to A. versicolor. Furthermore, exposure of beta-glucans on the surface of *C. cladosporioides* by heat killing the spores altered the immune response. Live C. cladosporioides induced robust airway hyperresponsiveness, eosinophilia, and a predominately Th2 response, while heat-killed C. cladosporioides induced a strong Th17 response and neutrophilic inflammation, but very mild AHR. 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 Th2 response analogous to live spores. Thus, the host immune response to C. cladosporioides is dependent on surface availability of beta-glucans rather than the total beta-glucan content. Finally, coexposure with A. versicolor and HDM or C. cladosporioides induced synergy in the development of AHR and airway inflammation. Mice co-exposed to A. versicolor and HDM developed a mixed Th2/Th17 response. Thus, co-exposure results in the development of a phenotype that mimics severe asthma. These data demonstrate that not all molds are created equal. The difference in beta-glucan exposure accounts for the distinct inflammatory phenotypes induced by each mold. Further, co-exposure results in synergistic inflammatory responses.

Preface

This thesis consists of an introduction (Chapter 1), two completed manuscripts (Chapters 2 and 4), two manuscripts near completion (Chapters 3 and 5), and a summary (Chapter 6). The work presented herein was performed by the author under the guidance of Dr. Gurjit Khurana Hershey in the Division of Asthma Research, Cincinnati Children's Hospital, Department of Pediatrics at the University of Cincinnati College of Medicine.

Chapter 2 has been accepted for publication in the Journal of immunology and Chapter 4 has been submitted to the Journal of Allergy and Clinical Immunology. Upon completion, Chapters 3 and 5 will be submitted for publication.

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List of Abbreviations

| AHR | Airway hyperresponsiveness |
|---------|------------------------------------|
| APC | Antigen presenting cell |
| A. ver | Aspergillus versicolor |
| BALF | Bronchoalveolar lavage fluid |
| BMDC | Bone marrow derived dendritic cell |
| C. clad | Cladosporium cladosporioides |
| CLR | C-type lectin |
| DC | Dendritic cells |
| HDM | House dust mite |
| IL | Interleukin |
| I.T. | Intratracheal |
| Th | T helper |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| | |

Chapter 1: General Introduction

<u>1. Fungi and Molds</u>

1a. Molds as a clinical problem

Fungi are eukaryotic organisms that compromise a large group of micro- and macroorganisms including mushrooms, yeast, and molds. Most fungi are saprophytic and rely on other organisms for their supply of nutrients. Due to their saprophytic nature, fungi play an important role in the ecosystem by recycling nutrients, but fungi can exploit all organic materials. This ability can lead to damage of food, wood, textiles, and buildings (8). Further, some fungi have the ability to invade living organisms and cause infections (14). Thus, fungi can impact human health.

It is estimated that up to 1.5 million species may exist within this kingdom, but only a fraction of these fungi are involved in human disease. Fungi involved in human disease are classified as either pathogenic or non-pathogenic. The pathogenic fungi are able to colonize the host and establish active infections (6). On the other hand the non-pathogenic fungi cause hypersensitivity reactions such as allergy and asthma without necessarily establishing an infection (15). In fact, in 2007, nearly half of the weekly requests received by the National Institute for Occupation Safety and Health concerned work-related asthma and mold exposure (16). In one study, workers in a water-damaged building reported numerous respiratory symptoms(17). After relocation from the water-damaged work environment most symptoms resided, suggesting that the molds acted as an irritant but did not grow and establish an infection. Other studies have found associations between mold exposure and the development of asthma (18-23).

In this dissertation the environmental molds *Aspergillus versicolor* and *Cladosporium cladosporioides* are used to establish a model of chronic exposure to non-pathogenic molds. We

will establish some of the mechanisms by which these two molds interact with the immune system and induce distinct pulmonary inflammation. In particular we will explore the contribution of these two molds to the development of allergic asthma. In Chapter 2 we will establish our model of chronic mold exposure to individual molds, and in Chapters 2 and 3 we will look at the role of the innate immune system in the development of the immune response. Chapter 4 explores the role of beta-glucans in modulating the immune response. Finally, in chapter 5 we establish a model of co-exposure.

1b. Life Cycle and Structure of Fungi

Fungi are able to reproduce both sexually and asexually. Most fungi are adapted to dispersal through the air, and production of spores promotes dispersal. Both sexual reproduction and asexual reproduction ultimately leads to the release of spores (14, 24). The asexual spores, also termed conidia, are produced in specialized structures that arise from hyphae (14). After being released into the air, spores can remain suspended for long periods of time due to their small size (14). Upon landing in a suitable place spores can germinate and give rise to hyphae (25), and when a large mass accumulates the collection of hyphae are termed mycelium (8). Figure 1 is a cartoon displaying the sexual and asexual life cycle of molds resulting in production of mold spores (5). Production of a high number of spores and aerial distribution is critical for the propagation of fungi. Therefore, it is not surprising that spores are regarded as the most prevalent fungal particles in the air (14), and that normal respiration can lead to spore deposition in the lungs on a daily basis.

Figure 1. The life cycle of molds (5). Spores are released into the air and upon landing in a suitable place begin to germinate. Spores can be released from sexual and asexual structures.



Fungal spores tend to have thick walls to protect the organism from the environment but growing hyphae have relatively thin walls. However, both spores and hyphae have similar compositions of the cell wall. The major components of fungal cell walls are polysaccharides (26). Most of the polysaccharides found in fungal cell walls is composed of chains of beta-1,3glucans. Beta-1,3-glucans serve as the main structural constituent and all other components are covalently attached to the beta-1,3-glucans. As a result beta-1,3-glucans are required for proper cell wall formation (26).

In addition to the carbohydrate components of cell walls, fungal cell walls also have surface glycoproteins (26), most often found in the outer layer of spore cell walls (14). Hydrophobins are a common glycoprotein found on the surface of fungal cell walls, and along with melanin are involved in the production of a structure termed the rodlet layer (12, 27). The outer rodlet layer can cover up the underlying beta-glucans (28), and provides protection to the fungi from environmental exposures such as ionizing radiation (29). In addition the rodlet layer the outer wall of fungi can contain lipids and an extracellular mucus layer to allow the fungus to attach to the host/substrate (14). Figure 2 is an electron micrograph of the cell wall of *C*. *cladosporioides*, displaying the outer rodlet layer and inner beta-glucan layer of mold spores (12).

Upon germination of spores the outer cell wall is shed and beta-glucans are uncovered (30). This change in display of the inner cell wall between spores and hyphae likely impact the host response to fungi. In Chapter 2 we will demonstrate that our two molds of interest, *A. versicolor* and *C. cladosporioides*, have significant differences in surface availability of beta-glucans, and this affects the type of immune response that develops. Then, in Chapter 4 we will demonstrate that altering the exposure of beta-glucans in mold spores can modulate the immune response to the spores.

Figure 2. Ultrastructure of the mold *C*. *cladosporioides* (12). The outer opaque layer is the rodlet layer and the inner layer is where beta-glucans are found.



2. Molds in Disease

2a. Pathogenic molds

While our focus is on two non-pathogenic fungi, the majority of mechanistic murine studies use pathogenic fungi. Since all fungi have similar cell wall compositions, understanding the interactions of the pathogenic fungi with the immune system may provide insights into how the immune system recognizes the non-pathogenic fungi associated with asthma.

Table 1 outlines examples of the four different types of fungal infection; 1) cutaneous, 2) subcutaneous, 3) systemic, and 3) opportunistic mycoses (6). The severity of disease caused by infection with these fungi ranges from mild for the cutaneous fungi to severe with the systemic and opportunistic fungi (6). Two of the best-studied fungi in murine models from this table are

Aspergillus fumigatus and *Candida albicans*, but several of the other systemic and opportunistic are also studied in murine models. Mechanistic studies of these species are described in the next section. In this dissertation we will show the non-pathogenic fungi we are studying also use several of the mechanisms used by the pathogenic fungi to interact with the immune system.

[•] Table 1. Features of important fungal diseases (6).

| Table 48–1 Features of Important Fungal Diseases | | | | |
|--|---------------------------|-------------------------------|---|---------------------------|
| Туре | Anatomic Location | Representative Disease | Genus of Causative Organism(s) | Seriousness of Illness |
| Cutaneous | Dead layer of skin | Tinea versicolor | Malassezia | 1+ |
| | Epidermis, hair, nails | Dermatophytosis (ringworm) | Microsporum, Trichophyton, Epidermophyton | 2+ |
| Subcutaneous | Subcutis | Sporotrichosis | Sporothrix | 2+ |
| | | Mycetoma | Several genera | 2+ |
| Systemic | Internal organs | Coccidioidomycosis | Coccidioides | 4+ |
| | | Histoplasmosis | Histoplasma | 4+ |
| | | Blastomycosis | Blastomyces | 4+ |
| | | Paracoccidioidomycosis | Paracoccidioides | 4+ |
| Opportunistic | Internal organs | Cryptococcosis | Cryptococcus | 4+ |
| | | Candidiasis | Candida | 2+ to 4+ |
| | | Aspergillosis | Aspergillus | 4+ |
| | | Mucormycosis | Mucor, Rhizopus | 4+ |

1+ = not serious, treatment may or may not be given; 2+ = moderately serious, treatment often given; 4+ = serious, treatment given especially in disseminated disease.

2b. Non-pathogenic molds

Spores are ubiquitous in the outdoor air and are found indoors in water damaged buildings (14). The respiratory tract is exposed to many spores throughout life, and while many of these spores may not cause infection, they can activate the immune system and cause hypersensitivity reactions. In one study, workers in a water-damaged building reported numerous respiratory symptoms. Upon investigation of the building several different species of mold were found, including *Aspergillus versicolor*, *Penicillium spp.*, and *Stachbotrys chartaum* (17). After relocation from the water-damaged work environment most symptoms subsided, suggesting that the molds acted as an irritant but did not grow and establish an infection. Other studies identify molds as possible contributors to the development and activity of asthma in both adults and children (18-20, 31, 32). Several studies have implicated *Alternaria alternata* in asthma (20-23). However, other molds such as *Cladosporium spp.*, *Aspergillus spp.*, and *Penicillium spp*. have also been associated with asthma (18-20, 32). Exposure to these molds does not necessarily result in colonization of the lungs, but they can still induce significant pathology as evidenced by their association with asthma.

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, patient studies are often complicated by the fact that most patients are exposed to multiple allergens, including several molds. Furthermore, it is difficult to do mechanistic studies in human patients. Effective interventions to reduce mold exposure cannot presently be designed since they may or may not successfully target the relevant mold or immunological pathways. In this study, we characterized the responses to two common mold species that have been implicated in asthma and attempt to define the immunological

mechanisms through which these molds induce inflammation. Our goal is to define if either of the two molds can contribute to murine asthma and through what mechanisms.

<u>3. Immune Responses to Molds</u>

3a. Innate Immune Response

The first part of the immune system to be activated upon infection is the innate immune system. It is composed of phagocytic cells, soluble proteins that bind the infectious agent, antimicrobial peptides, and cells that recognize the invading pathogen and phagocytize the microbe. Phagocytic cells, such as dendritic cells, can activate the adaptive immune system if the infectious agent is not cleared by innate system (33).

3a.i. Innate Immune Cells

3a.i.i. Monocytes/Macrophages

Monocytes and macrophages are phagocytes and play a central role in innate and adaptive immunity. Monocytes are the cells that enter the bloodstream from the bone marrow, and once these cells enter the tissues they mature and become macrophages (34). Macrophages are long lived cells and are the first line of cellular defense against invading microorganisms (33). Macrophages can phagocytize invading pathogens in a non-specific manner, but phagocytosis is much more efficient when receptors on the surface of macrophages are involved (33). Upon engagement of these receptors macrophages become activated, generate of reactive oxygen species (ROS) to kill the invading pathogen, and can secrete several pro-inflammatory cytokines including IL-1, IL-6, IL-12, and TNF α (33). Secretion of cytokines leads to further activation of the immune system. Several of these receptors are pattern recognition receptors, which will be discussed in the next section.

In the lungs the major resident phagocytic cells are alveolar macrophages (35). Due to their small size, fungal spores are often deposited in the small airways where alveolar macrophages readily ingest the spores (36). The uptake of *A. fumigatus* spores by macrophages is similar to the uptake of bacteria, and leads to killing of the spores (35). Macrophages can produce ROS in response to uptake of *A. fumigatus spores* (37), and this is likely one mechanism by which macrophages can kill fungal spores. Further, beta-glucans, such as zymosan, induce the activation of macrophages as measured by production of TNF α (38), indicating a role for pattern recognition receptors. Thus macrophages likely have an important role in clearing fungi from the lungs and activating the immune response through production of the cytokine TNF α . *3a.i.ii. Neutrophils*

Neutrophils are also a phagocytic cell, but unlike macrophages are not found residing in tissues. Instead neutrophils are short-lived cells that are recruited to site of inflammation (34). Neutrophils are potent killers and generate a respiratory burst, which is a combination of toxic oxygen metabolites, proteases, phospholipases, lysozyme, and antimicrobial peptides. This burst is effective at killing bacteria, fungi, and even some viruses (33). In fact neutrophils are essential for controlling fungal infections. Neutrophils are recruited to the lungs after inoculation with *A*. *fumigatus* spores and form aggregates around the spores (39). Mice deficient in the chemokine receptor necessary for recruiting neutrophils to tissues or in the gp91^{phox} gene, a gene necessary for the respiratory burst, cannot prevent germination of *A. fumigatus* spores, and thus have an increased fungal burden (39). Further, depletion of neutrophils prior to or up to 3 hours after infection with *A. fumigatus* is associated with high mortality in mice (40). Taken together, these studies suggest a critical role for neutrophils early in the immune response against fungi. *3a.i.iii. Dendritic Cells*

Dendritic cells are a third type of phagocytic cell and are related to monocytes and macrophages (34). In the lungs dendritic cells form an elaborate network that is able to constantly sample the airways for inhaled antigen. Figure 3 is a diagram of different subsets of dendritic cells that can be found in the lungs (11). In the diagram the cells are divided into those that are present in the lung during steady state conditions and the dendritic cells that are recruited during inflammation. The surface markers CD11c and CD11b are used to classify the different sets of dendritic cells found in the lung. As shown in Figure 1 there are three steady state subsets of dendritic cells: conventional dendritic cells (CD11c+CD11b+), plasmacytoid dendritic cells (CD11^{DIM}CD11b-), and alveolar macrophages (CD11c+CD11b-). The inflammatory dendritic cells are also classified by expression of CD11c and CD11b (11).

Figure 3. Dendritic cell subsets in the lungs (11). (a) The subsets of dendritic cells present during steady state conditions, and (b) the inflammatory dendritic cells that are recruited during inflammatory states.



Lung dendritic cells are the sentinels of the airways. They reside in the airways in an "immature" state and are specialized to take up inhaled particulates and antigens. Upon activation through triggering of pattern recognition receptors or through cytokines secreted by epithelial cells, dendritic cells migrate to the lymph nodes where they can activate naïve T cells (11). In order to activate T cells dendritic cells must up regulate MHCII to be able to present antigen and up regulate the co-stimulatory molecules CD80 and CD86 (41). Additionally, dendritic cells produce large amounts of cytokines that can further activate T cells and other arms of the immune response (42). The ability of dendritic cells to activate T cells allow them to bridge innate and adaptive immunity.

Studies suggest that each of the different subsets of dendritic cells in the lungs have separate functions. Antigen presentation by plasmacytoid dendritic cells appears to promote induction of pulmonary tolerance (43-45), and CD11c+CD11b+ dendritic cells induce Th2 and Th17 responses in the lungs (43, 46, 47). Historically, the CD11c+CD11b+ cells were classified as myeloid dendritic cells (47), but more recently a separate subset was identified that express the markers Ly6C and the high affinity IgE α chain receptor (Fc α RI α) (46). Dendritic cells additionally have a role in presenting antigen to and activating differentiated T cells in the bronchus associated lymphoid tissue (BALT). The majority of dendritic cells found in the BALT three days after infection were CD11c+CD11b+ (48). Older studies did not look for expression of the inflammatory dendritic cell markers so it is not clear whether these older studies where studying conventional or inflammatory dendritic cells. However, transfer of antigen loaded dendritic cells differentiated by GM-CSF that express both CD11c+CD11b+ dendritic cells into the lungs can induce murine asthma (43). It is likely that both the conventional and inflammatory dendritic cells are capable activating T cells.

As a phagocytic cell, dendritic cells are important for the uptake of fungi. One study reported a novel protrusion of dendritic cells termed the "fungipod" that is formed after contact with yeast cell walls, specifically *Candida parapsilosis* (49), suggesting that the fungipod is an important mechanism for phagocytosis of fungi. Further, dendritic cells are able to discriminate between the yeast form and hyphae of *C. albicans*. Phagocytosis of yeast occurred through coiling phagocytosis, but phagocytosis of the hyphae occurred through zipper phagocytosis (50). Dendritic cells have also been shown to transport the conidia of A. fumigatus to the lymph nodes. Similar to C. albicans, the dendritic cells were able to discriminate between spores and hyphae. Additionally, after mice were exposed through the airways to spores and hyphae were found in the draining lymph nodes. The dendritic cells found in the lymph nodes had up regulated MHCII, CD80, and CD86 (51). Not only do dendritic cells take up fungi and transport fungi to the lymph nodes, they have also been shown to produce inflammatory cytokines in response to fungi. Human dendritic cells can produce IL-12 and IL-23 in response to A. fumigatus (52). Murine dendritic cells also produce IL-12 and IL-23 after exposure to A. fumigatus (53). These studies indicate that phagocytosis of fungi by dendritic cells is important for initiation of adaptive immune responses against fungi.

3a.ii. Pattern Recognition Receptors

The innate immune system is able to discriminate self from non-self through the use of pattern recognition receptors (PRRs). These are a family of receptors that recognize common motifs on infectious agents called pathogen associated molecular patterns (PAMPs) (54). There are two families that are important for the recognition of fungi, the C-type lectin receptors (CLRs) and the Toll Like Receptors (TLRs) (1, 9). Table 2 lists the PRRs and their ligands that have been shown to be important in recognition of fungi (9).

^a Table 2. Fungal pattern-recognition receptors (9). PRRs are classified as either soluble (secreted), or membrane bound.

| Location | PRR | Selected fungal PAMP(s)/ligands ^a |
|----------|-------------------------------------|---|
| Soluble | Surfactant protein A ^b | Mannan, glycoprotein A ^{Pc} |
| | Surfactant protein D | Mannan, β-glucan |
| | Galectin-3 | β-1,2-mannosides ^{Ca} |
| | Mannose binding lectin ^b | Mannan |
| | Pentraxin-3 | Galactomannan ^{Af} |
| | Ficolin-2 | β-1,3 glucan, GlcNAc |
| | Complement | Fungal surfaces, mannan, β-1,6 glucan |
| | C-reactive protein | Phosphocholine |
| Membrane | TLR1 ^b | Unknown |
| | TLR2 ^b | Mannan, phospholipomannan ^{Ca} , GXM ^{Cn} |
| | TLR4 | Mannan, O-mannan ^{Ca} , GXM ^{Cn} |
| | TLR6 ^b | Unknown |
| | TLR9 ^b | Fungal DNA |
| | Dectin-1 ^b | β-1,3-glucan |
| | Dectin-2 | α-mannan |
| | CR3 | β-glucan, mannan, BAD-1 ^{Bd} , HSP60 ^{Hc} , GXM ^{Cn} |
| | DC-SIGN | Mannan, galactomannan ^{Af} |
| | Mannose receptor | Mannan, N-mannan ^{Ca} , GlcNAc, glycoprotein A ^{Pc} |
| | CD14 | Mannan, GXM ^{Cn} |
| | FcyR | Mannan, GXM ^{Cn} |
| | Mincle | α-mannose |
| | SCARF | Mannan, β-glucan |
| | CD36 | β-glucan |
| | CD5 | β-glucan |
| | Very late antigen-5 | Cyclophilin A ^{Hc} |
| | Langerin | Mannan, β-glucan |
| Unknown | Unknown | Chitin |

*Fungal-specific PAMPs are indicated in superscript as follows: Pc, P. carinii; Ca, C. albicans; Af, A. fumigatus; Cn,

C. neoformans; Bd, B. dermatitidis; Hc, H. capsulatum. Other abbreviations: GlcNAc, N-acetyl-D-glucosamine (monomer); GXM, glucuronoxylomannan.

^bPolymorphisms in encoding genes that have been associated with susceptibility to fungal infections in humans.

3a.ii.i. C-type Lectin Receptors

The CLRs are a family of proteins that are characterized by the presences of one or more C-type lectin-like domains (CTLDs), and are made up of the following receptors: Dectin-1, Dectin-2, MINCLE, DC-SIGN, CD161, CLEC-1, and the mannose receptor (1). Figure 4 is a schematic of the CLR family. While these receptors contain conserved domains they are a functionally diverse group (55). However, they have been broadly separated into two groups based on signaling motifs. The "Dectin-2" family of CLRs are associated with the Fc receptor γ chain (Fc γ R), and this chain signals through the immunereceptor tyrosine-based activation motif (ITAM) to promote recruitment of SYK (55). On the other hand, the "Dectin-1" family of CLRs contain an ITAM like domain in the intracellular portion that can promote recruitment of SYK (55). All of these receptors recognize carbohydrate moieties and have been implicated in the recognition of fungi (1), and thus likely have important roles to play in determining the host response to fungi.



Figure 4. Schematic of the CLR family of receptors (1).

One of the best-studied CLRs is Dectin-1, and it was first identified as a novel gene expressed in murine epidermis derived dendritic cells (56), and is also expressed on macrophages/monocytes, neutrophils, and dendritic cells (56-58). Additionally, the human form of Dectin-1 is functionally equivalent to the murine form, and is expressed on monocytes/macrophages, dendritic cells, neutrophils, and eosinophils (59, 60). Shortly after identification of Dectin-1, it was discovered that Dectin-1 binds to Zymosan, which is composed of mainly beta-1,3-glucans, (58, 61). It was also shown that Dectin-1 was able to bind to the yeasts *Saccharomyces cerevisiae* and *Candida albicans* (61). Additional characterization of the Dectin-1 established that Dectin-1 exclusively binds beta-1,3-glucans and the minimum length required is 10-11 gluco-oligomers (62), and the conserved carbohydrate recognition domain (CRD) is the binding site for beta-glucans (63).

Examination of the intracellular portion of Dectin-1 identified a novel ITAM-like motif that is necessary to phosphorylate Syk and induce downstream signaling such as recruitment of CARD9 (64, 65). Further, localization to lipid rafts and recruitment of SYK and PLC γ 2 are required for optimal activation of the downstream pathway of Dectin-1 (66). Dectin-1 has also been shown to activate the inflammasome (67, 68). Figure 5 is a schematic of the downstream signaling pathways Dectin-1 employs (3). The identification of Dectin-1 as a receptor for a major component of fungal cell walls, beta-1,3-glucans, indicate that it is likely an important innate immune receptor for recognition of fungi. This idea is supported by the fact that Dectin-1 is expressed on cells of the innate immune system. Figure 5. Downstream signaling pathways of Dectin-1. Dectin-1 signals through Syk-dependent and -independent pathways. Signaling through Syk can occur through CARD9, PLCγ2, and the inflammasome (3).



Dectin-1 has an important role in the function of innate immune cells. The process of phagocytosis and fungal killing appear to be dependent upon recognition of fungi by Dectin1. Uptake of *A. fumigatus* by murine and human macrophages was inhibited by pre-incubation with laminarin, a known inhibitor of Dectin-1, or by blocking antibodies against Dectin-1. Furthermore, this response was dependent upon the exposure of beta-glucans since resting conidia with little beta-glucan exposure were not as readily taken up as swollen conidia that had greater exposure of beta-glucans (37, 69). Additionally, production of reactive oxygen species (ROS) by human macrophages was dependent on Dectin-1 and surface exposure of beta-glucans (37). These studies suggest Dectin-1 has a role in killing and clearance of *A. fumigatus*.

Accordingly, mice deficient in Dectin-1 have an increased burden of *A. fumigatus* in their lungs (70). Further, the demonstration that recognition of mold spores was dependent upon exposure of beta-glucans indicates that the structure of the fungal cell wall can modulate immune response.

Phagocytosis and killing of invading microbes is not the only function of macrophages. Several studies found that Dectin-1 is critical for production of TNF α from macrophages and dendritic cells in response to Zymosan or *A. fumigatus* (30, 37, 71-73). Similar to the findings with phagocytosis, one study also demonstrated that production of TNF α was also dependent on exposure of beta-glucans since only germinating conidia could induce TNF α production (73). The production of several other pro-inflammatory cytokines, such as IL-1 α , IL-1 β , IL-6, and IL-23 were also dependent on Dectin-1 (53, 73, 74). The induction of IL-1 β after exposure to fungi is likely due to the ability of Dectin-1 and it's downstream adaptor molecule, Syk, to activate the inflammasome(67, 68). Additionally, stimulation of dendritic cells with the Dectin-1 agonist, zymosan, induces maturation through upregulation of co-stimulatory molecules is also dependent on Dectin-1 (66). The importance of Dectin-1 in production of pro-inflammatory cytokines and maturation of dendritic cells suggests that Dectin-1 has an important role in directing the development of the immune response to fungi.

Both murine studies and studies in patients deficient in Dectin-1 indicate it is critical in the development of the immune response against fungi. Two different groups independently generated mice deficient in Dectin-1 (75, 76). Although both groups found that Dectin-1 was required for control of fungal infection, their results differed by the fungal infections that Dectin-1 had a protective role. Taylor et al. demonstrated that mice deficient in Dectin-1 had disseminated *C. albicans* infection and a decreased survival rate after infection with *C. albicans*

(57). Further, murine macrophages from Dectin-1 deficient mice had decreased uptake of Zymosan, a decrease in production of ROS, and decreased production of TNF α in response to *C. albicans*. There was also decreased production of TNF α in Dectin-1 deficient macrophages after exposure to *A. fumigatus* (57). Another study using these same mice demonstrated that Dectin-1 was also required for protection against pulmonary infection with *A. fumigatus* (70). Thus, the mice generated by Taylor et al. suggest that Dectin-1 is critical for protection from systemic infections with *C. albicans* and *A. fumigatus*. However, the study by Saijo et al. demonstrated that Dectin-1 was necessary for protection from *Pneumocystis carinii* infection but not *C. albicans* infection. This study also found that there was no difference in production of cytokines from macrophages, but Dectin-1 deficient macrophages did demonstrate decreased ROS production (76), and thus would be less efficient at killing fungal spores.

The differences in results concerning *C. albicans* could be explained by differences in the strain used or differences in dose. Taylor et al. used a dose that was 5 times less than that used by Saijo et al. It is possible that the dose Saijo et al. used overwhelmed the immune system in both wild-type and Dectin-1 deficient mice and therefore no difference was observed. Another explanation could be differences in that strains of mice used. Saijo et al. used mice that were backcrossed for 8 generations to either a BALB/c or C57BL/6J background, but Taylor et al. used mice on a mixed 129/Sv and C57BL/6 background. It is possible that in different genetic backgrounds Dectin-1 has a different effect on the immune response to fungi. Despite the reported differences between the two groups, it is apparent that Dectin-1 does have an impact on control of fungal infections *in vivo*. The effect of Dectin-1 likely depends on the type of fungus and other genetic factors.

In a family with four women that had recurrent mucocutaneous candiasis a mutation in the Dectin-1 gene was discovered. The Y238X mutation resulted in an early stop codon (77). Monocytes from these patients had decreased production of IL-6 and binding to *C. albicans*, but did not display decreased phagocytosis (77). The authors did not report systemic spread of the fungus, and this is likely because the macrophages still retain normal phagocytic activity, thus preventing systemic spread of the fungus.

However, studies have shown that the Y238X mutation puts hematopoietic stem cell (HSCT) patients at increased risk of developing invasive bronchopulmonary aspergillosis (IBPA) (78, 79). Interestingly, Chai et al. did not find an association between risk of IBPA in HSCT patients and the mutation in Dectin-1, but did find an association between the Y238X mutation and IBPA in non-HSCT patients (80). These studies in patients demonstrate that Dectin-1 is critical for protection against systemic infection with *A. fumigatus*, especially in immunocompromised patients.

Dectin-1 is clearly protective against the opportunistic fungal pathogens *C. albicans* and *A. fumigatus* (57, 70, 76-80). It is likely the protective mechanisms are due to the role Dectin-1 has regulating phagocytosis and secretion of pro-inflammatory cytokines from phagocytic cells. However, it is not known if any of the non-pathogenic fungi that are involved in asthma signal through Dectin-1. We will show in Chapter 2 that the non-pathogenic fungi binds to Dectin-1, and that Dectin-1 has a protective role in murine asthma development after exposure to *A. versicolor*. However, the other mold we are studying, *C. cladosporioides*, is able to induce murine asthma independent of Dectin-1. Further, in Chapters 3 and 4 we demonstrate that Dectin-1 has role in dendritic cell production of the cytokines IL-6, IL-23, and TNF α . *3a.ii.ii. Toll Like Receptors*

The TLRs are the best-characterized family of PRRs. To date there are 10 functional human TLRs and 12 functional murine TLRs (81). The Toll gene was first discovered in *Drosophilia*, and after identification of human IL-1R functional similarities began to emerge between the two genes (82). It is now known that the TLRs and IL-1R all contain an intracellular Toll-interleukin 1 receptor (TIR) domain required for downstream signal transduction. The TIR domain recruits a combination of the adaptor molecules MyD88, TRIF, TRAM, and TIRAP depending on the TLR (81). All TLRs with the exception of TLR3 use MyD88, and TLR4 can signal through both TRIF and MyD88 (54). Figure 6 is a schematic of TLR signaling in using the IL-1R and TLR4 as examples (4). The TLR family can recognize a host of microbial components that are derived from various invading pathogens and the host (4). Importantly, several TLRs are known to recognize fungal PAMPs, and therefore have a role in shaping the immune response to fungi.



Figure 6. The TLR signaling pathway. Upon ligation with their respective ligands TLRs recruit the adaptor molecules MyD88, TIRAP, TRAM, or TRIF to initiate downstream signaling which results in transcription of inflammatory genes (4)

A few studies have found a role for the TLR pathway in production in pro-inflammatory cytokines in response to pathogenic fungi. The adaptor protein, MyD88, is important for phagocytosis of *C. albicans* and production of TNF α , but not *A. fumigatus* (83). The absence of TLR1 and TLR2 causes an impaired pro-inflammatory cytokine response (IL-6, TNF α , IL-12p40) but there is no effect on mortality (84). However, several studies examining the role of the TLR pathway focus on cooperation between TLRs and Dectin-1. These demonstrate synergy between TLRs and Dectin-1 in production of TNF α , IL-6, IL-23, and IL-12p40 by using specific

ligands for the receptors (71, 72, 74). Further, TLR2 and Dectin-1 cooperate to mediate efficient phagocytosis. Dectin-1 is necessary for binding of conidia to macrophages and TLR2 is necessary for efficient uptake of the conidia (85). These studies demonstrate the TLR family have important roles in the recognition of fungi and often cooperate with Dectin-1.

There are a few studies examining the role of TLRs in mold induced asthma, but these studies sensitize the mice with mold protein extracts systemically and then challenge the mice through the airways with conidia (86, 87). TLR9 negatively regulates cytokine production in *A*. *fumigatus* induced murine asthma but TLR9 deficient mice have an increased fungal burden after challenge with conidia (86). In a similar model of *A*. *fumigatus* induced asthma, TLR6 had a protective role in the development of murine asthma (87). However, the use of protein extracts to systemically sensitize mice is not a good model of how sensitization and exposure to molds occurs in human patients. Furthermore, these studies use a known pathogenic mold to induce allergic responses. It is not known how many of the other non-pathogenic molds associated with asthma interact with the TLR family. In Chapter 3 we will demonstrate in a model of chronic pulmonary mold exposure, the TLR pathway is protective from development of murine asthma induced by *A. versicolor*. The TLR adaptor protein, MyD88, is critical for the development of Th17 responses, and mice deficient in MyD88 develop Th2 responses and murine asthma after exposure to *A. versicolor*.

3b. Adaptive Immune Response to Molds

The innate immune response is a critical first line of defense against invading pathogens, and sets the stage for the development of the adaptive immune response. An adaptive immune response develops as a second line of defense if the innate response fails to remove the infectious agent in a timely manner (33). Initiation of the adaptive immune response begins with activation
of naïve T helper (CD4+T) cells in secondary lymphoid tissues by dendritic cells. Once activated the T cells can migrate to sites of inflammation, recognize antigen, and perform their effector functions (34). Figure 7 is a scheme of the currently recognized subsets of effector T helper (Th) cells. These encompass Th1, Th2, Th17, Th9, and Th22 cells (13).

Classically the field of immunology has recognized the TH1/Th2 paradigm, but more recently other the three other subsets have been recognized. Th1 cells protect against intracellular pathogens and have an important role in several autoimmune diseases. Differentiation of Th1 cells from naïve T cells requires IL-12, IFNy, and the transcription factor T-bet (88). They produce large amounts of IFNy, and this is important for induction of opsonizing and complement activating antibodies. Additionally, IFNy activates macrophages and increases their microbicidal activity (88). Th2 cells mediate immunity against parasitic infections, and are key mediators of allergies and asthma. Historically, IL-4 was recognized as the critical cytokine for T cells differentiation (88), but more recently other cytokines such as IL-25, TSLP, and IL-33 were shown to contribute to the development of Th2 cells (89). In addition, the transcription factor GATA3 is necessary for Th2 cell differentiation (88). They produce IL-4, 5, 9, 13, and 25. The effector functions of these cytokines include inducing B cells to produce IgE, recruiting eosinophils, and inducing smooth muscle cell contraction. Recently, a subset of Th cells that produce IL-9 have been identified as Th9 cells. Even though IL-9 was considered a Th2 cytokine, these cells do not produce any other Th2 cytokines (90). Th9 cells, like Th2 cells, have a role in development of allergic diseases and clearance of helminth infections (90). It was shown that IL-4 and TGFβ could induce T cells that produce IL-9 (91). PU.1 appears to be the critical transcription factor for differentiation of Th9 cells, but several other transcription factors have also been suggested to have a role in differentiation (90). Th17 cells mediate immunity

against extracellular bacteria and fungi, and like Th1 cells, are key players in several autoimmune diseases. The induction of Th17 cells from naïve T cells requires TGFB, IL-6, and IL-1β, and IL-23, and the transcription factor RORyT (92-95). They produce IL-17a, IL-17F, IL-21, and IL-22. IL-17A and IL-17F both recruit and activate neutrophils. Similar to identification of Th9 cells, another subset of Th cells that produce IL-22 but not any other Th17 cytokines are now classified as Th22 cells (96). IL-6, TNF α , and the aryl hydrocarbon receptor appear to be important for differentiation of these cells (13). Th22 cells may be critical for skin homeostasis since they appear important for keratinocyte proliferation and migration (97). Finally, a sixth set of effect CD4+ T cells are the regulatory T (Treg) cells. These cells are not listed in the schematic in Figure 6. Treg cells express CD25 and the transcription factor Foxp3. Treg cells can be divided into two main categories, those that are thymically derived and those that are induced in the periphery (98). Peripherally induced Treg cells are also known as adaptive Treg. cells and can be further divided into Th3 cells, TR1 cells, and induced Treg cells. All three express Foxp3 (98), indicating that Foxp3 is also necessary for the function of adaptive Treg cells. They have a critical role in regulating immune responses and maintaining self-tolerance (88).

Figure 7. Distinct Subsets of CD4+ Effector T Cells (13). The cytokines and transcription factors necessary for differentiation into distinct effecter T cell subsets are depicted. Tregs are missing from this schematic diagram.



Although there are currently six different subets of Th cells that recognized, only three of the subsets are induced in response to fungi. In general Th1 and Th17 cells are thought to be protective, and induction of Th2 cells leads to susceptibility to fungal infections and development of allergic responses (36). In fact protein extract from *Alternaria* has potent Th2 adjuvant activity, and induces OVA specific T cells to produce IL-4 and IL-13 (99). Hyphae or heat-killed spores of *A. fumigatus* also induce Th2 responses, but in the same studies live spores induced Th1 responses (51, 100). Thus, the immune system appears to differentiate between exposures that can or cannot lead to potential infection, and induce protective responses against infectious exposures. Further, induction of Th17/Th1 responses instead of Th2 responses after

Cryptococcus neoformans infection in mice promotes clearance of the fungi (101). These studies support the notion that Th1/Th17 responses are protective from infection.

Studies in patients also point to a protective role of Th17 responses. Patients with deficiencies in IL-17 or the IL-17 receptor have an increased risk of developing mucocutaneous candidiasis (102-104). Although fungi are able to induce multiple types of T helper cell responses, induction of Th17 responses appears to be most protective against infection.

The induction of a Th17 response by fungi appears to be dependent on Dectin-1. Dectin-1 can inhibit Th1 responses and promote Th17 responses (105), and mice deficient in Dectin-1 produce less IL-17 and are susceptible to invasive aspergillosis (70). Activation of dendritic cells with the Dectin-1 agonist, curdlan, converts Treg cells to IL-17 producers (106). Further, induction of Th17 responses after *C. albicans* is dependent on SYK and CARD9, both of which are downstream of Dectin-1 (107). These studies indicate that Dectin-1 has a critical role in induction of Th17 responses after exposure to fungi. Further they suggest that Th1 responses may not be as important as thought since Dectin-1 is inhibitory against Th1 development and Dectin-1 is critical for protection from fungal infections.

While it is clear that a Th17 response is protective against infection with pathogenic fungi, the type of T cell responses induced by the non-pathogenic fungi is unclear. Extract from the environmental mold *Alternaria* acts as a Th2 adjuvant (99), and some studies use extract from the pathogenic mold *A. fumigatus* to sensitize mice and induce Th2 responses (108-110). However, the use of extract is not the same as using mold spores since the cell wall is obliterated in making extracts and antigens in spores not otherwise seen by the immune system may be present in the extract. As a result, there is a need to develop models of chronic exposure to the non-pathogenic molds in order to delineate the mechanisms through which they induce or do not

induce asthma. In Chapter 2 we develop a model of chronic mold exposure and demonstrate that two environmental molds induce very distinct inflammatory phenotypes. Further we show, the development of a Th17 phenotype is dependent on in Dectin-1 and exposure of beta-glucans in Chapters 1 and 2. In Chapter 3 we demonstrate that the MyD88 pathway is also important for the development of Th17 responses. Additionally, in Chapters 2 and 3 we demonstrate that in the absence of Dectin-1 signaling, either in deficient mice or because of minimal exposure of beta-glucans, a Th2 response and murine asthma develop.

4. Asthma and Mold

As previously mentioned, there are an estimated 1.5 million species of fungi (25). Due to the aerial nature of fungal spores they are regarded as the most prevalent fungal particles in the air (14), and that normal respiration can lead to spore deposition in the lungs on a daily basis. Many molds have an impact on human disease, either as pathogenic molds that have the ability to grow within the human body and establish infection (6, 14), or as non-pathogenic molds that have the ability to induce hypersensitivity reactions without necessarily establishing an active infection (15).

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 (111), and in another pediatric cohort persistent childhood asthma was associated with sensitivity to mold (22). In adult asthma, allergic sensitization to molds was associated with more hospital and ICU admissions due to asthma (112, 113). These studies underscore the importance of mold exposure as a public health concern and the relevance of mold exposure to asthma.

4a. Classification of Asthma

Prevalence rates of asthma have doubled in the past two decades and asthma is now considered the most common chronic disease of childhood (114, 115). Asthma is a chronic inflammatory disorder caused by an overactive immune response, and is characterized clinically by variable airway obstruction (116). Histologically there is chronic airway inflammation including infiltration of the airways with lymphocytes, eosinophils and mast cells, goblet cell hyperplasia, and thickening of the submucosa (117). However, asthma is a heterogeneous disease, and consists of several overlapping phenotypes that can be classified by inflammatory subtype (118, 119) or severity (120-122).

The classical paradigm of asthma is atopic asthma characterized by Th2 cells and eosinophilia (123). Asthma can be classified as eosinophilic and non-eosinophilic. Noneosinophilic asthma is generally characterized by the presence of neutrophils (124). Further, asthma can be divided into four groups of asthma based on the type of inflammation: 1) eosinophilic asthma, 2) neutrophilic asthma, 3) mixed granulocytic (mixture of eosinophils and neutrophils), and 4) paucigranulocytic asthma (absence of inflammatory cells) (118). Neutrophilic inflammation in asthmatic patients is associated with chronic airway obstruction as measured by FEV_1 (125), and patients with mixed granulocytic asthma had greater use of inhaled corticosteroids (126). Patients with neutrophilic asthma demonstrated less improvement after steroid use than patients without neutrophilic inflammation (119). Additionally, patients with a mixed granulocytic phenotype demonstrate poor asthma control and lower lung function than that of patients with an eosinophilic phenotype (127). These studies indicate that neutrophils are an important component of non-eosinophilic asthma, and neutrophilic asthma is an important sub-group of asthma.

While severe asthma is recognized as a subset of asthma, it is unclear how to define this subset. The assessment of asthma severity often includes symptom scores, use of beta-agonists, bronchial responsiveness to methacholine, spirometric measurements of lung function, and biomarkers of inflammation (128). Some studies assert that severity assessment in asthma should include the consumption of health care resources (129), and the level of current clinical control (130). In 2009 the World Health Organization (WHO) proposed a definition of severe asthma, with three subgroups: 1) untreated severe asthma, 2) difficult to treat severe asthma, and 3) treatment resistant severe asthma (122). According to several of these suggestions on how to classify severe asthma, neutrophils are associated with severe asthma. As listed above neutrophilic or mixed granulocytic asthma is associated with decreased lung function, worse asthma control, and increased corticosteroid use.

In Chapter 5 we explore the contribution of mold to severe asthma. In human subjects we assess asthma severity using symptom scores (131), in the murine we studies we define severe asthma based on AHR was previously published in other murine models (43, 132).

4b. Immune Basis of Asthma

4b.i. Th2 Response

It is hypothesized that Th2 cytokine responses contribute to the pathogenesis of asthma (117). As previously mentioned, Th2 cells produce the cytokines IL-4, 5, 9, 13, and 25 (88). Il-4 is known to have a crucial role in differentiation of Th2 cells (117), Dendritic cells are the key antigen presenting cell for induction of Th2 cells, but the production of IL-4 by cells such as basophils and eosinophils is also important in the lymph nodes (46). Additionally, data indicates a role for TSLP, IL-25, and IL-33 produced by epithelial cells, basophils, and non-B non-T cells (also termed nuocytes) in the induction of Th2 cells (10). Figure 8 is a schematic of the role

epithelial cells, basophils, non-B non-T cells, and dendritic cells have differentiation of naïve T cells into Th2 cells (10).

[•] Figure 8. The role of epithelial cells, dendritic cells, basophils, and non-B non-T cells on Th2 differentiation (10).



Early studies determined depletion of CD4+ T cell prior to lung challenge with allergen prevented AHR and development of experimental murine asthma (133), and more specifically transfer of Th2 clones into the lungs of mice induces symptoms of murine allergic airway disease (134). Inhibition of IL-4 by either antibody or gene targeting prevents the development of allergic airway disease (135-137). One of the most important roles for IL-4 in asthma is induction of IgE synthesis from B cells (138), but IL-4 also has a role in recruitment of eosinophils to the airways (136). Further, IL-4 is critical for the induction of and expansion of Th2 cells during sensitization but does not have a role during the challenge phase (135). In addition, Th2 cells from IL-4 deficient mice are still able to induce AHR (139). These studies indicate IL-4 is critical for induction of asthma but has a minimal role in the effector phase of the asthma response.

Another Th2 cytokine, IL-13 has a critical role during the challenge phase of murine asthma. Blockade of IL-13 after allergen sensitization but before challenge greatly reduces development of AHR and allergen induced goblet cell hyperplasia. Additionally, administration of recombinant IL-13 is sufficient to AHR, eosinophilia, and production of IgE (140, 141). Mice deficient in IL-13 that have been backcrossed to the BALB/c background for at least 10 generations also display reduced AHR, eosinophils, IgE, and Th2 cells (142). As an important mediator of the effector phase of asthma, IL-13 has an important role in the development of subepithelial fibrosis, mucus hypersecretion, and induction of airway hyperresponsiveness (143). Thus IL-13 is essential for the effector phase of asthma, but IL-4 is essential for initiation of the Th2 response.

Additionally the cytokine IL-5 is involved in Th2 responses and asthma (117). Mice deficient in IL-5 do not develop eosinophilia or AHR, but reconstitution of these mice with

recombinant IL-5 restored airway eosinophilia and AHR (144). Administration of a neutralizing antibody against IL-5 also abolished eosinophilia and AHR (145). These studies indicate IL-5 has a role in recruitment of eosinophils to airways during an allergic response. Further the recruitment of eosinophils may have an effect on development of AHR.

Figure 9 is a schematic the roles of Th2 cytokines in the pathogenesis of asthma (2).

Figure 9. The effects of Th2 cytokines on mast cells, eosinophils, and B cells in the pathogenesis of asthma (2).



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As alluded to above, several other cells are involved in the allergic response induced by secretion of Th2 cytokines. B cells secrete IgE that then binds to mast cells. When allergens bind to and cause cross-linking of IgE pre-formed mediators such as histamine, cytokines, and leukotrienes are released from mast cells. The release of these mediators results in the symptoms of early response to allergen challenge such as micro vascular leakage, increased mucus production, and bronchoconstriction. Further mast cells can produce cytokines that contribute to late phase responses of asthma (117). Additionally, eosinophils likely induce AHR through the

release of several mediators including cytokines, eosinophils cationic protein, major basic protein, and lipid mediators (134). Finally, natural killer T cells (NKT) cells have also been implicated in allergic asthma. Mice deficient in NKT cells fail to develop AHR after antigen challenge (146), and mice exposed to lipid antigen, the ligand for NKT cells, develop AHR and eosinophilia (147).

Despite the amount of research delineating the contribution of the Th2 response to development of asthma there are very few studies examining the role of mold spores in induction of Th2 responses. As previously stated, the studies that examine the role of molds in asthma and Th2 responses use protein extracts (99, 108-110), and there is a need for a model of chronic mold exposure. In Chapter 2 we demonstrate that the environmental mold *Cladosporium cladosporioides* induces robust Th2 responses and murine asthma. In Chapter 3 we demonstrate that the induction of Th2 responses is dependent on surface exposure of beta-glucans. In the discussion we propose a hypothesis for a potential mechanism for induction of Th2 responses by *C. cladosporioides*.

4b.ii. Th17 Responses

The Th2 response in allergic asthma has been clearly established, but more recently studies have focused on the role of IL-17 and Th17 cells as contributors to asthma (148-150). As previously stated Th17 cells produce IL-17a, IL-17F, IL-21, and IL-22 (88). IL-17 is a pro-inflammatory cytokine produced by T cells, NKT cells, neutrophils, and macrophages which an important role in protection from extracellular pathogens and in chronic disease (151-154), and is important for recruitment of neutrophils (88). The induction of Th17 cells from naïve T cells requires TGFβ, IL-6, and IL-1β, and IL-23 (92-95).

IL-23, IL-17, and Th17 cells have been implicated in asthma, although the exact contribution to asthma remains controversial. IL-23 can enhance the development of an allergic phenotype. Overexpression of the IL-23 receptor enhances eosinophilic inflammation, but mice deficient in IL-23 have attenuated eosinophilic inflammation (155). Dendritic cells from mice that are more susceptible to development of severe AHR produce more IL-23 than those from mice that develop mild AHR, and this increased production of IL-23 is associated with a mixed Th2/Th17 response (43). Additionally, Th17 cells can enhance antigen specific T cell activation (156), and the effects of Th2 cytokines induced murine asthma (157). In fact, IL-13 and IL-17A administration directly to the airways results in a synergistic induction of AHR (132). IL-17 increases mucin production (158) and smooth muscle contraction (159), as does IL-13 (143), and these effects may explain the observed synergy. Thus, these studies suggest that IL-17 can enhance Th2 responses, and may lead to more severe experimental murine asthma.

Neutrophilic asthma is also associated with IL-17. Overexpression of IL-17F in the lungs of mice challenged with Ova results in recruitment of neutrophils and development of more robust AHR than mice that have normal expression of IL-17F (160), and blockade of IL-17 reduces the number of neutrophils recruited to the airways (161). Importantly, steroid treatment has no effect on Th17 responses or neutrophilia (162), indicating that the IL-23/Th17 cell axis may be an important target in steroid resistant asthma. Neutrophilic asthma is often considered severe, and IL-17 is likely the key mediator of neutrophilic asthma.

Patient studies also support a role for IL-17 in asthma. Increased IL-17 in induced sputum from asthmatics is associated with increased bronchial hyperreactivity to methacholine (150). Increased numbers of neutrophils in sputum from asthmatics is associated with persistent, steroid resistant asthma and increased expression of IL-17 (148). Further, increased expression

of IL-17 in sputum is associated with decreased lung function (163). Both eosinophils and T cells that produce IL-17 were found in the sputum of patients with severe asthma but not control subjects (149, 164, 165). Interestingly, two studies identified a unique subset of T cells that produce both IL-4 and IL-17A in the blood of asthmatic patients (166, 167). These studies clearly indicate that IL-17 is increased in asthmatic patients, and suggest a role for IL-17 in severe asthma.

However, the role of IL-17 and Th17 cells in asthma still remains controversial. Schnyder-Candrian et al. reported that IL-17 is a negative regulator of established asthma. IL-17 was required during the sensitization phase of asthma, but recombinant IL-17 administered during the effector phase of asthma attenuated the murine asthma phenotype (168). Further, macrophages that produce IL-17 are required for initiation of asthma in mice (153). IL-17 has been shown to regulate influx of granulocytes into the airways, specifically blockade of IL-17 increases IL-5 levels and recruitment of eosinophils while decreasing recruitment of neutrophils (161). The negative regulatory effect of IL-17 on established asthma is likely through decreased recruitment of eosinophils (168). These studies indicate that IL-17 may be necessary for initiation of asthma but can negatively regulate established asthma by decreasing recruitment of eosinophils. Thus, more studies on the kinetics of Th17 responses in asthma may clarify the role of IL-17.

As previously described, fungi are potent inducers of Th17 responses, and these responses are important for protection against fungal infections. Considering the evidence also supporting a role for Th17 responses in asthma it is surprising that the role of IL-17 in mold-induced asthma has yet to be explored. In Chapter 2 we demonstrate that the environmental mold *A. versicolor* induces a predominantly Th17 response accompanied by neutrophils.

Blockade of IL-17 results in decreased neutrophils and increased eosinophils, likely due to increased expression of IL-5. The increase in airway eosinophilia is correlated with increased AHR. Further, in Chapter 5 we demonstrate that when mice are exposed to *A. versicolor* and house dust mite extract there is a synergistic response. Both Th17 and Th2 cells characterize this response.

4c. Mold-Induced Asthma

4c.i. Patient Studies

As previously stated in 2007, nearly half of the weekly requests received by the National Institute for Occupation Safety and Health concerned work-related asthma and mold exposure (16). In one study, workers in a water-damaged building reported numerous respiratory symptoms that were relieved upon relocation to a non-water damaged building. Upon investigation of the building several different species of mold were found, including *Aspergillus versicolor*, *Penicillium spp.*, and *Stachbotrys chartaum* (17). This study underlines the effects mold exposure can have on respiratory health.

Mold exposure is significantly associated with asthma in multiple studies. Homes in Baltimore, where childhood asthma is common, had a significantly higher mold burden than the average burden of U.S. homes (169), and severely asthmatic children in Detroit lived in homes with a higher mold burden than children without asthma (19). Additionally, mold exposure is associated with wheezing in young children. Asthma is difficult to diagnose in infants and toddlers, and wheezing is often used as a risk factor for development of asthma (170). In infants mold exposure was a significant risk factor for recurrent wheezing (171), and in the same cohort was associated with a positive asthma predictive index at age 3 (172). Other cohorts of infants have also reported that exposure to molds was associated with respiratory symptoms and an

increased risk for developing asthma (111, 170, 173). In a cohort of adult patients, increased ambient fungal spore counts were associated with new asthma symptoms (18). Taken together these studies clearly demonstrate a correlation between mold exposure and development respiratory symptoms/asthma. However, it is difficult to determine which molds are contributing to asthma since there are often multiple molds in the home that patients are exposed to.

Examining the correlation between sensitization to mold and asthma may be a better indicator of which molds are contributing directly to asthma since sensitization indicates an immune response to mold. Several studies have demonstrated associations between sensitization to molds and asthma. In a longitudinal cohort, sensitization to Alternaria at age 6 was significantly associated with having chronic asthma at age 22 (174). Adults in New York City that were sensitized to molds were more likely to have asthma than those not sensitized. The pattern of sensitization was important; adults sensitized to *Cladosporium* or *Alternaria* were more likely to have asthma than adults sensitized to Aspergillus (175). Another study found that sensitization to A. fumigatus and C. herbarum was associated with new adult-onset asthma (176). Finally, Alternaria sensitization in children raised in a dessert environment was associated with both development of new asthma and persistent asthma (22). These studies demonstrate an important association between sensitization to specific molds and asthma. Further, some studies suggest that some molds are more likely to be associated with asthma than other molds, but not all of the studies are in agreement (175). One study suggests that sensitization to Aspergillus is not important in asthma, but another study suggests that it is (176). Thus, even sensitization data may not identify the causative molds.

Importantly, mold exposure and sensitization is associated with severe asthma. Skin prick test positivity to *Alternaria* was associated with respiratory arrest in children with asthma (177),

and increases in daily asthma admissions at a children's hospital are associated with a rise in ambient fungal spore counts (178). Severe asthma is also associated with fungal sensitization in an adult population (179). Recurrent hospital admissions are associated with sensitization to multiple allergens, including multiple molds (112), and patients admitted to the ICU due to asthma are more likely to have a positive skin test for one or more fungal allergens (113). Hospital admissions for asthma increase during months when mold spores counts peak (31, 112), and increase after thunderstorms when spore counts are high (20, 32, 180). Deaths from asthma increase during months when mold spore counts are high (181). Taken together, these studies suggest a potential role for molds in the development of severe asthma. Further examination of the role molds have in severe asthma may lead to better interventions and treatments, such as the use of anti-fungal agents, which have been show to have efficacy in asthmatic patients with sensitization to molds (182).

Despite the large amount of evidence associating mold exposure with severe asthma, there are few studies examining how molds contribute to severe asthma. Goplen et al. suggest that exposure to multiple allergens, ragweed, house dust mite, and mold, can overcome the development of tolerance and induce prolonged inflammation in a murine model of asthma. Further, the authors claim the addition of mold extract is critical for establishing the features of chronic asthma (183). Thus, this study points to a potential role of mold exposure in the development of severe asthma. However, there is still a need to develop models to study severe asthma, and in particular the contribution of mold spores, not mold extract, to severe asthma.

In Chapter 5 we demonstrate in a mouse model that mice exposed to both *A. versicolor* spores and HDM develop higher AHR and have significantly more cells in the airways than mice exposed to either HDM of *A. versicolor* spores alone. The increased AHR is associated with a

mixed Th2/Th17 response. Further, co-exposure to two molds spores, *A. versicolor* and *C. cladosporioides*, induces synergy in development of AHR and airway inflammation. Thus, we demonstrate that co-exposure to the Th17 inducing mold, *A. versicolor*, and either the Th2 inducing allergen HDM or the mold *C. cladosporioides*, results in development of more severe asthma than exposure to either antigen alone.

4c.ii Murine Models

A number of murine models exist to study mold-induced asthma. In general there are two different methods of fungal inhalation. Models employ either involuntary aspiration, where animals are forced to inhale suspension of fungal spores, or inhalation of aerosolized spores. Further, some studies employ systemic sensitization to fungi before pulmonary exposures. Often, systemic sensitization is done with fungal extracts as opposed to intact fungi, and occasionally are used as adjuvants to in an Ova model of murine asthma (184).

Mold extracts are potent inducers of Th2 responses. Protein extract from *Cladosporium herbarum*, *Penicillium chrusogenum*, and *Alternaria alternata* act as potent allergic adjuvants and increase the production of Ova specific IgE (99, 185). *A. alternata* extract induces dendritic cells to promote differentiation of Ova specific CD4+ T cells into Th2 cells (99). Additionally, *Penicillium chrysogenum* extract can induce eosinophilia and IgE dose dependent manner (186). Further, when compared to HDM extract, *P. chrysogenum* induced more robust allergic responses (187). *A. fumigatus* extract is commonly used in murine models of asthma, and systemic sensitization with *A. fumigatus* extract followed by challenge with spores induces chronic AHR, goblet cell hyperplasia, and fibrosis (109). Similar murine models have provided important insights into allergic mechanisms (108, 110). These studies provide models to study allergic mechanisms, but not interactions with mold spores.

There are murine models of mold-induced asthma that use primarily mold spores rather than protein extracts. *Alternaria alternata* and *Cladosporium herbarum* spores induce robust murine asthma characterized by increased IgE, AHR, airway eosinophilia, and production of the Th2 cytokines IL-4, IL-5, and IL-13 (188, 189), and *Aspergillus niger* spores induce murine asthma that is dependent upon fungal proteases (190). These murine studies indicate that *A*. *fumigatus*, in addition to several non-pathogenic molds, are potent inducers of murine asthma. However, only a few of these studies provide mechanistic insight into how molds contribute to the development of asthma.

There is a need to study the immunological mechanisms of mold-induced asthma in a physiological model. In the environment we are exposed to whole fungi through the airways, and not protein extracts. It is highly unlikely that we are sensitized systemically unless molds enter through an open wound. For the most part, sensitization to molds in patients occurs through the airways. In fact, chronic administration of *A. fumigatus* spores, with no use of mold extracts, results in a neutrophilic phenotype in BALB/c mice and an eosinophilic phenotype in C57Bl/6 mice (191). Murine exposure to *A. fumigatus* spores without prior systemic sensitization to mold extracts induces a drastically different inflammatory phenotype, dependent on the strain of mice used, than models using systemic sensitization with protein extracts. The discrepancy in models using exclusively fungal spores versus models that use mold extracts induces a function of the strain of mold sto asthma.

In Chapter 2 we develop a model of chronic mold exposure using intra-tracheal (IT) exposure to either *A. versicolor* or *C. cladosporioides* spores. Our model has several advantages including (a) utilization of whole spores rather than mold extracts; (b) continuous IT exposure over a period of 3 weeks without use of adjuvants or prior sensitization; and (c) the dose of 10^{6}

spores was based on air sampling data from the Cincinnati Childhood Allergy and Air Pollution Study (192), thus ensuring the use of a physiological dose.

5. Cincinnati Childhood Allergy and Air Pollution Study

The Cincinnati Childhood Allergy and Air Pollution Study (CCAAPS) is a wellcharacterized prospective birth cohort in the greater Cincinnati area. The central goal of CCAAPS is to determine how exposure to allergen and air pollution impacts the development of atopic respiratory disease in a high-risk cohort. The prospective cohort study recruited newborns from birth records from 2001-2003. All subjects had to have at least one parent with allergen sensitization and history of allergy symptoms in order to be enrolled in the study (193). Every year the children are seen by a physician and their parents fill out a questionnaire adapted from the International Study of Allergies and Asthma in Childhood (ISSAC) (194), and undergo skin prick testing to determine sensitization to allergens, and at age 7 they will undergo pulmonary function test (PFTs) and venipuncture. All homes were inspected at the time of enrollment. Each home underwent visual inspection for mold and water damage in addition to collecting dust samples to determine the quantity and type of allergens present in the homes. Dust samples were assayed for mold spores, house dust mite, cat, dog, and cockroach. Homes were then classified as either more moldy homes or less moldy homes (19). Additionally, outdoor pollen and fungal spore counts were continuously measured at 3 stations during the first year (195). Since there were no differences in fungal and pollen counts between the stations, counts were obtained from the Hamilton county Department of Environmental Services for the calendar years 2001-2009. A complete description of the sampling location and procedures is available elsewhere (196, 197).

Data from CCAAPS has found significant associations between mold exposure and respiratory health in children enrolled in the study. Children living in homes determined to have significant mold and water damage were more likely to develop upper respiratory infections (URIs) during the first year of life than children living in homes with little or no visible mold and water damage (198). Further, children living in the homes with significant mold and water damage were more likely to have recurrent wheezing (171). At age 3 children who lived in homes with high visible mold during infancy were significantly more likely to have a positive asthma predictive index than those who lived in homes with minimal visible mold (172). The investigators of CCAAPS developed a relative moldiness index to measure moldiness based on mold-specific quantitative PCR from dust samples rather than visible mold (199). This relative moldiness index was used to determine the impact of mold exposure on development of asthma at age 7 on patients in the cohort. It was found that an increased mold burden during the first year of life was associated with increased incidence of asthma at age 7 (200), indicating that mold exposure is a risk factor for development of asthma in the Greater Cincinnati area.

6. A. versicolor and C. cladosporioides

A. versicolor and *C. cladosporioides* were chosen for this study for three reasons. 1) They are commonly found in moldy homes in the CCAAPS cohort (199), 2) are different in size and shape, and 3) have different cell wall compositions (7). The difference in size and shape could potentially mean that the spores are distributed in different areas of the lung, and the cell wall composition may have an effect on interactions with the immune system. Table 3 shows the size of *A. versicolor* and *C. cladosporioides* spores in comparison to several other common mold spores (7). *A. versicolor* spores are spherical in shape and are significantly smaller than *C. cladosporioides* spores. *C. cladosporioides* spores are ellipsoidal in shape and the size listed in

Table 3 is the geometric mean of the width and length. Table 4 shows the average beta-glucan contents as measured by the Limulus Amebocyte lysate (LAL) assay of A. versicolor and C. *cladosporioides* in comparison to other common mold spores (7). The total beta-glucan content of C. cladosporioides is greater than that of A. versicolor. This is not due to the differences in spore size since the beta-glucan content when adjusted for spore size and volume is still greater in C. cladosporioides spores. Despite having differences in beta-glucan contents, it is not clear if the beta-glucans in both spores are exposed. As described in the first section of the introduction, mold spores often have surface glycoproteins (26) that are found in the outer layer of spore cell walls (14). Furthermore, a study of the cell wall of C. cladosporioides revealed that there is also a large amount of melanin found in the outer cell wall (12). It is therefore possible that despite the fact that C. cladosporioides has a greater beta-glucan content; the beta-glucans are not as exposed as those in A. versicolor. We demonstrate that this is indeed the case in Chapter 2. Table 3. Spores sizes (7).

| Fungal Species | Spore Size (µm) | Spore Surface Area (µm²) | Spore Volume (µm³) |
|--------------------|-----------------|--------------------------|--------------------|
| A. alternata | 17** | 643 | 1021 |
| A. chevalieri | 4.5 | 64 | 48 |
| A. flavus | 5 | 76 | 62 |
| A. penicillioides | 4.5 | 62 | 46 |
| A. unguis | 3 | 30 | 16 |
| A. versicolor | 3 | 28 | 14 |
| A. pullulans | 12** | 628 | 786 |
| C. cladosporioides | 5.6** | 83 | 60 |
| C. herbarum | 5** | 59 | 36 |
| E. nigrum | 28 | 2463 | 11494 |
| P. brevicompactum | 3 | 29 | 15 |
| S. chartarum | 7.5** | 148 | 172 |
| W. sebi | 4 | 49 | 32 |

Mean of n=30 spores for each fungal species. **Geometric mean of width and length for ellipsoidal spores. Spores of Aspergillus species, E. nigrum, P. brevicompactum and W. sebi are spherical. Spores of A. alternata, A. pullulans, Cladosporium species, and S. chartarum are ellipsoidal.

| Fungal Species | pg/Spore | pg/Spore Surface Area* | pg/Spore Volume** |
|--------------------|----------|------------------------|-------------------|
| A. chevalieri | 0.22 | 3.46 | 4.61 |
| A. flavus | 0.03 | 0.39 | 0.48 |
| A. penicillioides | 0.01 | 0.16 | 0.22 |
| A. unguis | 0.005 | 0.16 | 0.32 |
| A. versicolor | 0.03 | 0.91 | 1.84 |
| A. pullulans | 3.76 | 5.98 | 4.78 |
| C. cladosporioides | 0.25 | 3.00 | 4.19 |
| C. herbarum | 8.66 | 146.33 | 240.54 |
| E. nigrum | 241.57 | 98.08 | 21.02 |
| P. brevicompactum | 0.21 | 7.02 | 13.81 |
| S. chartarum | 0.004 | 0.03 | 0.33 |
| W. sebi | 0.12 | 2.40 | 3.72 |

Table 4. Beta-glucan content of spores (7).

* pg/µm²x10⁻³. ** pg/µm³x10⁻³.

†(1-3)-β-D-glucan content is an average of 4-6 experiments, CV=27%.

Further, 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 suggest that patients exposed to *C. cladosporioides* were more likely have elevated IgE titers (201) and develop asthma symptoms as compared to patients exposed to *A. versicolor* (175, 201). In addition, murine studies indicated that *A. versicolor* protein extract had weak Th2 adjuvant activity as compared to protein extract from *A. alternata* (99). However, to our knowledge there are no murine studies with *C. cladosporioides*. Taken together these studies indicate an important association between *C. cladosporioides* and development of asthma, but suggest that *A. versicolor* is not a potent inducer of asthma.

7. Summary

Fungi and molds are ubiquitous microorganisms that we are exposed to on a constant basis. The spores of fungi are easily aerosolized and can be found in high concentrations. The spore walls are made up of carbohydrates including beta-glucans, and sometimes an outer cell wall composed of hydrophobins and melanin. Only a few fungi are able to cause infectious

disease, but continuous exposure to non-pathogenic fungal spores may lead to respiratory irritation and asthma.

Several pathogenic fungi have been used to study the interactions of fungi with the immune system. From these studies Dectin-1 was identified as the receptor for beta-glucans and thus shown to be important for immune response to fungi. Additionally, the TLR and MyD88 pathway has been implicated in immune responses to fungi, often through cross talk with Dectin-1. The development of adaptive Th17 responses to fungi is downstream of Dectin-1, and Th17 responses are protective against fungal infections. However, it is not known if these immune responses are elicited in response to non-pathogenic fungi that are involved in asthma.

Asthma is a chronic inflammatory disorder of the airways and is characterized clinically by variable outflow obstruction. It is a heterogeneous disease and can manifest from a very mild disease with few exacerbations to persistent severe disease. Classically, asthma has been described as an allergic disease with a Th2 basis. More recently, Th17 cells and IL-17 have been implicated in severe asthma but the role still remains controversial.

Many studies have implicated mold exposure in the development of asthma and in particular severe asthma. Specifically in the Greater Cincinnati Area data from CCAAPS demonstrated that a high mold burden during the first year of life is significantly associated with development of asthma at age 7. However, the immune mechanisms underlying to mold-induced asthma is unclear.

A. versicolor and *C. cladosporioides* are two common molds identified in CCAAPS and are of different sizes and cell wall composition.

Based on the previously published data presented in this introduction we hypothesize

that A. versicolor and C. cladosporioides will induce distinct immune responses in the lungs

of mice through differential activation of the innate and adaptive immune systems.

In Chapters 2-4 we will test the hypothesis with the following aims. Figure 10 is a schematic of the aims we will test in chapters 2-4.

Figure 10. Aims tested in dissertation.



Aim 1: Characterize the immune responses induced by A. versicolor and C. cladosporioides.

We will develop a model of chronic mold exposure by exposing mice to a suspension of 10^6 mold spores by aspiration 3 times a week for 3 weeks. Two days after the last exposure we will measure AHR by Flexivent, determine the number and type of cells in the bronchoalveolar lavage fluid (BALF). We will also use intracellular staining for cytokines to determine the frequencies of Th2 and Th17 subsets in the lungs. We predict that *A. versicolor* and *C. cladosporioides* will induce distinct pulmonary inflammatory phenotypes.

<u>Aim 2:</u> Determine the role of innate and adaptive immune pathways in response to mold exposure.

We will expose Dectin-1 and MyD88 deficient mice to our model of chronic mold exposure and analyze the inflammatory phenotype as described in aim 1. We will also examine cytokine production in dendritic cells from these mice to determine the role that Dectin-1 and TLRs have on cytokine production in dendritic cells. We hypothesize that Dectin-1 will have a critical role in the development of mold-induced inflammation.

<u>Aim 3:</u> Define differences in the cell wall composition.

We will examine the beta-glucan exposure in *A. versicolor* and *C. cladosporioides* using antibodies against beta-glucans to visualize the beta-glucans by immunofluorescence. We will also measure binding to recombinant Dectin-1 to determine the relative levels of beta-glucan exposure. Further, we will determine the influence on the immune response by altering the exposure of beta-glucans. We predict that alterations in surface of beta-glucans will modulate the type of immune response that develops.

Based on the data presented in Chapter 2 and asthma literature, we further hypothesized that the Th17 response induced by *A. versicolor* would synergize with Th2 responses to induce severe asthma. We test this hypothesis in Chapter 5 by exposing mice to both *A. versicolor* and HDM. We also analyzed a patient population at Cincinnati Children's Hospital that are sensitized to HDM or HDM and molds to determine the impact on asthma severity scores. Figure 11 is a schematic of this hypothesis.



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Dectin-1 and IL-17A suppress murine asthma induced by *Aspergillus versicolor* but not *Cladosporium cladosporioides* due to differences in beta-glucan surface exposure¹

Running Title: Role of Dectin-1 in mold induced asthma.

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Abstract

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, while 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 to 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 beta-glucans on their surface and were able to bind Dectin-1. Thus, the host response to C. cladosporioides was IL-17A- and Dectin1-independent, while Dectin-1 and IL-17A-dependent pathways were protective against the development of asthma after exposure to A. versicolor.

Introduction

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 pathogensis, 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 Occupation Safety and Health concerned work-related asthma and mold exposure (3). In the Cincinnati Allergy and Air Pollution Study (CCAAPS) longitudinal birth cohort, mold exposure was associated with increased incidence of wheeze in infants (4), increased risk of developing asthma at age 3 (5), and was a predictor of asthma development at age 7 (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 CCAAPS 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 suggest that patients exposed to *C. cladosporioides* were more likely have elevated IgE titers (8) and develop asthma symptoms as compared to patients exposed to *A. versicolor* (8, 9). In addition, murine studies indicated that *A. versicolor* protein extract had weak Th2 adjuvant activity as compared to 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 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 the pathogenesis of asthma and can synergize with IL-13 to induce AHR (11, 12), but IL-17 has also been shown to be a negative regulator of established murine asthma (13). On the other hand, 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 mucocutanous candidiasis (15, 16). Furthermore, IL-17 is down stream of Dectin-1, the receptor for beta-glucans (17-22). Accordingly, deficiency in Dectin-1 has been associated with chronic mucocutanous candidiasis (23), and invasive pulmonary aspergillosis in hematopoietic transplant patients (24). Mice deficient in Dectin-1 develop fatal invasive pulmonary aspergillosis and do not clear Candidia as efficiently as wild type mice (25-27). Further, 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 moldinduced 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 predominately 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 generously 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 (CCHMC). All procedures were preformed 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 CCHMC.

Preparation of mold spores and spore challenge model.

Aspergillus versicolor isolate 52173 and Cladosporium cladosporioides isolate 6721 (American Type Culture Collection, Manassas, VA) were grown on malt extract agar for 4-6 weeks 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 $2x10^{7}$ spores/ml. Mice were lightly anesthetized with Isoflurane and exposed to saline, 10^{6} *A. versicolor* spores, or 10^{6} *C. cladosporioides* spores in 50ul intratracheally (IT) 3 times a week for 3 weeks, and then were assessed 2 days after the last exposure. The mold spore dose was chosen based on the concentrations of these molds found in homes of children in the CCAAPS 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 antibody were provided by Amgen, Inc (Seattle, WA). The rat M210 antibody was chimerized by fusing the variable region domain of the rat IgG to mouse IgG1 constant domains. Antibodies were administered IP 3 days a week, 250 ug/mouse, starting the day of the first spore administration. The dosing was determined from a previous publication using these antibodies (12).

In order to quantify mold burden, mice were exposed IT to 5x10⁶ of either *A. versicolor* or *C. cladosporioides*, mice were sacrificed 24 hours later, and the left lobe of the lung was fixed in paraformaldhyde, dehydrated, paraffin embedded, and cut into 5µm sections. Sections were stained with Grocott's methanmine 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 400x using a Nikon 90i Fully Automated Upright MicroscopeSystem with a Nikon DS-Ri1 12 Mega-Pixel Color Camera. The software used to aquire the images was Nikon Elements Quantitative AnalysisSoftware. Images were color enhanced using Adobe Photoshop CS4.

Assessment of asthma phenotype.

Airway hyperresponsiveness (AHR²) to methacholine (acetyl-b-methylcholinechloride; Sigma, St. Louis, MO) was assessed in mice using flexiVent, a mechanical ventilator system (SCIREQ, Montreal, PQ, Canada) (34). Mice were anesthetized with ketamaine, 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 1.25s, 2.5Hz volume-driven

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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 Ptr = RV + EV + Po, and Ptr = tracheal pressure, V = volume, E = elastance, Po is a constant and C = 1/E. The maximum R value and minimum C value with a coefficient of determination of 0.9 or greater (as determined by the flexiVent software) was used to determine the dose-response curve.

Bronchoalveolar lavage fluid (BALF³) collection and analysis

BALF was collected and analyzed as previously described (30). Briefly, the lungs were lavaged with 1 ml PBS + 2mM 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 2ml of RPMI 1640 containing Liberase DL (0.5 mg/ml; Roche Diagnostics, Indianapolis, IN) and DNAse I (0.5 mg/ml; Sigma, St Louis, MO). Lung cells were passed through a 70µm cell strainer with a syringe rubber and the strainer washed with 5ml of RPMI+DNAse I media. Cells were centrifuged and resuspended in 2ml of RPMI before counting. Cell viability was confirmed by trypan blue exclusion.

Approximately 10⁶ lung cells were transferred in to a V-bottom 96 well plate on ice,

centrifuged and resuspended in PBS containing FcBlock (2.4G2 mAb, Biolegend, San Diego, CA) after stimulation with PMA and Inomycin in the presence of Brefeldin A (eBioscience, San Diego, CA) and monensin (eBioscience, San Diego, CA). Cells were labeled with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit according to manufacturer's instructions (Invitrogen by Life Technologies, Carlsbad, CA). T cells were stained with CD3e-FITC and CD4-Pacific Blue (BioLegend, San Diego, CA). Intracellular staining for IL13-PE (eBioscience, San Diego, CA) and IL17A-AF647 (BioLegend, San Diego, CA) was done using reagents from eBioscience (San Diego,CA). All flow cytometric data were acquired using the FACS Canto 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, supported in part by NIH AR-47363, NIH DK78392 and NIH DK90971. Data was analyzed used 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), and purified with RNeasy MiniElute Kit (Qiagen, Valencia, CA). Reverse transcription was preformed using iScript Reverse Transcription Supermix (BioRad, Hercules, CA). Real-time PCR was done using the SYBR Green Master Kit and a LightCycler 480 instrument (Roche Diagnostics, Indianapolis, IN). Murine hypoxanthine phosphoribosyltransferase (HPRT) 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'.

Beta-glucan staining and Dectin-1 pulldown.

Binding to recombinant murine Dectin-1 (rDectin-1) (R&D Systems, Minneapolis, MN) was determined by a pull down assay. Mold spores were labeled with Alexa Fluor 488 dye (Life Technologies, Grand Island, NY), and 400,000 spores were incubated with rDectin-1 at a concentration of 1nM. The rDectin-1 was pulled down with magnetic nickel beads (Life Technologies, Grand Island, NY). 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-glucan on the spores, 10⁶ spores were incubated with murine anti-beta-glucan antibody, primary antibody, (Biosupplies, Bundoora, Australia) at a concentration of 1ug/ml in 1% goat serum in 0.01% PBS Tween-20, and the secondary antibody was goat anti-mouse DyLight 594 (BioLegend, San Diego, CA). Images were acquired at 1000x using a Nikon 90i Fully Automated Upright MicroscopeSystem with a Nikon DSQiMc Camera, with Z-sequence taken every 0.5um. Nikon Elements Quantitative AnalysisSoftware was used to aquire the images.

Statistical analysis.

All statistical analyses were done using PRISM software (GraphPad Software Inc., La Jolla, CA). Statistical significance was assessed using one-way ANOVA followed by a Neuman-Kohls post-test or a two-way ANOVA followed by a Bonferroni post-test 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 (Figure 1). C. cladosporioides induced robust AHR (Fig. 1a), while A. versicolor induced only mild AHR (Fig. 1A) suggesting that these two molds have distinct effects on lung physiology. While 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 predominately neutrophils, while C. cladosporioides induced recruitment of both eosinophils and neutrophils (Fig. 1b). Further, only mice exposed to C. cladosporioides had increased levels of total IgE (Fig 1c). In order to determine whether the observed differences were dose-dependent, mice were exposed to 10^4 vs. 10^6 spores. Mice exposed to 10^4 spores had less airway inflammation as compared to 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, while 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 (Supp Fig. 1). Thus, our data indicates that C. cladosporioides induces allergic inflammation even at a lower exposure level.

No difference in distribution of mold spores in the lungs.

In order 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 hours 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 antigen presenting cells 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 (Supp. Fig. 2), indicating that both molds were cleared from the lungs by 6 weeks after a single exposure. Thus, the molds did not actively grow and establish an infection in the lungs. These data indicate 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 (ICCS). *A. versicolor* induced predominately Th17 cells while *C. cladosporioides* induced predominately Th2 cells (Fig. 3a, b). 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, while *A. versicolor* induced expression of only IL-17. Molds have been reported to induce Th1 responses, however, we did

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not observe significant production of IFNγ (Supp. Fig. 3). Even though we observed fewer Th17 cells in the lungs of *C. cladosporioides* exposed mice than *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 predominately a Th17 response, but the T cell response to *C. cladosporioides* is predominately 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 vs. C. cladosporioides, we investigated the role of IL-17A in our model using a neutralizing antibody against IL-17A. Mice were exposed to A. versicolor or C. cladosporioides as before and also administered 250ug of isotype control or anti-IL17A antibody intraperitoneally over the course of the experiment. Surprisingly, blockade of IL-17A in A. versicolor exposed mice resulted in development of robust AHR compared to animals receiving isotype antibody (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 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 (Supp. Fig 4). Despite a report that IL-17A downregulates eotaxin and goblet cell hyperplasia (13), we did not observe any differences in eotaxin expression (Supp. Fig 4c) or goblet cell hyperplasia (data not shown) in the lungs of isotype versus anti-IL-17A 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.

On the other hand, blockade of IL-17A in *C. cladosporioides* exposed mice resulted in development of higher AHR (Fig. 4a,b). 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 two days after the last exposure we determined the types of T effector cells in the lungs by ICCS. As expected, Dectin-1 -/- mice had fewer Th17 cells in the lungs compared to wild type mice following *A. versicolor* exposure (Fig. 5a, b). In contrast, Th2 cells were increased in the Dectin-1-/- mice compared to 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, c). Our data supports a role for Dectin-1 in development of Th17 responses to mold, and 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.

Since *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 hours 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 and 6a), indicating that our phenotype is not specific to one strain. However, in the absence of Dectin-1 -/- and wild type mice exposed to *C. cladosporioides* were similar in terms of AHR and BALF eosinophilia (Figs. 6c, d). 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 beta-glucans on the spore surface and binding to Dectin-1 when compared to *C. cladosporioides*.

Our data support that Dectin-1 inhibits AHR and allergic inflammation following *A*. *versicolor* exposure while *C. cladosporioides* acts in a Dectin-independent manner. This suggested that *A. versicolor* but not *C. cladosporioides* spores bind to Dectin-1. Yet a previous report from our group revealed that *C. cladosporioides* spores have a greater beta-glucan content than *A. versicolor* spores (32). Therefore, we wanted to determine if there is a difference in the exposure and availability of beta-glucans on the surface of these spores. Staining for betaglucans on *C. cladosporioides* spores indicated that the beta-glucans are only minimally exposed

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at the ends of the spores where they were connected in a growing chain before harvesting the spores (Fig. 7a). On the other hand, *A. versicolor* spores displayed strong staining for beta-glucans throughout the spore surface (Fig. 7a), indicating that the beta-glucans are more exposed in *A. versicolor* than *C. cladosporioides*.

We next determined the binding of each spore to Dectin-1 using recombinant Dectin-1 in a pull down 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 while *C. cladosporioides* does not, and this is likely due to the differences in surface exposure of beta-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 since 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 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 (a) utilization of whole spores rather than mold extracts; (b) continuous IT exposure over a period of 3 weeks without use of adjuvants or prior sensitization; and (c) the dose of 10^6 spores was based on air sampling data from the CCAAPS 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. On the other hand, exposure to *A. versicolor* induced predominately 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. Further, it is unlikely that the observed phenotypes are due to differences in spore size (32) since we observed only quantitative, but not qualitative, differences in the phenotype with a lower exposure level (10^4 spores) as compared to 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 has also been shown to be a negative regulator of established murine asthma (13). While the role of IL-17 is controversial in asthma, it is well established that signaling though 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 studyfound 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 wild type cells (35). Both of these studies suggest a possible role for Dectin-1 in mold-induced asthma. In the current 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 is supported by previous reports suggesting that IL-17 prevents eosinophil recruitment in asthma (13, 34, 35). In addition, arecent study by Werner et al. demonstrated decreased IL-17A and increased airway eosinophils in Dectin-1 -/- mice exposed to A. fumigatus (28) 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 *C. cladosporioides*. The mechanism underlying the distinct inflammatory phenotypes induced by *A. versicolor* and *C. cladosporioides* is likely due to the differences 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 betaglucans in *A. versicolor* and *C. cladosporioides*; with *A. versicolor* having greater beta-glucan exposure and binding to Dectin-1 than *C. cladosporioides*. Our data now add to the current 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 beta-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 beta-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 utilized, which promotes a Th2 response and the development of an asthma phenotype (Fig. 8). In contrast, *C. cladosporioides* spores have minimal surface availability of beta-glucans and do not bind to Dectin-1. As a result *C. cladosporioides*-induced asthma is independent of IL-17A and

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Dectin-1. A recent study suggested that melanin in fungal spores blocks signaling through pattern recognition receptors (36). It is interesting to speculate that *C. cladosporioides* spores contain a substance, which covers the beta-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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Footnotes

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²AHR: airway hyperresponsiveness

³BALF: bronchoalveolar lavage fluid

⁴A. ver: *A. versicolor*

⁵C. clad: *C. cladosporioides*

Figure Legends.

Figure 1: *A. versicolor* and *C. cladsoporioides* induce distinct inflammatory phenotypes. (A) BALB/c mice were exposed IT to 10^6 *A. versicolor* (A. ver⁴) or *C. cladosporioides* (C. clad⁵) 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 are expressed as mean and SEM. N=6-8 mice per group. p<0.05, **p<.01, and ***p<0.001 as compared to saline. #p<0.05, ##p<0.01, and ###p<0.001 as compared to *A. versicolor*.

Figure 2: Similar Distribution of A. versicolor and C. cladosporioides in the lungs of

exposed mice. (A) Silver stained sections from mice 24 hours following a single exposure to 10^6 spores at 400x magnification. Arrows indicate pulmonary antigen presenting cells containing mold spores. Scale bars are 50um (B) Quantification of spores per high power field from silver stained sections. Data are representative of 2 independent experiments and are expressed as mean and SEM. N=3-4 mice per group.

Figure 3: *A. versicolor* induces Th17 cells while *C. cladsoporioides* 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 3 independent experiments are expressed as mean and SEM. N=6-8 mice per group. *p<0.05, **p<0.01, and ***p<0.001 as compared to saline. ##p<0.01 and ###p<0.001 as compared to *A. versicolor*.

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 antibody or anti-IL-17A and 48 hours after the last challenge AHR. (B) Comparison of AHR data at 100mg/ml of methacholine. (C) BALF total and differential counts. (D) Relative levels of mRNA for IL-5 in whole lung. Data are representative of 2 independent experiments and expressed as mean and SEM. N=5-6 mice per group. *p<0.05 and **p<0.01, and ***p<0.001 as compared to saline. #p<0.05, ##p<0.01, and ###p<0.001 as compared to saline. #p<0.05, ##p<0.01, and ###p<0.001 as compared to A.

Figure 5: Increased Th2 cells and decreased Th17 cells in Dectin-1 -/- mice exposed to *A*. *versicolor*. (A) Contour plots and (B,C) 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 3 independent experiments and expressed as mean and SEM. N=4-6 mice per group. *p<0.05, **p<0.01, and ***p<0.001 as compared to saline. ##p<0.01 and ###p<0.001 as compared to *A*. *versicolor* wild type mice.

Figure 6. Dectin-1 protects against allergic inflammation induced by *A. versicolor*. (A, 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 hours later. (B, D) Total and differential counts of BALF for *A. verisoclor* (B) or *C. cladosporioides* (D) exposed mice. Data are representative of 3 independent experiments and expressed as mean and SEM. N=4-6 mice per group. *p<0.05, **p<.01, and ***p<0.001 as compared to saline. #p<0.05, ##p<0.01, and ###p<0.001 compared to exposed Dectin-1 -/- mice.

Figure 7. *A. versicolor* spores have increased beta-glucan surface availability and Dectin-1 binding compared to *C. cladosporioides* spores. (A) Immunofluorescence images of beta-glucan staining taken at 1000x for *A. versicolor* and *C. cladosporioides* spores. Scale bar is

10um. (B) Dectin-1 binding assay for *A. versicolor* and *C. cladosporioides* spores. Data are representative of 2 independent experiments and are expressed as mean and SEM. N=3 samples per group.

Figure 8: Working model. Exposure of beta-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. Beta-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.


A. ver

C. clad



Β.

Α.



Figure 3



IL-13







Β.

C.











Saline

A. ver







100

Α.



Β.





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 IFNg 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

Α.



Β.



Supplemental Figure 2

A. ver





Supplemental Figure 3

Α.



CD4



Supplemental Figure 4





MyD88 signaling contributes to the Th17 response induced by the environmental mold *A*. *versicolor*.

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Abstract

Significant evidence supports a role for mold exposure in the pathogenesis of asthma. Very few mechanistic studies explore the responses to molds that have been implicated in asthma, but there is considerable evidence implicating both TLR and Dectin-1 signaling in innate immune response to the pathogenic molds A. fumigatus and C. albicans. In this study we examined the role of Dectin-1 and MyD88 in dendritic cells in the development of murine asthma. In the absence of Dectin-1 or MyD88 dendritic cells expressed significantly less TNFa, IL-23, and IL-6. Further, this decrease in pro-inflammatory cytokines correlated with a decrease in the Th17 response in vivo. After repeated exposure to A. versicolor spores, mice deficient in MyD88 developed murine asthma including, increased Th2 cells, eosinophilia, and AHR. Wild-type mice developed a predominantly Th17 response characterized by mild AHR and neutrophilia. Further, we observed an increase in Dectin-1 expression in mice deficient in MyD88 after exposure to A. versicolor. Thus both Dectin-1 and MyD88 are important for production of proinflammatory cytokines by dendritic cells, and protect from the development of murine asthma. Our data indicate a role for the TLR pathway as well as Dectin-1 in protection against murine asthma in response to the environmental mold A. versicolor.

Introduction

Sensitization to common environmental allergens results in allergic diseases such as asthma. It is estimated that the prevalence of sensitization to at least one allergen is between 16% to 46% depending on the country, with the highest prevalence rates in developed countries (1). Asthma is characterized histologically by chronic airway inflammation including infiltration of the airways with lymphocytes, eosinophils and mast cells, goblet cell hyperplasia, and thickening of the submucosa. These histological changes lead to the critical symptoms of variable airflow obstruction and airway hyperresponsiveness (AHR) (2). CD4+ T cells are critical mediators of the allergic response. Depletion of CD4+ T cell prior to lung challenge with allergen prevented AHR (3). In particular, T helper type 2 cells (Th2) are responsible for development of asthma. Transfer of Th2 clones into the lungs of mice induces symptoms of murine allergic airway disease (4), and targeting of IL-4, a critical Th2 cytokine, by either antibody or gene targeting prevents the development of allergic airway disease (5-7). While Th2 cells are critical for the development of asthma, it is becoming increasingly clear that there are several other cell types that have important roles in development and maintenance of disease.

As professional antigen presenting cells, dendritic cells play a key role in activation and differentiation of T cells. In the lungs dendritic cells form a complex network beneath the epithelial cells, and can extend their processes between epithelial cells to sample inhaled antigens in the airways (8, 9). In fact a subset of dendritic cells, myeloid dendritic cells, are critical in the development of asthma. Transfer of antigen loaded myeloid dendritic cells to the airways of mice can induce development of murine asthma (10, 11). In order for dendritic cells to activate T cells they undergo a maturation process after taking up antigens (9). Pattern recognition receptors (PRRs) are a family of receptors that recognize common motifs on infectious agents

called pathogen associated molecular patterns (PAMPs) (12). Engagement of PRRs can induce maturation of dendritic cells (9, 13). The toll-like receptors (TLRs) are the best studied of the all PRRs. They consist of 10 and 12 functional receptors in humans and mice (12). Each receptor recognizes a different microbial PAMP and induces signaling through a set of adaptor molecules including MyD88 and TRIF. Upon recruitment of the adaptor molecules, downstream signaling occurs through the NFkB and mitogen-activated protein kinase pathways resulting in transcription of pro-inflammatory genes and activation of antigen presenting cells (14).

More recently the C-type lectin family, in particular Dectin-1, has been getting more attention. Dectin-1, a PRR which recognizes beta-glucans (15), is expressed on macrophages/monocytes, neutrophils, and dendritic cells (16, 17). Examination of the intracellular portion of Dectin-1 identified a novel ITAM-like motif that is necessary to phosphorylate Syk, recruit CARD9, and induce transcription of pro-inflammatory genes and activation of dendritic cells (18, 19). Signaling through Dectin-1 promotes fungal immunity, specifically by inducing dendritic cells to secrete proinflammatory cytokines to polarize naive T cells towards Th17 cells (19-22). Accordingly, deficiency in IL-17A or Dectin-1 has been associated with an increased risk for fungal infections in humans (23-28). Results from animal studies also demonstrate that the absence of Dectin-1 leads to increased susceptibility to fungal infections (29-31) and decreased production of IL-17A (31, 32). Furthermore, our own study recently demonstrated that Dectin-1 is protective from development of murine asthma induced by A. versicolor, likely through induction of downstream Th17 responses (33). A similar study employing a model of A. fumigatus induced murine asthma, also demonstrated that Th17 responses are protective from mold-induced asthma. However, this study demonstrated that the responses were downstream of TLR6 (34). Evidence is beginning to suggest that there is

significant cross talk between Dectin-1 and TLRs in response to pathogenic fungi such as *A*. *fumigatus* and *C. albicans* (35). However, many environmental fungi have been associated with mold-induced asthma (36-40), and there is a need to elucidate the mechanisms underlying this subset of asthma, including interactions with the innate immune system.

In this study we examined the role of Dectin-1 and MyD88 in dendritic cells. We hypothesized that MyD88, similar to Dectin-1, is required for induction of Th17 responses. Our data reveal that dendritic cells lacking Dectin-1 or MyD88 have lower expression of Th17 skewing cytokines after exposure to *A. versicolor*. Further, there is a decrease in Th17 cells and increase in Th2 cells in the lungs of mice lacking MyD88, which correlates with an asthma phenotype.

Materials and Methods

Mice.

Dectin-1 -/- mice have been previously described and were generously provided by Dr. Gordon Brown (29). MyD88 -/- mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and breed and housed in a specific-pathogen free animal facility at Cincinnati Children's Hospital Medical Center (CCHMC). Wild type C57Bl/6 mice were purchased from Harlan Laboratories (Indianapolis, IN), and housed in a specific-pathogen free environment in the animal facility at CCHMC. All procedures were preformed 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 CCHMC. *Preparation of mold spores and spore challenge model.*

A. versicolor isolate 52173 (American Type Culture Collection, Manassas, VA) was grown on malt extract agar for 4-6 weeks 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 $2x10^7$ spores/ml. Mice were lightly anesthetized with Isoflurane and exposed to saline, $10^6 A$. *versicolor* spores in 50ul intratracheally (IT) 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 (41).

Assessment of asthma phenotype.

AHR to methacholine (acetyl-b-methylcholinechloride; Sigma, St. Louis, MO) was assessed in mice using flexiVent, a mechanical ventilator system (SCIREQ, Montreal, PQ, Canada) (34). Mice were anesthetized with ketamaine, 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, 75, 150, and 300 mg/ml. Measurements were made using a 1.25s, 2.5Hz volumedriven 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 Ptr = RV + EV + Po, and Ptr = tracheal pressure, V = volume, E =elastance, Po is a constant and C = 1/E. The maximum R value and minimum C value with a coefficient of determination of 0.9 or greater (as determined by the flexiVent software) was used to determine the dose-response curve.

Bronchoalveolar lavage fluid (BALF²) collection and analysis

BALF was collected and analyzed as previously described (42). Briefly, the lungs were lavaged with 1 ml PBS + 2mM 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. *Bone Marrow Derived Dendritic cells (BMDC)*.

BMDC were generated as previously described (11). Briefly, 1.5×10^7 bone marrow cells in RPMI media (RPMI 1640 medium with 10% FBS, L-glutamine, and antibiotics) in the presence of GM-CSF (10 ng/ml; Miltenyi, Auburn, CA). On day 4, fresh media supplemented with GM-CSF was added. On day 8-9 BMDC were collected, plated, and stimulated with *A. versicolor* spores at a 1:2 cell to spore ratio. After 24 hours supernatants and RNA were collected.

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 2ml of RPMI 1640 containing Liberase DL (0.5 mg/ml; Roche Diagnostics, Indianapolis, IN) and DNAse I (0.5 mg/ml; Sigma, St Louis, MO). Lung cells were passed through a 70µm cell strainer with a syringe rubber and the strainer washed with 5ml of RPMI+DNAse I media. Cells were centrifuged and resuspended in 2ml of RPMI before counting. Cell viability was confirmed by trypan blue exclusion.

Approximately 10⁶ lung cells were transferred in to a V-bottom 96 well plate on ice, centrifuged and resuspended in PBS containing FcBlock (2.4G2 mAb, Biolegend, San Diego, CA) after stimulation with PMA and Inomycin in the presence of Brefeldin A (eBioscience, San Diego, CA) and monensin (eBioscience, San Diego, CA) . Cells were labeled with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit according to manufacturer's instructions (Invitrogen by Life Technologies, Carlsbad, CA). T cells were stained with CD4-Pacific Blue (BioLegend, San Diego, CA). Intracellular staining for IL13-PE (eBioscience, San Diego, CA) and IL17A-AF647 (BioLegend, San Diego, CA) was done using reagents from eBioscience (San Diego,CA). All flow cytometric data were acquired using the LSR Fortessa (Becton Dickinson, Mountain View, CA), maintained by the Research Flow Cytometry Core in the Division of Rheumatology at Cincinnati Children's Hospital Medical Center, supported in part by NIH AR-47363, NIH DK78392 and NIH DK90971. Data was analyzed used FlowJo software (Tree Star, Ashland, OR).

RT-PCR.

RNA was extracted from the lungs or from BMDC using TRIzol reagent (Life Technologies, Grand Island, NY). Reverse transcription was preformed using iScript Reverse Transcription

Supermix (BioRad, Hercules, CA). Real-time PCR was done using the SYBR Green Master Kit and a LightCycler 480 instrument (Roche Diagnostics, Indianapolis, IN). Murine hypoxanthine phosphoribosyltransferase (HPRT) 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-6 was specifically amplified 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 TNFa was specifically amplified using forward primer5'-TGT GCT CAG AGC TTT CAA CAA-3' and reverse primer 5'-CTT GAT GGT GGT GCA TGA GA-3'. cDNA for murine IL-23 was specifically amplified 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'. cDNA for murine Dectin-1 was specifically amplified using forward primer 5'-CGT CAC AGC TTG CAA AGA AG-3' and reverse primer 5'- CGA TCC AGG TTG GTC CTT TA-3'.

Statistical analysis.

All statistical analyses were done using PRISM software (GraphPad Software Inc., La Jolla, CA). Statistical significance was assessed using one-way ANOVA followed by a Neuman-Kohls post-test or a two-way ANOVA followed by a Bonferroni post-test for dose response data.

Results

A. versicolor induced expression of Th17 skewing cytokines in DCs is Dectin-1 dependent.

We previously reported that *A. versicolor* exposure induces a Th17 response in mice that is dependent on Dectin-1, and we hypothesized that altered upstream Th17 skewing cytokines account for decreased Th17 cells in Dectin-1 deficient mice after exposure to *A. versicolor*. Dendritic cells are the sentinels of the airways and are constantly scanning the airways for allergens. Signaling through Dectin-1 on dendritic cells has been shown to induce pro-Th17 factors including TNF α , IL-6, and IL-23 (21, 29, 43, 44). Therefore, wished to determine the role of Dectin-1 in expression of these cytokines in dendritic cells. We examine the expression of IL-6, IL-23, and TNFa in BMDCs. II-6 and IL-23 have been shown to be important in induction of Th17 cells (45-48), and TNFa has been shown to support development of Th17 responses (49). After exposure to *A. versicolor* dendritic cells (Fig. 1a-c). Our data indicates that Dectin-1 is necessary for the production of Th17 skewing cytokines from dendritic cells after exposure to *A. versicolor*, and suggests that dendritic cells are important determinants of the T cell response in Dectin-1 deficient mice.

A. versicolor induced expression of Th17 skewing cytokines in DCs is also MyD88 dependent.

Since the TLR signaling pathway can also regulate immune response to molds, we wanted to determine the role of TLRs in our model. Several TLRs have been implicated in the response to molds (13), and are important in generation of Th17 responses after exposure to mold (34). Based on our data indicating Dectin-1 regulates expression of Th17 skewing cytokines in dendritic cells, we wished to determine if the TLR pathway had a similar regulatory

role in expression of Th17 skewing cytokines. Rather than investigate each TLR separately we chose to use MyD88 deficient mice to generate BMDC. We analyzed the expression of IL-6, IL-23, and TNFa in wild-type and MyD88 deficient BMDCs exposed to *A. versicolor*. After exposure to *A. versicolor* we observed decreased expression of IL-6, IL-23, and TNFa in dendritic cells deficient in MyD88 as compared to wild dendritic cells. Thus, our data indicates that the TLR family, through MyD88, also has an important role for production of Th17 skewing cytokines by dendritic cells after exposure to *A. versicolor*.

Decreased Th17 cells and increased Th2 cells in mice deficient in MyD88.

Since it is known that TLR6 is necessary for induction of Th17 responses in responses to *A. fumigatus* (34), and we observed that MyD88 signaling regulates the expression of Th17 skewing cytokines in dendritic cells, we wanted to determine MyD88 is important for induction of Th17 cells *in vivo*. We characterized the T cells in the lungs of wild-type and Dectin-1 -/- mice following repeated exposure to *A. versicolor* spores We observed fewer Th17 cells in MyD88 deficient mice and increased Th2 cells (Fig. 3a, b). Further, expression of IL-17A in the lungs of exposed mice was decreased in MyD88 deficient mice as compared to wild-type mice and IL-4 expression was increased (Fig. 3c, d). Further, similar to our previously published data indicating that Dectin-1 does not have a role in development of T cell responses after exposure to *C. cladosporioides*, we did not observe any difference in T cells in MyD88 -/- *C. cladosporioides* exposed mice (data not shown). Thus, MyD88 is important for the development of Th17 responses.

Increased AHR and eosinophilia in MyD88 deficient mice.

We previously reported that Dectin-1 deficient mice exposed to *A. versicolor* develop an allergic asthma phenotype characterized by AHR and eosinophilia (33). Based on our findings

that MyD88 -/- mice exposed to *A. versicolor* have increased Th2 cells, we wished to determine if MyD88 is also important for inhibiting an asthma phenotype in *A. versicolor* exposed mice. AHR and airway inflammation were characterized in mice exposed chronically to *A. versicolor*. We observed a significant increase in AHR in MyD88 deficient mice exposed to *A. versicolor* as compared to wild-type mice (Fig 4a). Additionally, mice lacking MyD88 developed robust eosinophilia, but wild-type mice developed very little eosinophilia (Fig 4b). Additionally, when MyD88-/- mice were exposed to *C. cladosporioides* there was no difference in phenotype compared to wild type mice (data not shown). Thus, these data indicate that MyD88 is also an important regulator of allergic asthma in response to *A. versicolor* exposure.

Dectin-1 expression is unregulated after exposure to A. versicolor in MyD88 deficient mice.

TLRs and Dectin-1 can interact with each other to induce immune responses (12), and TLR6 can regulate the expression of Dectin-1 (34). Since we observed similar phenotypes in MyD88-/- and Dectin-1 -/-, mice we wanted to determine if MyD88 could regulate the expression of Dectin-1 after exposure to *A. versicolor*. We found that in mice lacking MyD88 expression of Dectin-1 was increased after exposure to *A. versicolor* as compared to wild-type mice (Fig. 5). Thus MyD88 regulates the expression of Dectin-1 in our model of chronic mold exposure.

Discussion

These data demonstrate a role for the TLR signaling pathway in the induction of Th17 responses after exposure to *A. versicolor*. In the present study we demonstrate that Dectin-1 and MyD88 are necessary for expression of the Th17 skewing cytokines IL-6 and IL-23, in addition to expression of TNFa. Furthermore, this decrease in Th17 skewing cytokines correlates with a decrease in Th17 cells in Dectin-1 deficient (33) and MyD88 deficient mice. There is a concurrent increase in Th2 cells, leading to development of AHR and eosinophilia in MyD88 deficient mice, similar to that observed in Dectin-1 deficient mice. Thus, both the TLR pathway and Dectin-1 are critical for induction of Th17 responses after exposure to *A. versicolor*.

Dectin-1 and TLR2 ligands synergize to activate monocytes and macrophages (50, 51), and cooperate for maximal production of TNF α (52), IL-23 (21), and uptake of spores (53). Further, in response to *A. fumigatus* dendritic cells deficient in Dectin-1 or MyD88 make less TNF α than wild-type dendritic cells (49). In our study we examined the role of Dectin-1 and MyD88 in dendritic cells. We found that deficiency in either Dectin-1 or MyD88 resulted in a decrease in production of the proinflammatory cytokines TNF α , IL-23 and IL-6, suggesting that both PRR pathways are critical for the innate immune response against *A. versicolor*.

Further, we observed a decrease in Th17 cells and increase in eosinophils in MyD88 deficient mice after exposure to *A. versicolor*, and we previously reported similar results in mice deficient in Dectin-1 (33). Consistent with our data TLR6 deficient mice have decreased Th17 cells and increased eosinophils (34). Our data highlight the role of both Dectin-1 and MyD88 in production of proinflammatory cytokines from dendritic cells, and in development of Th17 cells. Furthermore, our data also suggest a role for TNF α from dendritic cells as a regulator Th17 vs. Th2 immune responses, consistent with a previous study demonstrating TNF α produced by

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dendritic cells is a critical regulator of IL-17 and IL-5 levels (49). Dendritic cells from Dectin-1 and MyD88 deficient mice produce significantly less TNF α after exposure to *A. versicolor*, and *in vivo* these mice have significantly fewer Th17 cells and increased numbers of Th2 cells. It would be interesting to take dendritic cells exposed to *A. versicolor* from MyD88 or Dectin-1 deficient mice and transfer them to wild type mice and vice versa to further delineate the role of pro-inflammatory cytokines produced by dendritic cells in the inflammatory phenotype induced by *A. versicolor*.

Studies suggest that TLR2 or TLR6 can positively regulate the expression of Dectin-1 (34, 54). However, we observed an increase in Dectin-1 expression in the lungs of mice deficient in MyD88 after exposure to A. versicolor. While this is seemingly contradictory to published literature, it is possible that other TLRs besides TLR2, 6 may regulate the expression of Dectin-1. Several other TLRs are also important for recognition of molds such as TLR1, 4, and 9 (55-57), and may have a role in regulating expression of Dectin-1. Furthermore, the up regulation of Dectin-1 expression we observed may be a compensatory mechanism. By using mice deficient in MyD88 all but TLR3 and TLR4 signaling is abolished (12). It is conceivable that as a result of knocking out one innate pathway (TLRs) for recognition of mold spores, that the other major innate pathway (Dectin-1) is up regulated to compensate. It would be interesting to examine the regulation of the TLR pathway by Dectin-1. We hypothesize that in the absence of Dectin-1, components of the TLR pathway would be upregulated to compensate for the loss of Dectin-1. However, in the absence of MyD88 or Dectin-1 there is a significant change in inflammatory phenotype, indicating that Dectin-1 and MyD88 have non-redundant roles in promoting a Th17 response after exposure to A. versicolor. In light of all the data supporting a collaborative role between Dectin-1 and TLR signaling (21, 50-53), it is interesting to speculate that there is

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synergy between the two pathways. While there is significantly fewer Th17 cells Dectin-1 (33), the response it is not completely abolished. It is possible that in these mice the MyD88 pathway is compensating for the loss of Dectin-1, and that the absence of both pathways would result in complete abolishment of Th17 responses.

In conclusion, we demonstrate that a non-pathogenic mold can signal through either Dectin-1 or MyD88 to induce production of the pro-inflammatory cytokines TNFa, IL-23 and IL-6. The production of these pro-inflammatory cytokines is correlated with a Th17 response, and in the absence of Dectin-1 (33) or MyD88 a Th2 response and murine asthma develop. Importantly, our data now add to the field by demonstrating that non-pathogenic molds also activate the innate immune system through Dectin-1 and TLR signaling pathways. These innate signaling pathways are important to prevent the development of asthma in response to *A. versicolor*.

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Figure Legends

Figure 1: *A. versicolor* induced expression of pro-inflammatory cytokines in DCs is Dectin-1 dependent. (A) Expression of TNFa, (B) IL-6, and (C) IL-23 in BMDC after exposure to *A. versicolor* spores. Data are representative of 3 independent experiments and are expressed as mean and SEM. N=4 samples per group. *p<0.05 and ***p<0.001 as compared to saline. #p<0.05 and ###p<0.001 as compared to *A. versicolor* exposed Dectin-1 -/- BMDC.

Figure 2: A. versicolor induced expression of pro-inflammatory cytokines in DCs is MyD88

dependent. (A) Expression of TNFa, (B) IL-6, and (C) IL-23 in BMDC after exposure to *A*. *versicolor* spores. Data are representative of 3 independent experiments and are expressed as mean and SEM. N=4 samples per group. *p<0.05 and ***p<0.001 as compared to saline. #p<0.05 and ###p<0.001 as compared to *A. versicolor* exposed MyD88 -/- BMDC.

Figure 3: Induction of Th17 cells by *A. versicolor* is MyD88 dependent. (A) Contour plots and (B) quantification of intracellular cytokine staining for IL-17A or IL-13 in CD4+ cells isolated from the lungs of exposed mice. (C) Expression of IL-17A and IL-4 in the lungs of exposed mice. Data are representative of 1 experiments and are expressed as mean and SEM. N=3-5 mice per group. *p<0.05 and ***p<0.001 as compared to saline. #p<0.05 and ###p<0.001 as compared to *A. versicolor* exposed MyD88 -/- mice.

Figure 4: MyD88 protects against allergic inflammation induced by *A. versicolor*. (A) Wild type C57Bl/6 orMyD88 -/- mice were exposed to *A. versicolor* spores and AHR was assessed 48 hours later. (B) Total and differential counts of BALF for *A. versicolor* exposed mice. Data are representative of 1 experiment and expressed as mean and SEM. N=43-5mice per group. *p<0.05 and **p<.01 as compared to saline. #p<0.05 and ##p<0.01 compared to exposed MyD88 -/- mice.

Figure 5: Dectin-1 expression is increased in MyD88-/- mice after exposure to A. versicolor.

Expression of Dectin-1 in wild type C57Bl/6 or MyD88 -/- mice after exposure to *A. versicolor* spores. Data are representative of 1 experiment and expressed as mean and SEM. N=3-5 mice per group. *p<0.05 as compared to saline and #p<0.05 compared to exposed MyD88 -/- mice.




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Surface availability of beta-glucans is critical determinant of host immune response to *C*. *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. *C. cladosporioides* have a high beta-glucan content, but the beta-glucans are not available on the surface of the live spores.

Objective: We sought to determine if 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 measurement of airway hyperresponsivness, airway inflammation, and cytokine production. Pro-inflammatory cytokines from dendritic cells were measured by quantitative PCR and ELISA.

Results: Live *C. cladosporioides* induced robust airway hyperresponsiveness, eosinophilia, and a predominately Th2 response, while heat-killed *C. cladosporioides* induced a strong Th17 response and neutrophilic inflammation, but very mild AHR. 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 Th2 response analogous to live spores. Further, the production of Th17 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 surface availability of beta-glucans rather than the total beta-glucan content.

Key Messages

- Heat killing *C. cladosporioides* spores effectively exposes 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 Th17 responses.

Capsule Summary

Alterations in exposure of beta-glucans on mold spores can change the host response to molds. Surface availability of beta-glucans on mold spores is more important in determining the immune response than the total beta-glucan content of spores.

Key Words

Mold *Cladosporium cladosporioides* Beta-glucans Asthma Dectin-1

Abbreviations

AHR: airway hyperresponsiveness

BALF: bronchoalveolar lavage fluid

C. clad: C. cladosporioides

HK: heat-killed

Introduction

Fungal spores are ubiquitously distributed in both the indoor and outdoor environment, and are often associated with respiratory disease¹. 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 wheeze², and in another pediatric cohort persistent childhood asthma was associated with sensitivity to mold³. In adult asthma, allergic sensitization to molds was associated with more hospital and ICU 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 cells walls is (1-3)-beta-D-glucans, and are commonly used as a marker of mold exposure in the environment⁶. Beta-glucans account for up to 60% of the weight of the cell wall ⁷, and are important for recognition of molds by the immune system. Dectin-1, the receptor for beta-glucans⁸, 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 towards Th17 cells¹¹⁻¹⁴. Accordingly, deficiency in IL-17A or Dectin-1 has been associated with an increased risk for fungal infections in humans¹⁵⁻²⁰. 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 *A. fumigatus* spores increases activation of dendritic cells and increases binding of Dectin-1²⁵. Heat- killed *Candida albicans* has greater binding to Dectin-1 than 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 heatkilled mold spores²⁸, suggesting that exposure of beta-glucans on the surface of spores may alter the immune response. Further, recognition of different stages of mold growth is dependent upon accessibility of beta-glucan and binding to Dectin-1²⁹. 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 two spores and, therefore, differences in binding to Dectin-1³⁰. 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 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 live spores. Further, heat-killed *C. cladosporioides* spores induced an attenuated asthma phenotype compared to live spores. The inflammatory phenotype induced by heat-killed *C. cladosporioides* spores was predominately a Th17 response marked by neutrophilic inflammation in the airways, and this response was dependent on Dectin-1.

Materials and Methods

Mice.

Dectin-1 -/- mice have been previously described and were generously provided by Dr. Gordon Brown²¹. 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 (CCHMC). All procedures were preformed 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 CCHMC.

Preparation of mold spores and spore challenge model.

Cladosporium cladosporioides isolate 6721 (American Type Culture Collection, Manassas, VA) was grown on malt extract agar for 4-6 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 $2x10^7$ 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 50ul intratracheally (IT) 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 published³⁰.

Assessment of AHR.

AHR to methacholine (acetyl-b-methylcholinechloride; Sigma, St. Louis, MO) was assessed in mice using flexiVent (SCIREQ, Montreal, PQ, Canada) as previously described ³⁰.

Bone Marrow Derived Dendritic cells (BMDC).

BMDC were generated as previously described ³¹. BMDC 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 (BALF) collection and analysis

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, CA) in undiluted BALF. IL-6 and TNFa were also detected according to manufacturer instructions (BioLegend, San Diego, CA) in supernatants from BMDC 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 25-30 min in 2ml of RPMI 1640 containing Liberase DL (0.5 mg/ml; Roche Diagnostics, Indianapolis, IN) and DNAse I (0.5 mg/ml; Sigma, St Louis, MO). Lung cells were passed through a 70µm cell strainer. Cells were centrifuged and resuspended in 2ml of RPMI. Cell viability was confirmed by trypan blue exclusion.

Approximately 10⁶ lung cells were transferred in to a V-bottom 96 well plate on ice, centrifuged and resuspended in PBS containing FcBlock (2.4G2 mAb, Biolegend, San Diego, CA) after stimulation with PMA (50 ng/ml); Sigma, St Louis, MO) and ionomycin (500 ng/ml; Sigma, St Louis, MO), in the presence of Brefeldin A (eBioscience, San Diego, CA) and monensin (eBioscience, San Diego, CA) 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, CA). T cells were stained with CD4-Pacific Blue (BioLegend, San

Diego, CA). Intracellular staining for IL13-PE (eBioscience, San Diego, CA) and IL17A-AF647 (BioLegend, San Diego, CA) was done using reagents from eBioscience (San Diego, CA). All flow cytometric data were acquired using the LSR Fortessa (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 was analyzed used FlowJo software (Tree Star, Ashland, OR).

RT-PCR.

RNA was extracted from the lungs or from BMDC using TRIzol reagent (Life Technologies, Grand Island, NY). Reverse transcription was preformed using iScript Reverse Transcription Supermix (BioRad, Hercules, CA). Real-time PCR was done using the SYBR Green Master Kit and a LightCycler 480 instrument (Roche Diagnostics, Indianapolis, IN). Murine hypoxanthine phosphoribosyltransferase (HPRT) 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-6 was specifically amplified 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 TNFa was specifically amplified using forward primer5'-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 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 (rDectin-1) (R&D Systems, Minneapolis, MN) was determined as previously described³³. Briefly, mold spores were labeled with Alexa Fluor 488 dye (Life Technologies, Grand Island, NY), and incubated with rDectin-1 (R&D Systems, Minneapolis, MN), and pulled down with magnetic nickel beads (Life Technologies, Grand Island, NY). 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-glucan on the spores, 10⁶ spores were incubated with murine anti-beta-glucan antibody, primary antibody, (Biosupplies, Bundoora, Australia) at a concentration of 1ug/ml in 1% goat serum in 0.01% PBS Tween-20, and the secondary antibody was goat anti-mouse DyLight 594 (BioLegend, San Diego, CA). Images were acquired at 1000x using a Nikon 90i Fully Automated Upright MicroscopeSystem with a Nikon DSQiMc Camera, with Z-sequence taken every 0.5um. Nikon Elements Quantitative AnalysisSoftware was used to acquire the images. For measurement of mean fluorescence intensity, the fluorescence of each spore in 10 fileds of view was measured using. Nikon Elements Quantitative AnalysisSoftware was used to determine the fluorescence intensity. 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 using PRISM software (GraphPad Software Inc., La Jolla, CA). Statistical significance was assessed using one-way ANOVA followed by a Neuman-Kohls post-test or a two-way ANOVA followed by a Bonferroni post-test for dose response data.

Results

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

Our previous data indicates 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-1³⁰. Previous studies reported that heat killing *Candia albicans* results in exposure of beta-glucans $^{26, 27}$. Therefore, we wanted to determine if 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-glucans³⁰. 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 reported³⁰. In contrast, exposure of mice to heat-killed *C. cladosporioides* spores resulted in 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 Th2 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 vs. heat-killed *C. cladosporioides* spores. While both live and heat-killed spores were able to induce Th2 cells (Fig 2b), only the heat-killed spores induced Th17 cells (Fig. 2a). The gating strategy is demonstrated in Supplemental Figure 1. However, we found by analyzing IL-17A and IL-4 in the BALF by ELISA (Fig. 2c) or in the lungs by quantitative PCR (Fig. 2d) that heat-killed spores induced significant amounts of IL-17A and

very little IL-4 compared to live spores (Fig. 2c, d). Thus, heat-killed spores induce a strong Th17 response, and a relatively weak Th2 response compared to live spores.

Heat killing of *C. cladosporioides* exposes beta-glucans.

In order 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 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. 3a). Importantly, the heat-killed spores were intact and the process of heat killing did not disrupt the spores (Fig. 3a). Further, we did not observed any autofluorscence in the AF488 channel since only spores stained with an AF488 dye demonstrated any flourscence (Supp. Fig. 2). The staining intensity was stronger in the heat-killed spores supporting that there are more beta-glucans exposed following heat killing (quantified in Fig. 3b). In order to confirm this, we directly examined the ability of the heat-killed spores to bind recombinant Dectin-1. As shown in Fig. 3c, heat-killed spores demonstrated increased binding to recombinant Dectin-1 than live spores (Fig. 3c) 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 to exposed to heat-killed *C. cladosporioides* spores developed robust AHR and eosinophilia. Further, 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 C57Bl/6 mice developed more robust eosinophilia than BALB/c mice. Thus, these data indicate the inflammatory phenotype induced by heat-killed *C. cladosporioides* spores is dependent on Dectin-1.

Th17 response induced by heat-killed C. cladosporioides spores is dependent on Dectin-1.

We next determined if the development of a Th17 response following exposure to heatkilled 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 Th17 cells in Dectin-1 -/- mice compared to wild type mice (Fig. 5a, 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. 5c). Further, there was an increase in Th2 cells in Dectin-1 -/- mice compared to wild type mice exposed to heat-killed C. cladosporioides spores (Fig. 5a, 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 -/- vs. wild type mice following exposure to heat-killed C. cladosporioides spores (Fig. 5d). Induction of Th2 cytokines by live C. cladosporioides spores is independent of Dectin-1 (data not shown and previously published³⁰). Thus, the Th17 response induced by heatkilled spores is dependent on Dectin-1. In the absence of Dectin-1, the frequency of Th2 cells and IL-4 expression in the lungs were increased.

Th17 skewing cytokines are decreased in the lungs of Dectin-1 deficient mice.

Since the Th17 response induced by heat-killed *C. cladosporioides* spores is dependent on Dectin-1, we hypothesized that altered upstream expression of Th17 skewing cytokines by heat-killed versus live spores may be the mechanistic basis for our observations. IL-6 is important for priming Th17 cells in the mucosal tissues ³⁴, and TNF α production by dendritic cells is important for regulating IL-17 levels in the lungs ³⁵. 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* had a significant increase in expression of IL-6 as compared to saline exposed mice, and this increase was independent of Dectin-1. There was not a significant difference between wild type and Dectin-1 -/- live *C. cladosporioides* exposed mice (Fig. 6a). There was only a slight, non-significant, induction of TNF α by live *C. cladosporioides* spores in both wild type and Dectin-1 -/- mice (Fig. 6b). On the other hand, heat-killed *C. cladosporioides* spores induced expression of both IL-6 and TNF α in a Dectin-1 dependent manner (Fig. 6c, d). In the absence of Dectin-1 expression of IL-6 and TNF α were significantly reduced (Fig. 6c, d). Thus, Dectin-1 promotes the induction of Th17 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.

Since we observed altered expression of IL-6 and TNF α in response to heat-killed spores in Dectin-1 -/- mice, we wanted to determine if expression of Dectin-1 in dendritic cells could contribute to altered production of Th17 skewing cytokines. Signaling through Dectin-1 on dendritic cells has been shown to induce pro-Th17 factors including TNF α , IL-6, and IL-23 ^{13, 21, ^{36, 37}. Therefore, we investigated the role of Dectin-1 in the response of dendritic cells to heatkilled versus live *C. cladosporioides* spores. When bone-marrow derived dendritic cells (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, upon exposure to heat-killed spores, BMDCs from Dectin-1 -/- mice showed significantly attenuated expression of IL-6, IL-23, and} TNF α compared to wild type mice (Fig. 7a, b, c). Similarly, in the absence of Dectin-1, BMDCs secreted significantly less IL-6 and TNF α (Fig. 7d, 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 C. *cladosporioides* indicating that Dectin-1 inhibits eosinophilic inflammation and AHR in response to A. versicolor³⁰. In this study, we demonstrate that exposure of beta-glucans in C. *cladosporioides* by heat killing induces a neutrophilic inflammatory phenotype characterized by Th17 cytokines similar to that induced by A. versicolor. In the absence of Dectin-1, heat-killed C. cladosporioides induce strong AHR, eosinophilia, and Th2 cytokines. 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 pro-inflammatory cytokines IL-6, IL-23, and TNF α , all of which have been shown to be important in the development of Th17 inflammatory responses ^{34, 35, 38, 39}, was significantly attenuated in the absence of Dectin-1 following exposure to heat-killed C. cladosporioides spores. 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.

Quantification of beta-glucans in the environment is often used as a surrogate for mold exposure ⁴⁰⁻⁴². Several studies have examined the direct relationship between beta-glucans and

health effects (reviewed in ⁷) with inconsistent results. One study found that low levels of betaglucans were positively correlated with a positive asthma predictive index (API), but high levels of beta-glucans were negatively correlated with a positive API⁴³. A study in Germany similarly found a negative association between beta-glucan levels in mattress dust and sensitization to aeroallergens ⁴². Another study also found a negative correlation since there was a trend towards increased IFNy in patients exposed to beta-glucans rather than Th2 cytokines ⁴¹. 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 A. versicolor 44 , 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-1³⁰. It is the surface availability of beta-glucans that is important for determining the immune response, not the betaglucan 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 betaglucan 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 exposure of surface beta-glucans in the same species of mold can alter the immune response in a Dectin-1 dependent manner. Additionally, our data suggest that when quantifying mold exposure, it is important to consider 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.

Acknowledgements

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Figure Legends Figure 1. Heat killing *C. cladosporioides* prevents development of murine asthma. (A)

BALB/c mice were exposed IT 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=4-6 mice per group. p<0.05, **p<.01, and ***p<0.001 as compared to saline. #p<0.05, ##p<0.01, and ###p<0.001 as compared to C. clad HK.

Figure 2. Induction of a Th17 response by heat-killed *C. cladosporioides.* (A) Contour plots and (B) quantification of intracellular cytokine staining for IL-13 or IL-17A in CD4+ lung cells. (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=4-6 mice per group. p<0.05, **p<.01, and ***p<0.001 as compared to saline. #p<0.05, #p<0.01, and ##p<0.001 as compared to C. clad HK.

Figure 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 1000x for live and heat-killed *C. cladosporioides* spores. Scale bar is 10um. (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. *p<0.05

Figure 4. Heat-killed *C. cladosporioides* **prevents murine asthma in a Dectin-1 dependent manner.** (A) Wild type C57Bl/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 two independent experiments and are expressed as mean and SEM. N=4-6 mice per group. *p<0.05, **p<.01, and ***p<0.001 as compared to saline.

Figure 5. In the absence of Dectin-1 heat-killed *C. cladosporioides* induces a Th2 response.

#p<0.05, ##p<0.01, and ###p<0.001 as compared to C. cladosporioides exposed wild-type mice.

(A) Contour plots and (B) quantification of intracellular cytokine staining for IL-13 or IL-17A in CD4+ lung cells. (C) Levels IL-17A in BALF and mRNA in whole lung. (D) Levels IL-4 in BALF and mRNA in whole lung. Data are representative from two independent experiments and are expressed as mean and SEM. N=4-6 mice per group. p<0.05, **p<.01, and ***p<0.001 as compared to saline. #p<0.05, ##p<0.01, and ###p<0.001 as compared to *C. cladosporioides* exposed wild-type mice.

Figure 6. Expression of Th17 skewing cytokines in the lungs is dependent on Dectin-1. (A,

C) Expression of IL-6 in the lungs of mice exposed to live (A) or heat-killed (C) C.

cladosporioides. (B, D) Expression of TNFa in the lungs of mice exposed to live (B) or heatkilled (D) *C. cladosporioides*. Data are representative from two independent experiments and are expressed as mean and SEM. N=4-6 mice per group. p<0.05, **p<.01, and ***p<0.001 as compared to saline. ###p<0.001 as compared to *C. cladosporioides* exposed Dectin-1 -/- mice.

Figure 7. Proinflammatory cytokine production in BMDCs is dependent on Dectin-1. (A)

Expression of IL-6, (B) TNFa, and (C) IL-23 in BMDC. (D) level of IL-6 and (E) in culture supernatants of BMDC. Data are representative of 3 independent experiments and are expressed as mean and SEM. N=4 samples per group. *p<0.05, **p<.01, and ***p<0.001 as compared to saline. ##p<0.01 and ###p<0.001 as compared to *C. cladosporioides* exposed Dectin-1 -/- BMDC.



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Figure 7



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Supplemental Data.

Supplemental Figure 1: (A) Live cells gating with Live Dead Stain (Invitrogen), (B) gating of lymphocytes in the live population based on FSC and SSC, and (C) CD4 gating from the lymphocyte population.

Supplemental Figure 2: 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).

Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 2: C. clad or C. clad HK spores were stainined with an AF488 dye (green) or not stained and then incuabated with anti-beta glucan and a secondary antibody (red).

The environmental mold *A. versicolor* synergizes with HDM extract to induce severe asthma

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Abstract

There is strong evidence to support a role for mold exposure in the development of severe asthma, but the mechanisms of how molds induce severe asthma are poorly understood. Molds are known to induce Th17 responses, and IL-17 has been implicated in severe asthma. Our objective was to determine in a co-exposure model if induction of a Th17 response by *Aspergillus versicolor* can synergize with Th2 responses induced by either HDM or *Cladosporioides* to induce severe asthma. Mice were exposed to *A. versicolor* +/- house dust mite (HDM) or *C. cladosporioides*. Airway inflammation, bronchoalveolar lavage fluid (BALF) cytokine levels, and lung T cells were assessed. Asthma severity was determined among childhood allergic asthmatics sensitized to HDM +/- mold. Co-exposure to *A. versicolor* and HDM or *C. cladosporioides* induced more severe AHR and airway inflammation that either exposure alone. This synergy was associated with a mixed Th2/Th17 response in the lungs. Further, asthmatic children sensitized to both mold and HDM had higher mean symptom scores than those sensitized to HDM alone. Thus, our data indicate that molds can induce severe asthma, likely through induction of Th17 responses.

Introduction

Nearly 23 million people in the United States, including 7 million children, have been diagnosed with asthma (1-3). Classically allergic asthma is characterized histologically by chronic airway inflammation including infiltration of the airways with lymphocytes, eosinophils and mast cells, goblet cell hyperplasia, and thickening of the submucosa. These histological changes lead to the critical symptoms of variable airflow obstruction and airway hyperresponsiveness (AHR) (4). The current paradigm suggests that allergic asthma is dependent on Th2 cells and their cytokines IL-4, IL-13, and IL-5 (5-9). However, asthma is a heterogeneous disease, and consists of several overlapping phenotypes that can be classified from mild to severe (10-12) or by etiology such as obesity associated asthma, ozone induced asthma, or aspirin induced asthma (13-15). The many phenotypes of asthma suggest there may be other underlying etiologies besides the classic Th2 paradigm.

In the past 10-15 years there has been a focus on defining severe asthma. Some of these studies have focused on asthma exacerbations, lung function, asthma control, corticosteroid use, and consumption of health care resources (12, 16). Other studies have focused on the underlying inflammatory etiology, in particular the role of IL-17 and neutrophils as components of severe asthma (17-19). IL-17 is a pro-inflammatory cytokine produced by T cells, NKT cells, neutrophils, and macrophages which an important role in protection from extracellular pathogens and in chronic disease (20-23). The role of IL-17 and Th17 in asthma remains controversial. Several studies have found IL-17A and IL-17F are associated with increased neutrophilic inflammation, decreased lung function, increased airway hyperresponsiveness to methacholine, and overall asthma severity (17, 19, 24-27). Some murine models of asthma demonstrate that IL-17A contributes the pathogenesis of allergic asthma and can synergize with IL-13 to induce

AHR (28, 29). Further, in a model of ozone exposure, IL-17 was necessary for development of AHR and neutrophilic recruitment (14), suggesting a role for IL-17 in non-allergic asthma. However, murine studies have also shown a negative role for IL-17 in asthma. In an established ova-model of murine asthma, IL-17 negatively regulated AHR and eosinophil recruitment (30). Further, our own study demonstrated that IL-17 is protective against development of murine asthma after exposure to a single species of mold, *A. versicolor* (31). These studies suggest that IL-17 likely has a role in severe asthma, but the exact role is still unclear and may depend on the type of exposure.

The type of exposure may be an important contributor to severity of disease. Many studies suggest that molds are associated with asthma, in particular severe asthma. Recurrent hospital admissions are associated with sensitization to multiple allergens, including multiple molds (32), and patients admitted to the ICU due to asthma are more likely to have a positive skin test for one ore more fungal allergens (33). Hospital admissions for asthma increase during months when mold spores counts peak (32, 34), and increase after thunderstorms when spore counts are high (35, 36). Deaths from asthma increase during months when mold spore counts are high (37). It is possible that the increased severity of mold-induced asthma is due to expression of IL-17. Molds are potent inducers of IL-17 and Th17 cells (23, 31, 38), and in fact patients with deficiencies in IL-17 signaling, either due to inborn errors in IL-17 signaling (39) or autoantibodies against IL-17 cytokines, develop chronic mucocutanous candidiasis (40, 41). These studies suggest the increased exposure/sensitization to molds increases asthma exacerbations and thus severity. Further, a possible explanation for the increased severity may be the induction of IL-17 by exposure to molds.

We hypothesize that mold exposure contributes to severe asthma through induction of IL-17. In this study we demonstrate co-sensitization to both house dust mite (HDM) and molds in a pediatric population is associated with an increase in symptom scores. Further, in a murine model of asthma co-exposure to both HDM and the mold *A. versicolor* induce more severe AHR than exposure to either HDM or *A. versicolor* alone. This increase in AHR is associated with a mixed Th17/Th2 response. Additionally, co-exposure to molds that induce distinct immune response also results in synergistic worsening of airway restriction.

Materials and Methods

Subjects

The children included in this study are enrolled in the Greater Cincinnati Pediatric Clinic Repository (42) Children ages 5-18 years with asthma (diagnosed according to ATS criteria (43)) who were skin prick test (SPT) positive to HDM or HDM and molds. Asthma severity was determined by questionnaire. Children must have had at least one respiratory symptom frequency score to be included. For children with multiple symptom scores (from multiple questionnaires completed over time), the worst symptom score was included in this analysis. The symptom frequency scores were determined from symptom frequency questions for wheezing, coughing, shortness of breath, and chest tightness. Possible frequency answer choices included 0/never having symptoms on average over the last 12 months (score 0), having symptoms less than 1 time a week on average over the last 12 months (score 0), or having symptoms on average 1-2 (score 1), 3-5 (score 2), or 6-7 (score 3) times a week over the past 12 months. Children with severe asthma are those with 1 or more symptoms that occur on average at least 6-7 days a week over the past 12 months (maximum score of 4 for one or more symptoms).

Mice.

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 (CCHMC). All procedures were preformed 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 CCHMC.

Preparation of mold spores and spore challenge model.

Aspergillus versicolor isolate 52173 and Cladosporium cladosporioides isolate 6721 (American Type Culture Collection, Manassas, VA) were grown on malt extract agar for 4-6 weeks 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 $2x10^{7}$ spores/ml. House dust mite extract (*Dermatophagoides pteronyssinus*) was purchased from Greer Laboratories (Lenoir, NC). Mice were lightly anesthetized with Isoflurane and exposed to 50µl of saline, HDM (10µg) in 50ul of saline, 10^{6} spores in 50ul of saline, both HDM and *A. versicolor* spores in 50ul of saline, or both *C. cladosporioides* and *A. versicolor* spores in 50ul of saline intratracheally (IT) 3 times a week for 3 weeks, and then were assessed 2 days after the last exposure. The mold spore dose was chosen based on the concentrations of these molds found in homes of children in the CCAAPS birth cohort (44).

Assessment of asthma phenotype.

Airway hyperresponsiveness (AHR²) to methacholine (acetyl-b-methylcholinechloride; Sigma, St. Louis, MO) was assessed in mice using flexiVent, a mechanical ventilator system (SCIREQ, Montreal, PQ, Canada) (34). Mice were anesthetized with ketamaine, 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 1.25s, 2.5Hz volume-driven oscillation applied to the airways by the flexiVent system (SnapShot perturbation). Twelve

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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 Ptr = RV + EV + Po, and Ptr = tracheal pressure, V = volume, E = elastance, Po is a constant and C = 1/E. The maximum R value and minimum C value with a coefficient of determination of 0.9 or greater (as determined by the flexiVent software) was used to determine the dose-response curve.

Bronchoalveolar lavage fluid (BALF³) collection and analysis

BALF was collected and analyzed as previously described (45). Briefly, the lungs were lavaged with 1 ml PBS + 2mM 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. *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 2ml of RPMI 1640 containing Liberase DL (0.5 mg/ml; Roche Diagnostics, Indianapolis, IN) and DNAse I (0.5 mg/ml; Sigma, St Louis, MO). Lung cells were passed through a 70µm cell strainer with a syringe rubber and the strainer washed with 5ml of RPMI+DNAse I media.

Approximately 10⁶ lung cells were transferred in to a V-bottom 96 well plate on ice, centrifuged and resuspended in PBS containing FcBlock (2.4G2 mAb, Biolegend, San Diego, CA) after stimulation with PMA and Inomycin in the presence of Brefeldin A (eBioscience, San Diego, CA) and monensin (eBioscience, San Diego, CA). Cells were labeled with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit according to manufacturer's instructions (Invitrogen by Life Technologies, Carlsbad, CA). T cells were stained with CD3e-FITC and CD4-Pacific Blue (BioLegend, San Diego, CA). Intracellular staining for IL13-PE (eBioscience, San Diego, CA) and IL17A-AF647 (BioLegend, San Diego, CA) was done using reagents from eBioscience (San Diego,CA). All flow cytometric data were acquired using the LSR Fortessa (Becton Dickinson, Mountain View, CA), maintained by the Research Flow Cytometry Core in the Division of Rheumatology at Cincinnati Children's Hospital Medical Center, supported in part by NIH AR-47363, NIH DK78392 and NIH DK90971. Data was analyzed used FlowJo software (Tree Star, Ashland, OR).

Statistical analysis.

Human subject analysis was performed in SAS Software version 9.2 (SAS Institute, Inc, Cary, NC). The Shapiro-Wilk test was done to analyze normality. A one-sided Wilcoxen test was used to compare the means between groups. All statistical analyses for animal experiments were done using PRISM software (GraphPad Software Inc., La Jolla, CA). Statistical significance was assessed using one-way ANOVA followed by a Neuman-Kohls post-test or a two-way ANOVA followed by a Bonferroni post-test for dose response data.

Results

Sensitization to mold increases severity of childhood asthma

Mold exposure is associated with severe asthma (32, 34-37), and recurrent hospital admissions due to asthma are associated with sensitization to multiple allergens, one of which is often mold (32, 33). To determine the effect of mold sensitization in a pediatric cohort we identified 51 patients in the GCPCR who were sensitized to HDM alone, and 44 patients who were sensitized to HDM and mold. Comparison of the mean severity scores between the two groups revealed that the patients sensitized to HDM and mold had a higher mean symptom score than the patients sensitized to HDM alone (Fig. 1). Although, the difference did not reach statistical significance, the data suggest a trend towards greater asthma severity when sensitized to both HDM and mold.

Murine asthma is exacerbated upon exposure to both mold and HDM.

Since our data from the GCPCR demonstrated a trend towards more severe asthma in patients sensitized to both mold and HDM, we wanted to determine if mold and HDM would synergize to induce severe asthma in a murine model. We have previously reported that the mold *A. versicolor* induces a Th17 response in a murine model of chronic mold exposures (31), and Th17 responses have been implicated in severe asthma (17, 19, 24-27). Using a model of chronic *A. versicolor* exposure combined with HDM exposure we observed significantly increased AHR in mice exposed to both HDM and *A. versicolor*, but exposure to either HDM alone or *A. versicolor* alone only induced mild AHR (Fig. 2a). Further, there was significantly more cells recruited to the airways in mice exposed to both *A. versicolor* and HDM than mice exposed to either HDM or *A. versicolor* alone (Fig. 2b). Mice co-exposed to *A. versicolor* and

HDM developed mixed neutrophilic/eosinophilic inflammation (Fig. 2b). Our data demonstrate development of severe asthma after co-exposure to HDM and *A. versicolor*.

Induction of both Th2 and Th17 after co-exposure to HDM and A. versicolor

We have previously reported that the mold *A. versicolor* induces a Th17 response in a murine model of chronic mold exposures (31), and Th17 responses have been implicated in severe asthma (17, 19, 24-27). Therefore, we wished to determine the types of T cells present in the lungs of mice after co-exposure to HDM and *A. versicolor*. We determined the frequency of IL-13 producing cells (Th2) and IL-17A (Th17) cells in the whole lung were determined by intracellular cytokine staining (ICCS). Exposure to *A. versicolor* alone induced a Th17 response, and exposure to HDM alone induced a Th2 response. However, co-exposure to both *A. versicolor* and HDM induced a mixed Th2/Th17 response (Fig. 3). Thus, co-exposure to HDM and *A. versicolor* induces a mixed T cell response.

Co-exposure to both A. versicolor and C. cladosporioides exacerbates murine asthma.

C. cladosporioides induces Th2 responses in a murine model of chronic mold exposure (31). Since *A. versicolor* synergizes with HDM to induce severe airway restriction, we wanted to determine if *A. versicolor* could also synergize with *C. cladosporioides*. Mice exposed to both spores developed more severe AHR than mice exposed to either *A. versicolor* or *C. cladosporioides* alone (Fig. 4a). Further, co-exposed mice had more inflammatory cells recruited to the airways as opposed to mice exposed to either mold alone (Fig. 4b). This increase in cells was due primarily to an increase in eosinophils. These data suggest that *A. versicolor* can synergize with *C. cladosporioides* to induce severe asthma.

Discussion

Pathogenic fungi are potent inducers of Th17 responses (38, 46, 47), and several species of molds have been associated with severe asthma (32, 34-37). In this study we analyzed the relationship between mold and severe asthma in the GCPCR, a pediatric cohort (42), and in a murine model of asthma. Our data demonstrated that patients with asthma in the GCPCR sensitized to both mold and HDM have higher mean symptom scores than patients sensitized to HDM alone, and thus in agreement with previous studies. Further, we demonstrated that *A*. *versicolor* could synergize with HDM or with *C. cladosporioides* to induce severe experimental murine asthma.

These data demonstrate that environmental exposures capable of inducing a Th17 or Th2 response independently can synergize upon co-exposure to induce a severe asthma phenotype. Our previous work demonstrated that the environmental molds *A. versicolor* and *C. cladosporioides* induce distinct patterns of pulmonary inflammation; *A. versicolor* induces a Th17 response, but exposure to *C. cladosporioides* induces a Th2 response (31). HDM is associated with asthma in patient studies (48-51), and is commonly used in murine models of asthma (29, 45, 52-54). In this study we show that co-exposure to either *A. versicolor* and HDM, or *A. versicolor* and *C. cladosporioides* induces a synergistic response in the development of AHR and airway inflammation. Furthermore, co-exposure to *A. versicolor* and HDM induces a mixed Th17/Th2 response. Neither exposure is capable of inducing a mixed response on its own.

Several patient studies have found an association between IL-17 and overall asthma severity (17, 19, 24-27). However, the role of IL-17 in murine studies remains controversial. IL-17A has been implicated in the pathogenesis of asthma and can synergize with IL-13 to

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induce AHR (28, 29), but has also been shown to be a negative regulator of established murine asthma (30). Further, we have previously shown that IL-17 can negatively regulate development of AHR and eosinophil recruitment to the airways after exposure to *A. versicolor*. The data in this study are seemingly contradictory to our previous findings. We demonstrate synergy between an IL-17 inducer, *A. versicolor*, and a Th2 inducer, HDM or *C. cladosporioides*. Based on our previous observations, we would have predicted that *A. versicolor* would inhibit AHR and eosinophil recruitment through induction of Th17 cells. However, IL-13 and IL-17A administration directly to the airways results in a synergistic induction of AHR (29). It is likely that in our current study we are observing similar synergism between IL-17 and IL-13, and it would be interesting to test this hypothesis by neutralizing IL-17 in co-exposed mice.

In conclusion we have demonstrated that the environmental mold *A. versicolor* can synergize with either HDM or *C. cladosporioides* to induce severe murine asthma. Co-exposure to *A. versicolor* and HDM induces a mixed Th2/Th17 response. This mixed response is likely responsible for the development of severe murine asthma. Thus, it is likely that molds contribute to severe asthma through induction of Th17 responses that are able to synergize with Th2 responses.

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Figure Legends

Figure 1: Severe asthma in mold sensitized patients. The mean symptom score for patients in the GCPCR sensitized to HDM alone or HDM and mold were calculated. The p value was calculated by a one-sided Wilcoxon Ranked Sums test.

Figure 2: Synergy between *A. versicolor* and HDM. (A) BALB/c mice were exposed IT to 10^{6} *A. versicolor* (A. ver), 10ug of HDM, or both 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. Data are representative of 1 independent experiment and are expressed as mean and SEM. N=4-6 mice per group., ***p<0.001 as compared to saline. ##p<0.01 and ###p<0.001 as compared to *A. versicolor* or HDM alone.

Figure 3: Co-exposure to A. versicolor and HDM induce a mixed Th2/Th17 response.

(A) Contour plots and (B) quantification of intracellular cytokine staining IL-17A or (C) IL-13 in CD4+ lung cells. Data are representative of 1 independent experiments and are expressed as mean and SEM. N=4-6 mice per group. *p<0.05, and ***p<0.001 as compared to saline. #p<0.05 and ###p<0.001 as compared to *A. versicolor* or HDM alone.

Figure 4: Synergy between *A. versicolor* and *C. cladosporioides*. (A) C57Bl/6 mice were exposed IT to 10^{6} *A. versicolor* (A. ver) spores, 10^{6} *C. cladosporioides* (C. clad) spores, or both 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. Data are representative of 1 independent experiment and are expressed as mean and SEM. N=4-6 mice per group., *p<0.05 and ***p<0.001 as compared to saline. ##p<0.01 as compared to *A. versicolor* or *C. cladosporioides* alone.

Figure 1



Figure 2

Α.



Methacholine mg/ml

Β.



Figure 3



Figure 4

Α.









Chapter 6: Summary and Discussion

General Summary of Studies

Fungal spores are ubiquitous in the environment. Due to their aerial distribution and small size, they are inhaled and deposited in the lungs on a daily basis. Depending on the environment a person lives/works in, daily exposure can range from 10^4 spores/m³ outdoor up to 10^{10} spores/m³ indoors in mold-damaged buildings (1). Therefore it is not surprising that fungi are involved in human disease. In 2007 nearly half of the weekly requests received by the National Institute for Occupation Safety and Health concerned work-related asthma and mold exposure (2), indicating that mold exposure has a key role in asthma development. 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.

Despite the number of studies implicating molds in asthma there are very few mechanistic studies exploring the interactions between the immune system and these molds. The majority of studies that define the mechanistic interactions between fungi and the immune system use pathogenic molds in murine models. Further, many of the studies that use murine models to study mold-induced asthma use systemic sensitization, rather than airway sensitization, or they studies use protein extracts from molds either alone or in combination with mold spores (3-10). The combination of a lack of mechanistic studies on molds implicated in asthma, and the use of fungal extracts for systemic sensitization lead us to develop a more physiological model of chronic mold exposure.

The work in this dissertation uses two molds that are common in the Ohio River Valley. Based on the reports from CCAAPS implicating mold exposure in development of asthma in pediatric patients (11), we wanted to use mold spores identified in this study. *A. versicolor* and

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C. cladosporioides were identified in the homes of patients in the CCAAPS cohort (12). Further, they are different sizes and contain different amounts of beta-glucans (13). Both the size and beta-glucan content of the spores could influence inflammation due to possible differences in distribution in the lungs and interactions with the innate immune system.

Based on the reported differences in the two mold spores and the review of the literature presented in the introduction we hypothesized that *A. versicolor* and *C. cladosporioides* will induce distinct immune responses in the lungs of mice through differential activation of the innate and adaptive immune systems. In Chapters 2-4 we test this hypotheses with the following aims:

Aim 1) Characterize the immune responses induced by A. versicolor and C. cladosporioides.

In Chapter 2 we characterize our model of chronic mold exposure using *A. versicolor* or *C. cladosporioides* spores. 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. On the other hand, exposure to *A. versicolor* induced predominately 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.

Aim 2) Determine the role of innate and adaptive immune pathways in response to mold exposure.

In Chapters 2 and 3 our data reveal IL-17A, Dectin-1, and MyD88 dependent pathways suppress asthma development in mice exposed to A. versicolor spores; blockade of IL-17A or deficiency in Dectin-1, or the TLR adaptor protein, MyD88, in mice exposed to *A. versicolor* spores mice resulted in development of robust AHR and eosinophilia. In contrast, *C. cladosporioides*-induced asthma was independent of Dectin-1, MyD88, and IL-17A. These data suggest that the Dectin-1, MyD88, and downstream IL-17A pathway inhibit allergic inflammation in response to *A. versicolor*, but not *C. cladosporioides*.

Additionally, our data support a mechanistic role for dendritic cells in the observed differences in mold-induced inflammation. Dectin-1 and MyD88 are necessary for expression of the Th17 skewing cytokines IL-6 and IL-23, in addition to expression of TNF α after exposure to *A. versicolor*. Dectin-1 is also important for production of these cytokines from dendritic cells exposed to heat-killed *C. cladosporioides* spores, but live spores induce these cytokines independent of Dectin-1. The production of IL-6, IL-23, and TNF α by dendritic cells correlates with the development of a Th17 response, suggesting an important role for PRRs on dendritic cells in the phenotype induced by *A. versicolor*.

Aim 3) Define the differences in the cell wall composition.

Our data in Chapter 2 and 4 attempt to define the mechanism underlying the distinct inflammatory phenotypes induced by *A. versicolor* and *C. cladosporioides*. We observed differences 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 beta-glucans in *A. versicolor* and *C. cladosporioides*; *A. versicolor* had greater beta-glucan exposure and binding to Dectin-1 than *C. cladosporioides*. Furthermore, we demonstrate that exposure of beta-glucans in *C*.

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cladosporioides increases the ability of these spores to bind to Dectin-1. Further, heat-killed *C. cladosporioides* spores induce a neutrophilic/Th17 phenotype similar to that induced by *A. versicolor*. In the absence of Dectin-1, heat-killed *C. cladosporioides* induce strong AHR, eosinophilia, and Th2 cytokines. Thus, the availability of beta-glucans on the surface of a mold is an important determinant in the type of inflammatory response that ensues.

In the final data chapter we test the **hypothesis that the Th17 response induced by** *A*. *versicolor* would synergize with Th2 responses to induce severe asthma. T

In Chapter 5 we demonstrated that *A. versicolor* is able to synergize with either HDM or *C. cladosporioides* to induce severe asthma. Mice exposed to both *A. versicolor* and HDM or *C. cladosporioides* develop significantly more severe AHR and have greater recruitment of inflammatory cells to the airways as compared to mice exposed to a single antigen. Further, co-exposure to *A. versicolor* and HDM induces a mixed Th2/Th17 response, suggesting a role for induction of Th17 responses in mold-induced severe asthma.

In Chapters 2-5 we have discussed our conclusions and in this chapter we will discuss our findings in the larger context of the current field of knowledge reviewed in the introduction.

Model of Chronic Mold Exposure

In Chapter 2 we developed a model of chronic mold exposure using the molds A. *versicolor* and *C. cladosporioides*. This model has several advantages including (a) utilization of whole spores rather than mold extracts; (b) continuous IT exposure over a period of 3 weeks without use of adjuvants or prior sensitization; and (c) the dose of 10^6 spores was based on air sampling data from the CCAAPS birth cohort (14), and thus ensures a physiological dose. Several other murine models of mold-induced asthma utilize systemic sensitization before challenge through the airways (4, 7, 8, 15). Yet in other murine models, protein extracts from molds are used exclusively (6, 9, 10, 16). Thus, we believe our model better mimics physiological exposure to mold spores due to the use of intratracheal exposure.

In this model, exposure to *C. cladosporioides* induced robust AHR, mixed eosinophilic/neutrophilic airway inflammation, Th2 cells, and elevated levels of serum IgE. Thus, *C. cladosporioides* induces an inflammatory phenotype characteristic of allergic asthma. On the other hand, exposure to *A. versicolor* induced predominately neutrophilic airway inflammation and Th17 cells. There were minimal changes in lung physiology or induction of IgE. Studies suggest that *A. versicolor* and *C. cladosporioides* are capable of inducing distinct immune responses. A study in Finland and another in New York City suggest that patients exposed to *C. cladosporioides* were more likely have elevated IgE titers (17) and develop asthma symptoms as compared to patients exposed to *A. versicolor* (17, 18). In addition, murine studies indicated that *A. versicolor* protein extract had weak Th2 adjuvant activity as compared to protein extract from *Alternaria alternata* (10). Thus, our data are in agreement with the literature, and indicate that *A. versicolor* and *C. cladosporioides* induce distinct pulmonary inflammatory phenotypes.

A. fumigatus and *A. niger* spores are capable of germinating in the lungs of mice (19, 20), but we did not observe germination of either *A. versicolor* or *C. cladosporioides* spores in the lungs. In fact, by six weeks after 1 exposure to spores there were no detectable spores in the lungs of mice. Thus, neither mold establishes infection in the lungs. Further, there was no difference in mold burden or distribution of mold spores. Additionally, it is unlikely that the observed phenotypes are due to differences in spore size (13) since we observed only

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quantitative, but not qualitative, differences in the phenotype with a lower exposure level (10^4 spores) as compared to a higher exposure level (10^6 spores). Strain differences have been reported in chronic models of *A. fumigatus* exposure; BALB/c mice develop neutrophilia and C57BL/6 mice develop eosinophilia (21). However, the asthma phenotype in our model was not strain-dependent as we observed similar findings in both BALB/c and C57BL/6 mice. Therefore, the observed differences in phenotype are not due to differences in mold burden, establishment of infection, spore size, or due to differences in mouse strains.

Basic and Clinical Implications

In the environment we are exposed to whole fungi through the airways, and not protein extracts as some models use. It is highly unlikely that we are sensitized systemically unless molds enter through an open wound. For the most part, sensitization to molds in patients occurs through the airways. Thus, it follows that the best murine model of mold-induced asthma should employ exposure exclusively through the airways, and exposure to mold spores rather than protein extracts. This becomes evident when comparing studies using A. fumigatus mold spores versus protein extracts. In BALB/c mice A. fumigatus extracts induce murine asthma characterized by eosinophilic inflammation, but spores induce neutrophilic inflammation (21, 22). Thus, it becomes difficult to compare different studies using A. fumigatus since many use different combinations of mold extract and spores. Further, knowing extracts and spores can induce distinct inflammatory phenotypes makes it difficult to compare studies since no two studies use the same model. In our model we are confident the distinct inflammatory phenotypes induced by A. versicolor and C. cladosporioides are due to differences in the mold spores and not due to differences in the model such as using extracts versus mold spores. Our model allows confidence that when comparing of different species of mold spores the observed differences are

due to differences the spores. Thus, if future studies of chronic mold exposure use our model, it would ensure accurate comparisons between species.

Our data also have potential clinical implications. 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 since they may or may not successfully target the relevant mold species. We have now developed a murine model of mold-induced asthma that allows for direct comparison of different species of mold. Our data demonstrate that different mold species are capable of inducing distinct inflammatory phenotypes in the lungs, and are in agreement with epidemiologic studies suggesting exposure to *Cladosporium species* is more likely to induce asthma symptoms in patients than exposure to *Aspergillus species* (17, 18). Thus, our data may indicate that it may be more important to prevent exposure to *C. cladosporioides* than *A. versicolor*. Further, our model will allow identification of inflammatory mechanisms in response to these molds, and these pathways could be potential therapeutic targets.

Innate and Adaptive Immune Mechanisms

Pattern Recognition Receptors and IL-17

IL-17A has been implicated in the pathogenesis of asthma and can synergize with IL-13 to induce AHR (23, 24), but has also been shown to be a negative regulator of established murine asthma (25). While the role of IL-17 is controversial in asthma, it is well established that signaling though Dectin-1 induces Th17 cells (26-29), and that both Dectin-1 and IL-17A are necessary for optimal protection from fungal infections (19, 30-34). In a recent study, TLR6
protected from development of *A. fumigatus* induced asthma through induction of IL-17, possibly by regulating Dectin-1 expression (15). Another study, also using *A. fumigatus*, 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 wild type cells (21). Both of these studies suggest a possible role for Dectin-1 in mold-induced asthma.

In Chapter 2 we provide direct support for the role of Dectin-1 in mold-induced asthma, and in Chapter 3 we demonstrate a role for MyD88. Our data reveal IL-17A, Dectin-1, and MyD88 dependent pathways suppress asthma development in mice exposed to A. versicolor spores. Both Dectin-1 and MyD88 contribute to the development of a Th17 response after exposure to *A. versicolor*, and in the absence of Dectin-1 and MyD88 mice exposed to *A. versicolor* develop a Th2 response, eosinophilia, and AHR. Furthermore, blockade of IL-17A resulted in development of robust AHR and eosinophilia, although there was no increase in Th2 cells. However, *C. cladosporioides* induced asthma independent of Dectin-1, MyD88, and IL-17. Our data is supported by previous reports suggesting that IL-17 prevents eosinophil recruitment in asthma, and that IL-17 production is downstream of Dectin-1 and TLR6 (15, 21, 25). In addition, a recent study by Werner et al. demonstrated decreased IL-17A and increased airway eosinophils in Dectin-1, the TLR adaptor protein MyD88, and downstream IL-17 can inhibit eosinophil recruitment and asthma development following exposure to *A. versicolor*.

It would be interesting to further investigate the mechanisms underlying IL-17 blockade of AHR and eosinophilia. IL-13 has been reported to inhibit differentiation of Th17 cells *in vitro* (35), and signaling through the IL-4 receptor inhibits IL-17 production after airway challenge with Ova (25). These studies suggest an inhibitory role of Th2 cytokines on the

development of Th17 cells. The possibility exists that IL-17 may have a similar effect on the development of Th2 cells. One study suggests that IL-17 inhibits Th2 responses by acting on dendritic cells to reduce the Th2 chemoattractant, TARC (25). However, we did not observe an increase in Th2 cells producing IL-13 or expression of IL-4 in the lungs after blockade of IL-17 in *A. versicolor* exposed mice. It is possible that systemic administration of neutralizing IL-17A antibodies did result in complete blockade in the lungs, or that other IL-17 family members were still active, and thus we did not observe an increase in Th2 cells. The use of IL-17 or IL-17 receptor deficient mice would result in more complete blockade of IL-17. We would expect to see an increase in Th2 cells. Another possibility could be the observed increased levels of IL-5 are simply due to increased eosinophil numbers since eosinophils are capable of producing IL-5 (36), and that there is another chemoattractant/cytokine that IL-17 influences and we have not looked at. Thus, our IL-17 data are in agreement with the literature but further studies are necessary to determine how IL-17 inhibits eosinophil recruitment.

Dendritic Cells

Since the Th17 response induced by *A. versicolor* spores is dependent on Dectin-1 and MyD88, we hypothesized that altered upstream expression of Th17 skewing cytokines by *A. versicolor* may be the mechanistic basis for our observations. Stimulation of dendritic cells with curdlan, a Dectin-1 agonist, induces secretion of IL-23, IL-6, and TNF α (37). IL-6 is critical for induction of Th17 cells in the skin and mucosal tissues (38), and IL-23 is needed for survival, expansion, and pathogenicity of Th17 cells (39, 40). The production of TNF α by dendritic cells has a crucial role in regulating IL-17 levels (21). In our studies we observed an increase in production of IL-6, IL-23, and TNF α in dendritic cells after stimulation with either *A. versicolor*

(Chapter 3) or *C. cladosporioides* (Chapter 4), but only in response to *A. versicolor* was production of these cytokines dependent on Dectin-1. Additionally, the production of IL-6, IL-23, and TNF α by dendritic cells after *A. versicolor* exposure was dependent on MyD88. Thus our data suggest a role for dendritic cells in the differentiation of Th17 cells after exposure to *A. versicolor*.

TNF α production by dendritic cells after exposure to *A. fumigatus* may serve as a key regulator in the balance between Th17 and Th2 cells, and dendritic cells from Dectin-1 -/- or MyD88 -/- mice exposed to *A. fumigatus* produce significantly less TNF α than dendritic cells from wild type mice (21). We observed a significant reduction in the frequency of Th17 cells in Dectin-1 -/- mice and MyD88 -/- mice after exposure to *A. versicolor* and increased eosinophilia and Th2 cells. These differences correlate with decreased production of TNF α from dendritic cells deficient in Dectin-1 or MyD88. It would be interesting to explore the role of TNF α in our model of chronic mold exposure. We hypothesize that it is the production of TNF α from dendritic cells that dictates the balance of Th2 and Th17 responses after exposure to *A. versicolor*, and in the absence of PRRs that recognize *A. versicolor* there is a failure of dendritic cells to produce TNF α .

Further experimentation is needed to test this hypothesis, and we propose two experiments. We would propose to neutralize TNF α in our model. By using antibodies against TNF α , we would block the actions of TNF α . If our hypothesis were correct, then we would expect that upon neutralization of TNF α we would observe fewer Th17 cells and an increase in Th2 cells. Additionally, there would be increased eosinophils in the airways and development of AHR. In fact, neutralization of TNF α in a model of chronic *A. fumigatus* exposure decreased production of IL-17 and increased IL-5, resulting in enhanced eosinophil recruitment to the airways (21). Thus, this study provides evidence to support our hypothesis.

Additionally, we propose to test the direct role of dendritic cells. To do this we would take dendritic cells exposed to *A. versicolor* from MyD88 or Dectin-1 deficient mice and transfer them to wild type mice and vice versa. If our hypothesis were correct we would expect that Dectin-1 -/- or MyD88 -/- dendritic cells upon transfer to wild type mice would induce a Th2, eosinophilic phenotype. Further, transfer of wild type dendritic cells to Dectin-1 -/- or MyD88 -/- dendritic phenotype. To demonstrate that it is indeed the decreased production of TNF α from the Dectin-1 -/- and MyD88 -/- dendritic cells, we could also transfer dendritic cells exposed to *A. versicolor* from TNF α -/- mice to wild type mice. We would expect that in this experiment there would be a Th2, eosinophilic phenotype. Thus, data from these proposed experiments would implicate TNF α production by dendritic cells as a critical regulator of the Th2/Th17 balance in response to *A. versicolor* exposure.

Potential Pathways for C. cladosporioides

Even though we have identified the innate immune pathways that *A. versicolor* signals through, namely Dectin-1 and the MyD88 signaling pathway, we did not identify the pathway responsible for the phenotype induced by *C. cladosporioides*. Natural killer T (NKT) cells are a subset of lymphocytes with limited diversity. They recognize glycolipids in the context of CD1, and are considered part of both the innate and adaptive immune system (41). Upon activation NKT cells produce large quantities of IL-4 and interferon (IFN) γ (42-44). As a result NKT cells can shape the immune response, and are critical in the development of asthma. Mice deficient in NKT cells fail to develop AHR, eosinophilia, and Th2 cells in an Ova model of murine asthma

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(45). Further, NKT ligands act as Th2 adjuvants to Ova, and induce an allergic phenotype that is dependent upon NKT cells (46). Lipids are a component of the outer cell wall of *C. cladosporioides* (47), and it is intriguing to speculate that these lipids may activate NKT cells in response to *C. cladosporioides* in our model of mold-induced asthma. In fact, chronic exposure to *C. cladosporioides* induces allergic granulomas (data not shown), similar to those induced by pulmonary exposure to NKT ligands (46). In order to determine the role of NKT cells in *C. cladosporioides* induced asthma we would propose to use NKT cell deficient mice such as the CD1 -/- mice in our model. If NKT cell recognize lipids in the cell wall of *C. cladosporioides*, and promote an allergic response, CD1 -/- mice would fail to develop murine asthma.

Another potential pathway that *C. cladosporioides* may utilize is Dectin-2. As mentioned in the introduction Dectin-2 is a member of the CLR family and it has been shown to recognize α -mannans (48). Several fungi contain mannose residues in their cells walls (49), and ultrastructural analysis of *C. cladosporioides* revealed that the cell wall contained mannose residues (47). It is possible that these mannan residues are α -mannans and are exposed in the cell wall of *C. cladosporioides*, unlike the beta-glucans. The availability of mannoses may allow for recognition of the spores by Dectin-2. Studies have shown that Dectin-2 may have a role in development of allergic responses. Production of cysteinyl leukotrienes by dendritic cells in response to HDM occurs through Dectin-2, and promotes development of allergic responses (50, 51). To test the role of Dectin-2 in *C. cladosporioides*. If Dectin-2 were responsible for the allergic phenotype induced by *C. cladosporioides*, then we would expect mice deficient in Dectin-1 would have an attenuated allergic phenotype. One final pathway that *C. cladosporioides* could potentially utilize is the TLR4 pathway. The TLR4 ligand, lipopolysaccharide (LPS), acts as Th2 adjuvant in a murine model of Ovainduced asthma (52). As already mentioned, the cell wall of *C. cladosporioides* is known to contain lipids (47). It is possible that a lipid in the cell wall could be functionally similar to LPS and trigger signaling through TLR4. Even though we demonstrated that MyD88 mice still develop an allergic phenotype in response to *C. cladosporioides*, it is possible that *C. cladosporioides* could signal through the TRIF pathway that is also utilized by TLR4. To test this we propose exposing TLR4 and TRIF deficient mice to *C. cladosporioides*. If *C. cladosporioides* utilizes TRIF signaling upon TLR4 engagement we would expect that both TLR4 and TRIF deficient mice would display an attenuated asthma phenotype after exposure to *C. cladosporioides*.

Implications

Our data demonstrate that *C. cladosporioides* induces an allergic asthma phenotype that is independent of Dectin-1, MyD88, and downstream IL-17, but in contrast *A. versicolor* induces a Th17 response that can inhibit recruitment of eosinophils to the airways and development of AHR. The development of a Th17 response in *A.* versicolor exposed mice is dependent upon Dectin-1 and MyD88 signaling. These data demonstrate that induction of Th17 responses by *A. versicolor* inhibits the development of an allergic phenotype. They are in agreement with previous reports suggesting that IL-17 prevents eosinophil recruitment in asthma, and that IL-17 production is downstream of Dectin-1 and TLR6 (15, 21, 25, 33). These data add to the current literature and demonstrate that in addition to a protective role in fungal infections, Dectin-1, MyD88, and IL-17 are also protective against mold-induced asthma after exposure to another *Aspergillus* species. It is likely that these pathways are utilized by several other mold species present in the environment.

The role of Dectin-1 in protection from mold-induced asthma may have potential clinical implications. In a family with four women that had recurrent mucocutaneous candiasis a mutation in the Dectin-1 gene was discovered. The Y238X mutation resulted in an early stop codon (53). Further, hematopoietic transplant patients with the Y238X polymorphism in Dectin-1 are at increased risk of developing invasive aspergillosis (54). This polymorphism may also increase the risk of invasive aspergillosis in healthy patients (55). It is conceivable that patients with the Y238X polymorphism may also be at an increased risk of developing asthma after exposure to molds, and thus Dectin-1 may be a possible therapeutic target in asthma.

Differences in Composition of the Cell Wall

A major component of fungal cells walls is beta-glucans, and beta-glucans are commonly used as a marker of mold exposure in the environment (56). Beta-glucans account for up to 60% of the weight of the cell wall (57), and are important for recognition of molds by the immune system. Dectin-1, the receptor for beta-glucans (58), is expressed on macrophages, monocytes, neutrophils, and dendritic cells (59, 60). Signaling through Dectin-1 promotes fungal immunity, specifically by inducing dendritic cells to polarize T cells towards Th17 cells (26, 28, 29, 61). In Chapter 2 we demonstrated that *A. versicolor* induced Th17 cells in a Dectin-1 dependent manner. However, to exposure to *C. cladosporioides* failed to induce Th17 cells, and induced murine asthma independently of Dectin-1. *C. cladosporioides* spores contain significantly more beta-glucans in the cell wall than *A. versicolor* spores (13). In Chapter 2 we showed staining for beta-glucans on *C. cladosporioides* spores indicated that the beta-glucans are only minimally

exposed on the ends of spores at the bud scars. On the other hand, *A. versicolor* spores displayed strong staining for beta-glucans throughout the spore surface. Additionally, we observed strong binding of *A. versicolor* spores to Dectin-1, but could not detect binding of *C. cladosporioides* spores. Thus, our data demonstrate that despite *C. cladosporioides* spores having a greater beta-glucan content than *A. versicolor* spores, the beta-glucans in *C. cladosporioides* spores are mot exposed to the immune system. Therefore, *C. cladosporioides* spores are unable to bind to Detin-1, and induce an inflammatory phenotype independent of Dectin-1.

Several studies indicate that the beta-glucans in mold spores are important for the type of immune response that develops. Removal of the outer rodlet layer, either genetically or chemically, from A. fumigatus spores increases activation of dendritic cells and increases binding of Dectin-1 (62). Heat killed *Candida albicans* spores have greater binding to Dectin-1 than live spores, and the heat-killed spores are better at activating antigen-presenting cells (63, 64). Taken together, these studies indicate manipulation of spore cell walls, either through removal of the outer rodlet layer or through heat killing, exposes the underlying beta-glucans and modulates the immune response. In Chapter 4, we further demonstrated that exposure of beta-glucans can alter the immune response. Heat-killed C. cladosporioides spores display significantly more betaglucans on their surface and thus have greater binding to Dectin-1. Further, heat-killed C. cladosporioides spores induce a neutrophilic inflammatory phenotype characterized by Th17 cytokines similar to the phenotype induced by A. versicolor. In the absence of Dectin-1, heatkilled C. cladosporioides induce strong AHR, eosinophilia, and Th2 cytokines. Thus our data, are in agreement with previous studies, and indicate that altering the surface exposure of betaglucans on mold spores can modulate the immune response.

Furthermore, our data support a mechanistic role for dendritic cells in the observed differences in mold-induced inflammation. In the whole lungs, the presence or absence of Dectin-1 did not impact the induction of pro-inflammatory cytokines by live *C. cladosporioides* spores, but induction of pro-inflammatory cytokines IL-6, IL-23, and TNF α was significantly attenuated in the absence of Dectin-1 following exposure to heat-killed *C. cladosporioides* spores. Dendritic cells deficient in Dectin-1 produced significantly less IL-6, IL-23, ad TNF α in response to heat-killed *C. cladosporioides* spores, similar to the response to *A. versicolor* spores. Thus, the data in Chapter 4 agree with the data from Chapter 3, and further support a role for dendritic cell production of TNF α in the Th17 phenotype.

Implications

Quantification of beta-glucans in the environment is often used as a surrogate for mold exposure (65-67). Several studies have examined the direct relationship between beta-glucans and health effects with inconsistent results (57). One study found that low levels of beta-glucans were positively correlated with a positive asthma predictive index (API), but high levels of betaglucans were negatively correlated with a positive API (68). A study in Germany similarly found a negative association between beta-glucan levels in mattress dust and sensitization to aeroallergens (67). Another study also found a negative correlation since there was a trend towards increased IFNγ in patients exposed to beta-glucans rather than Th2 cytokines (66). Studies examining airway function and inflammation also contradict each other (57). The inconsistencies in the literature may be explained by the fact that it is not the amount of betaglucans present, but rather the surface availability of beta-glucans on the mold spores in the samples taken. Our data from Chapters 2 and 4 now add to the current literature and demonstrate that differences in exposure of surface beta-glucans in the same species of mold can alter the immune response in a Dectin-1 dependent manner. Additionally, our data suggest that when quantifying mold exposure, it is important to consider development of 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. In fact, in the environment it is likely that exposure of beta-glucans in the cell walls of fungi change during adaptation to stress. Fungi are adept at adapting to environments with differing levels of ionizing radiation, and produce melanin to protect from stressors in the environment such as radiation (69). Fungi growing in environments where they are exposed to greater radiation, such as high altitude regions, produce significantly more melanin than their counterparts growing in environments with less radiation (70). Further, many fungi can grow on space craft where they are exposed to high levels of radiation (71), and it is likely these fungi produce significantly more melanin than the same species growing on earth. As mentioned in the introduction, this layer of melanin is incorporated into the rodlet layer and covers the underlying beta-glucans (47, 62). These studies lead one to speculate that people living in high altitudes, or astronauts, develop different immune responses to fungi than people living at lower altitudes due to differences in beta-glucan exposure in the mold spores. This could have important implications when assessing environmental exposures and their contributions to disease.

Severe Asthma

Pathogenic fungi are potent inducers of Th17 responses (19, 27, 37), and several species of molds have been associated with severe asthma (72-76). Further, there is an association between IL-17 and overall asthma severity in patients (77-82), but as previously mentioned, the

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role of IL-17 in murine studies remains controversial. IL-17A has been shown to contribute to murine asthma, possibility through synergy with IL-13 (23, 24), but has also been shown as a negative regulator of murine asthma (25). In Chapter 2 we demonstrated that *A. versicolor* induces a predominantly Th17 responses, which can inhibit development of AHR and eosinophilia. This data is in agreement with a previous study also showing that IL-17 is a negative regulatory of eosinophilia (25). However, given that IL-17 can also synergize with IL-13 when administered directly to the airways to induce AHR (24), we hypothesized that molds that induce Th17 responses, in particular, *A. versicolor*, could synergize with antigens that induce Th2 responses.

In Chapter 5 we first took advantage of the Greater Cincinnati Pediatric Clinical Repository (GCPCR) to examine the relationship between mold sensitization and severe asthma. The GCPCR is a large pediatric cohort that has previously been described (83). Comparison of patients from this cohort that were sensitized to HDM alone or HDM and molds revealed that patients with co-sensitization had higher mean symptom scores. This data is consistent with studies in adult populations demonstrating an association between mold sensitization and hospital admissions (74, 84), and thus symptom severity. Our analysis reveals that even in pediatric patients molds may contribute to severe asthma.

However, as mentioned in the introduction, it is unclear how molds contribute to severe asthma. In Chapter 5 we employed a murine model of co-exposure to *A. versicolor*, a Th17 inducer, and either HDM or *C. cladosporioides*, both of which induce Th2 responses. Co-exposure to *A. versicolor* and HDM or *C. cladosporioides* induced greater AHR and airway inflammation compared to either exposure alone. These data are consistent with a study demonstrating that the addition of *A. fumigatus* extract to HDM and ragweed exposure in a

murine model of asthma was able to overcome development of tolerance and induce sustained inflammation (85). Thus, exposure to molds in murine models is critical to development of severe murine asthma.

Additionally, mice co-exposed to both *A. versicolor* and HDM developed a mixed Th2/Th17 responses. Our data are consistent with a report that development of severe murine asthma is associated with production of both Th17 and Th2 responses, and blockade of IL-17A attenuates development of severe asthma (24). It would be intriguing to further test the contribution of IL-17 to the severe phenotype by blocking IL-17 either through neutralizing antibodies or through the use of IL-17 cytokine or receptor deficient mice. We have also shown that the production of IL-17 is downstream of Dectin-1 and the TLR pathway (Chapter 2 and 4), and we could also test the role of Dectin-1 and the TLR pathway in severe mold-induced asthma.

Implications

Our data demonstrating that *A. versicolor* is able to synergize with both *C. cladosporioides* and HDM is seemingly contradictory to the data reported in Chapter 2 that IL-17 inhibits eosinophil recruitment and AHR. However, the literature concerning the role of IL-17 in asthma is still controversial. In *A. fumigatus* models of murine asthma TLR6 protected from development of asthma through induction of IL-17, possibly by regulating Dectin-1 expression (15). 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 wild type cells (21). Both of these studies suggest a possible role for Dectin-1 and IL-17 in mold-induced asthma. Thus our data in Chapter 2 is in agreement with this literature. We directly demonstrate that Dectin-1 inhibits eosinophilia and AHR, likely through IL-17.

On the other hand, Th17 cells can enhance antigen specific T cell activation (86) and enhance the effects of Th2 cytokines induced murine asthma (87). IL-13 and IL-17A administration directly to the airways results in a synergistic induction of AHR (24), suggesting that this synergy accounts IL-17 enhancement of Th2 responses. Our data in Chapter 5 seems consistent with these studies. Co-exposure to *A. versicolor*, a Th17 inducer, and either HDM or *C. cladosporioides*, both of which are Th2 inducers, promotes synergistic AHR and inflammatory responses. Further, the observed synergy in mice co-exposed to *A. versicolor* and HDM is associated with a mixed Th2/Th17 responses. A mixed Th2/Th17 response in experimental murine asthma is associated with a more severe phenotype than mice that develop only a Th2 response (24, 88). Thus, our data in Chapter 5 are also consistent with already published literature.

Our data reveal that there is a need for further investigation of the role of IL-17 in asthma. Perhaps, IL-17 is necessary for initiation of asthma but can inhibit established asthma as suggested in the study by Schnyder-Candrian et al (25). It may be that the presence of *A*. *versicolor* is only necessary during the sensitization phase of experimental asthma. If exposure only occurs during the challenge phase then it is plausible that the Th17 response induced by *A*. *versicolor* will attenuate experimental murine asthma. In order to test this we propose during our three-week model to only co-expose mice during either the first week or the last week of exposure. If the Th17 response is critical during the sensitization but not the challenge phase then mice co-exposed only during the first week will develop severe asthma. Mice co-exposed during the last week will not develop severe asthma, and will possibly have attenuated asthma. This could also be tested through the use of IL-17 blocking antibodies. Blocking antibodies

during the first week of exposure should attenuate the severe asthma in co-exposed mice, but blockade during the last week of exposure should result in a more severe phenotype.

Dectin-1 can inhibit development of Th1 responses after exposure to *A. fumigatus* (27); it is almost as if the Th1 pathway is the default pathway and differentiation of Th1 cells only occurs if the Dectin-1 pathway is absent. Our data suggest something similar for Th2 cells. Dectin-1 inhibits development of Th2 responses when signaling is triggered either by *A. versicolor* or heat-killed *C. cladosporioides* spores. When Dectin-1 is absent, the spores induce a Th2 response by default. In the case of live spores, it is likely that Dectin-1 is never triggered due to lack of exposure of beta-glucans. However, in the case of co-exposure of *A. versicolor* with HDM or *C. cladosporioides* there is a strong trigger to induce the Th2 and the Dectin-1/Th17 pathways and therefore a mixed response develops. WE believe that synergy develops because there is induction of both types of responses. It would be intriguing to study the role of Dectin-1 in our co-exposure model. We would hypothesize that Dectin-1 contributes to the development of the Th17 response, and in Dectin-1 deficient mice there would not be a synergistic response due to the lack of Th17 responses.

There are significant potential clinical implications of our data in Chapter 5. Increased levels of IL-17 are associated with increased neutrophilic inflammation, decreased lung function, increased airway hyperresponsiveness to methacholine, and overall asthma severity (77-82). These patient data are consistent with our murine co-exposure model of severe asthma demonstrating increased IL-17 and neutrophils in mice co-exposed to both *A. versicolor* and HDM. Recently, the CCAAPS cohort identified three mold species that were significantly associated with asthma: *A. ochraceus, A. unguis, and P. variabile* (89). Interestingly, there was no association between exposure to *C. cladosporioides* and asthma in this cohort (89), even

though our data show that exposure to *C. cladosporioides* induces murine asthma. However, the study also did not find associations between house dust mite and asthma (89), which is also associated with asthma (90-93), and is commonly used in murine models of asthma (6, 24, 88, 94, 95). The possibility exists that in this population HDM and *C. cladosporioides* are inducing Th2 responses in the patients, but exposure to the three molds identified induce Th17 responses, and the two responses synergize to induce asthma. It would be intriguing to measure IL-17 levels in the sputum from patients exposed to these three molds to test this possibility.

Glucocorticoids (steroids) are the first-line treatment for asthma, but there is a subset of patients who exhibit disease that is resistant to steroid treatment (96). Patients with either neutrophilic or mixed granulocytic asthma appear to have steroid resistant asthma since they greater use of inhaled corticosteroids and demonstrate less improvement after steroid use than patients without neutrophilic inflammation (97, 98). Importantly, steroid treatment has no effect on Th17 responses or neutrophilia (99). Our data suggest that mold exposure contributes to the development of the Th17/neutrophil axis in asthma. Co-exposure to A. versicolor and HDM or C. cladosporioides results synergistic induction of severe asthma, and in the case of co-exposure to A. versicolor and HDM in a mixed Th2/Th17 phenotype that is also associated with mixed granulocytic (both neutrophils and eosinophils) airway inflammation. Further, patients in the GCPCR that were sensitized to both mold and HDM had higher mean symptoms scores than patients that were sensitized to HDM alone, suggesting that in this pediatric patient population mold contributes to severe asthma. Thus, our data indicates that when assessing patient severity it may be important to determine environmental exposures, and attempt to treat those exposures. Anti-fungal use in asthma patients with mold exposure has shown efficacy (100). Our data from Chapter 5 also suggests that A. versicolor may be an important contributor to asthma. Therefore,

avoidance of *A. versicolor* may be just as important as avoidance of *C. cladosporioides* unlike what we discussed in reference to our data in Chapter 2.

Putting it all Together:

In this thesis we demonstrated that the ability of mold spores to bind to Dectin-1 can modulate the type of immune response that develops. *A. versicolor* has greater surface exposure of beta-glucans than *C. cladosporioides* and therefore binds to Dectin-1 with greater affinity. Binding to Dectin-1 leads to the development of a Th17 response in the lungs of *A. versicolor* exposed mice. On the other hand, *C. cladosporioides* induces a predominately Th2, allergic response through a yet unidentified pathway. However, when surface exposure of beta-glucans in *C. cladosporioides* spores is increased through heat killing these spores are able to bind to Dectin-1 and induce a Th17 response. Finally, we demonstrated that activation of both the Th2 and Th17 pathways through exposure to both *C. cladosporioides* (or HDM) and *A. versicolor* induces a synergistic airway response resulting in a more severe asthma phenotype.

Molds have been implicated in the development of severe asthma (74, 84, 101, 102). In fact, in Chapter 5 we demonstrated the trend that pediatric patients sensitized to both mold and HDM have more severe asthma than patients sensitized to HDM alone. Further, a murine model demonstrated that the administration of mold extract in addition to ragweed and HDM extract broke through the development of tolerance in an asthma model (85), suggesting that molds may also be involved in more severe asthma in murine models. Based on the data in this dissertation we hypothesize that molds are important in the development of severe asthma due to their ability to induce Th17 responses, since Th17 cells and IL-17 have been implicated in severe asthma in both patients and animal models. (77, 79, 103, 104).

We clearly demonstrated that co-exposure to both *C. cladosporioides* and *A. versicolor* induced a synergistic response in AHR and airway inflammation. Additionally, HDM, which also induces Th2 responses, can also synergize with *A. versicolor*. However, we did not determine if induction of Th17 responses by another exposure besides *A. versicolor* could also synergize with *C. cladosporioides*. Since we demonstrated that heat-killed *C. cladosporioides* spores induce a Th17 response it would be interesting to determine if co-exposure to live and heat-killed *C. cladosporioides* spores would also result in a synergistic induction of a severe asthma phenotype. We would hypothesize that co-exposure to both live and heat-killed *C. cladosporioides* that co-exposure to both live and heat-killed *C. cladosporioides* spores would also result in a synergistic induction of a severe asthma phenotype. We would hypothesize that co-exposure to both live and heat-killed *C. cladosporioides* would result in synergistic development of severe asthma through triggering of both the Th2 and Th17 pathways.

It is interesting to note that the response to heat-killed spores was not a solely Th17 response. There was still a residual Th2 response, although not as robust as live *C. cladosporioides*, and no IgE response to heat-killed *C. cladosporioides*. It may be that this residual Th2 response is not strong enough to synergize with the Th17 response to induce severe asthma. Exposure to *A. versicolor* spores can also induce some mild eosinophilia and Th2 cells, albeit the induction of Th2 cells is significantly lower that induction of Th2 cells after exposure to live *C. cladosporioides* spores (Chapter 2; Figs. 1 and 3). Yet, mice exposed to *A. versicolor* do not develop robust AHR unless IL-17 is neutralized (Chapter 2; Figs 1 and 4). Interestingly, neutralization of IL-17 does not affect induction of Th2 cells for the development of AHR in our model of mold-induced asthma. Therefore, it is only in the setting of a robust eosinophil response, such as co-exposure to both *C. cladosporioides* and *A. versicolor*, that a synergistic response develops.

These observations have led us to speculate the on effect that heat killing has on C. *cladosporioides* spores. Even though heat killing exposes beta-glucans and thus increases the Th17 response through Dectin-1, as previously mentioned there is still a residual Th2 response. This response appears to be greater than the mild Th2 response induced by *A. versicolor*. Thus by heat killing the *C. cladosporioides* spores we are not changing the cell- wall composition to exactly match the composition of the cell of A. versicolor, but we are changing it from the normal composition. Surface hydrophobins, melanin, lipids, and an extracellular mucus lay are all important components of the outer fungal cell wall (1, 47, 49, 105). This outer layer can cover up the underlying beta-glucans (62). It is entirely possible that in the process of heat killing the spores components of the outer cell wall are denatured and can no longer cover up the betaglucans, which remain stable during the heat killing process. However, simply uncovering the beta-glucans is likely not sufficient to induce a Th17 response. Melanin is an important component of the cell wall of C. cladosporioides (47), and likely covers the beta-glucans in cell wall. We have data demonstrating inhibition of melanin formation by growing C. *cladosporioides* on a medium containing tricyclazole exposes the beta-glucans in the cell wall of C. cladosporioides. However, melanin deficient C. cladosporioides spores do not induce a Th17 response dependent on Dectin-1 (data not shown). Thus, it is likely that another process is occurring during heat killing that contributes to the observed differences in phenotype.

Many fungi contain a number of different proteases, and proteases are important for moldinduced asthma in an animal model using *Aspergillus niger* (20). While we have not analyzed the protease content of *C. cladosporioides* or *A. versicolor* it is likely that both of these spores do contain proteases, and it is possible that the proteases contained in the *C. cladosporioides* spores contribute to the asthma phenotype while the ones in *A. versicolor* do not. However, heat killing may denature the proteases in *C. cladosporioides* making them inactive. Thus, it is the combination of inactive proteases and beta-glucan exposure that accounts for the induction of Dectin-1 dependent Th17 responses in heat-killed *C. cladosporioides* spores.

Thus our data now add to the currently literature and demonstrate that not all molds are created equal. We propose a model (Figure 1) in which exposure of beta-glucans on *A. versicolor* promotes 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 utilized, which promotes a Th2 response and the development of an asthma phenotype Furthermore, co-exposure to mold capable of inducing Th17 responses can synergize with other allergens, which induce Th2 responses, to induce severe asthma. Yet, there are still many unanswered questions, such as determining what is changing in the heat-killed *C. cladosporioides* spores to induce an inflammatory response that is Dectin-1 independent. Further, applying the models developed in this dissertation to other molds could provide important insights into the mechanisms through which other molds act to induce inflammation. Thus, assessment of environmental exposures, in particular to different types of mold, will be important when determining the correct treatments for severe asthma. Exposure to mold could mean that anti-fungal agents are a viable treatment option.

^a Figure 1. Exposure of beta-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. Beta-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. When both Th2 and Th17 responses are triggered there is synergistic induction of severe asthma.



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