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Immunoprotection Versus Immunopathogenesis Associated With Aspergillus Fumigatus Exposure

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IMMUNOPROTECTION VERSUS IMMUNOPATHOGENESIS ASSOCIATED WITH *ASPERGILLUS FUMIGATUS* EXPOSURE

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ABSTRACT

Opportunistic fungus *Aspergillus fumigatus* can cause a wide range of disorders depending on the immunological state of the host. In individuals lacking a robust innate immune response, invasive aspergillosis (IA) within the lungs can occur. In contrast, patients with a hyperreactive immune response can develop an allergic disorder called Allergic Bronchopulmonary Aspergillosis (ABPA). Using mouse models, we investigated contributing factors of host defense in IA and fungal asthma induced by *A. fumigatus*.

The connection between fungi and the development of allergic disease has long been of interest. Previously, we have shown that beta-glucan receptor, dectin-1, is required for clearance during an invasive *A. fumigatus* infection. In this study, we show that dectin-1 and dectin-1-dependent IL-22 contribute to immunopathogenesis during chronic *A. fumigatus* exposure. Dectin-1-deficient mice displayed better lung function post-exposure that correlated with a reduction in pro-inflammatory and pro-allergic mediators in the lung. Assessment of purified CD4+ T cells revealed that Th2 cytokines and IL-17A were produced in a dectin-1-dependent manner. In contrast, Th17-related cytokine IL-22 was produced in a dectin-1-dependent manner in unfractionated lung cell cultures. Challenge of IL-22 knockout mice resulted in a phenotype that mimicked the dectin-$1^{-/-}$ mice. Moreover, neutralization of IL-22 improved lung function. Together,

the data suggest that dectin-1 and IL-22 contribute to pathology during a model of fungal asthma.

As neutrophils have been shown to be critical in host defense during invasive aspergillosis, we sought to determine the importance of other cell types during infection. We show that during an *A. fumigatus* infection, eosinophils are recruited to the lungs. Using eosinophils-deficient dblGATA-1 knockout mice, we demonstrate a requirement for eosinophils in optimal clearance. Elevated fungal burden observed in dblGATA-1-deficient mice was accompanied with reduced levels of *Epx* and *Prg2*. As a defect in other cellular recruitment was not observed in eosinophil-deficient mice, we investigated the killing ability of eosinophils generated from bone marrow. We show that eosinophils are able to respond to *A. fumigatus* through pro-inflammatory and Th2 cytokine production and control fungal growth in vitro. Collectively, our data shows a crucial role of eosinophils in host defense during a mouse model of IA.

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DEDICATIONS

I dedicate this dissertation to my awesome parents. Without their never-ending support, I never would have known what I am capable of achieving.

I also dedicate this paper to my best friend, my brother, who is always near me even when he isn't, and to the two best young men in the world, my nephews.

I love you guys.

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INTRODUCTION

Aspergillus fumigatus

Characteristics of A. fumigatus.

Aspergillus species are globally ubiquitous, filamentous fungi. With approximately 200 species in the genus, *Aspergillus* is a leading cause of human fungal ailments with *Aspergillus fumigatus* as the main causative agent of invasive fungal diseases. Although found most prevalently in soil and compost, *A. fumigatus* is a fairly omnipresent organism and can be found in pepper, tea, tap water as well as indoor air of homes and offices, thus further increasing the risk of exposure [1-3]. Indeed, with a conidial concentration of 1- 100 m^3 in the air and a size of 2-3 μm, individuals are exposed to hundreds of conidia on a daily basis [4].

Growing in a distinctive branching pattern, *A. fumigatus*, a saprophytic, asexual fungus, has two forms: the conidial and hyphal form. The conidia, spherical in shape, is the reproductive agent while the hyphae, with its stalk-like appearance, is responsible for the tissue damage commonly seen in disease. A robust organism, *A. fumigatus* can growth in a wide temperature range of 22ºC-55ºC with optimal growth occurring at 37ºC, which is the temperature of the human body [5, 6].

A. fumigatus Virulence Factors

Melanin. Although lacking sophisticated virulence and evasion factors of traditional pathogens, *A. fumigatus* uses the cooperation of seemingly innocuous properties to avoid and survive within the human body. In fungi, melanin, the compound

responsible for pigment, has been ascribed as a pathogenic factor. *A. fumigatus* conidia typically appear grey-green in color due to pigment 1, 8-dihydroxynapthalene-melanin [7, 8]. The identification of the polyketide synthase (*pskP/alb1*), the gene responsible for pigment, has led to the development of a white, mutant strain of *A. fumigatus.* Through UV mutagenesis or deletion of the *pskP/alb1* gene, melanin has been shown to be a contributor to pathogenesis; compared to a complemented wildtype strain, mutant conidia displayed structural cell wall differences and increased susceptibility to H_2O_2 and NaOCl [9]. Moreover, mutant conidia stimulated greater production of reactive oxygen species (ROS) from human monocytes and neutrophils with melanin being shown to have the ability to scavenge ROS from the culture media. Additional studies further showed that human macrophages infected with the *pskP* mutant strain had increased killing due to increased acidification of the phagosome [10]. Moreover, in vivo infection models, both systemic and intranasal, indicated an overall reduction in virulence for the melanin-mutated strains [10, 11].

The Rodlet Layer. The surface of *Aspergillus* conidia is a complex structure of proteins, a polysaccharide matrix, as well as glycosylphosphatidylinositol (GPI)-linked proteins (Figure 1). The outer surface, the rodlet layer, is composed of proteins RodA and RodB encoded by *rodA* and *rodB* genes, respectively. The rodlet proteins protrude from the surface and contribute hydrophobicity to the conidia, allowing for conidial dispersion. In addition to increasing dispersion and ultimately inhalation by hosts, both in vitro and in vivo studies suggest that rodlets may directly contribute to virulence. Deletion of *rodA* in conidia, but not the *rodB* gene, resulted in conidial hypersensitivity to killing by alveolar macrophages; however, this enhanced killing did not impact outcome

during a mouse model of pulmonary infection [12, 13]. The *rodA* mutant also exhibited decreased binding to collagen and albumin, but not laminin, fibrinogen or pulmonary epithelial cells [4, 13]. Importantly, RodA plays a role in the masking of beta-glucans in conidia; *rodA* mutants had enhanced surface exposure of β -1, 3-glucans and enhanced dectin-1-mediated responses in a mouse model of *Aspergillus* corneal infection. [14].

Glycans and Polysaccharides. Underneath the rodlet layer lies a collection of glycans including beta- and alpha-glucans, chitin, galactomannan, and sialic acid that comprises the cell wall [15]. Various studies have associated these molecules with adhesion. Wasylinka et al demonstrated that *A. fumigatus* conidia bound to the glycosaminoglycan binding domain of fibronectin. In the same study they demonstrated that treatment with negatively charged carbohydrates blocked observed binding, suggesting that negatively charged polysaccharides may play a role in adhesion. Furthermore, in addition to adhesion, sialic acid has been associated with dispersion and alveolar deposition [15, 16]. Treatment of *A. fumigatus* with sialidase resulted in agglutination and decreased fibronectin adherence. Moreover, sialic acids may be a correlate of virulence; sialic acids are present in a higher density on *A. fumigatus* as compared to less virulent *Aspergillus* species [17].

Toxins. *A. fumigatus* can produce a variety of toxins depending on its environment with the best characterized toxin being gliotoxin. Gliotoxin is a defense of the hyphae and has long been thought to possess pathogenic activity. Indeed, gliotoxin-producing strains of *A. fumigatus* appear more virulent those that lack gliotoxin expression [18]. In vitro, gliotoxin has inhibiting properties such as blocking macrophage phagocytosis, mitogen-activated T-cell proliferation, mast cell activation,

and cytotoxic T-cell responses [19-23]. In addition to immune cells, gliotoxin can target epithelial cells, slow ciliary beating and induce cellular damage [24]. Furthermore, gliotoxin has been shown to prevent the formation of the NAPDH oxidase complex in human neutrophils [25, 26].

In vivo, mutant strains were employed to ascribe a role for gliotoxin. The deletion of two genes involved in gliotoxin biosynthesis, *gliZ* and *gliP*, yields mutants that lack gliotoxin expression [27, 28]. In a cortisone-treated non-neutropenic mouse model of invasive aspergillosis, reduced disease was observed with the *gliP* mutant strain as compared to wildtype. Moreover, mice infected with the mutant *gliP* strain displayed reduced neutrophil apoptosis [29, 30]. Conversely, in a neutropenic model of invasive aspergillosis, *gliZ* and *gliP* mutant strains were shown to be as virulent as wildtype [31]. Collectively, the data suggest that gliotoxin contributes to virulence through impacting the role of neutrophils during defense, most likely by promoting neutrophil apoptosis. The role of other toxins in the pathogenesis of invasive aspergillosis has not been well-characterized. Restrictocin is a ribonucleotoxin encoded by the *res* gene. While deletion of the *res* gene did not have an effect in vivo in mouse models of IA, hyphae from mutant *res* strains exhibited more neutrophil-mediated damage as compared to wildtype strains [32]. Thus, like gliotoxin, restrictocin may contribute to virulence in non-neutropenic models.

Diseases of *A. fumigatus*

Ubiquitous but also largely benign, humans are exposed to and inhale hundreds of *A. fumigatus* conidia on a daily occurrence. In immunocompetent individuals, the innate immune system is sufficient to provide clearance. Disease occurs, however, when the

host response is aberrant; response to inhaled conidia is either too strong or too weak. *Aspergillus* can cause a wide range of diseases including saprophytic, allergic, and invasive disorders depending on the immunological state of the host as well as the virulence of the encountered pathogen (Figure 2). Moreover, due to the advent of immunosuppressive and myeloablative therapies, cases of *Aspergillus*-related disorders are on the rise [33].

Invasive Aspergillosis

The use of chemotherapy and immunosuppressive drugs has led to an increased incidence in IA [34, 35]. From 1978 to 1992 the rate of invasive mycoses increased by 2.7% as observed in autopsies [36]. With a mortality rate exceeding 50% in neutropenic patients and 90% in hematopoietic stem-cell transplantation (HSCT) recipients, invasive pulmonary aspergillosis is increasingly becoming a problem. Immunodeficiencies are the main risk factors for disease, including neutropenia, organ transplantation, prolonged corticosteroids, advanced AIDS and chronic granulomatuous disease with neutropenia being the most important (Table 1). Indeed, the risk of developing IA in neutropenic patients increases by 1% each day for the first 3 weeks; after 3 week, the risk of disease increases by 4%. Treatment for IA includes first-line defense voriconazole as well as caspofungin, itraconazole and posaconazole.

Soluble Factor Immune response. The immune response to inhaled conidia is largely driven by the components of the innate immune responses (Figure 3). Soluble antimicrobials released by immune and epithelial cells include surfactant proteins, lactoferrin, lysoszyme and defensins [reviewed in [37]]. Iron scavenger lactoferrin inhibits conidial germination by sequestering free iron required for growth [38].

Moreover, surfactant proteins SP-A and SP-D, produced by type II pneumocytes, act as opsonins during infection, promoting phagocytosis and modulation of the inflammatory cytokine milieu [39]. Specifically, administration of SP-D to immunosuppressed mice protected mice from a fatal dose of *A. fumigatus* [40].

Conidia that escaped these soluble factors go on to swell and germinate, recruiting a response from innate cells such as alveolar macrophages, dendritic cells, and neutrophils.

Alveolar Macrophages. Upon inhalation of conidia into the lungs, alveolar macrophages are the first line of defense. Engagement of receptors generates production of inflammatory cytokines and chemokines such as TNF-α, IL-1β, IL-6, IL-8, MIP-1α, and MCP-1 [reviewed in [41]]. Moreover, the significance of pattern recognition receptors (PRRs) present on alveolar macrophages including toll-like receptors and dectin-1 have been uncovered through various in vivo and in vitro studies. In vitro recognition studies revealed that both conidia and hyphae can bind TLR2 while TLR4 has been shown to recognize only the hyphal form of *A. fumigatus* [42]. Studies utilizing TLR-deficient mice demonstrate a role for both TLR2 and TLR4 in vivo; neutropenic $TLR2^{-/-}$ and $TLR4^{-/-}$ mice display higher fungal loads compared to wild-type mice [43, 44].

Another receptor expressed on alveolar macrophages, dectin-1, has been shown to be required for host defense against *A. fumigatus* [45]. Dectin-1 binds beta-glucans present on the fungal wall; β-1, 3-glucan is increasingly exposed as the conidia swell and germinate conidia (Figure 4). Engagement of dectin-1 promotes killing partly through phagocytosis and activation by macrophages with alveolar macrophages being the main cell type that phagocytose inhaled conidia. Killing of inhaled conidia is mediated partly

through acidification of the phagolysosome and ROS production [46-48]. While ROS has been shown to play a role in killing, in contrast, reactive nitrogen species have not been shown to be required for killing [49].

Neutrophils. In the event that the macrophage is overloaded and cannot prevent conidial swelling and germination, neutrophils are recruited by CXCL2 (MIP-2) and CXCL1 (KC). Neutrophils, through use of multiple receptors including TLRs and dectin-1, bind and kill hyphae through degranulation, release of oxidatives mediated by NADPH oxidase and nonoxidative elements [50]. Nonoxidative mediators include antimicrobials from neutrophilic granules, including defensins, pentraxin-3, lysozyme, and lactoferrin. Recruited in the lungs by swollen conidia, neutrophils can aggregate around both conidia and hyphae [51]. Binding and phagocytosing of swollen conidia leads to ROS release as well as degranulation. While hyphae are too large to be phagocytosed, neutrophils can bind and destroy hyphae by both oxidative and nonoxidative mediators.

Oxidative mediators have been shown to play a protective role during infection as mice deficient in gp91phox are unable to control infection in a model of invasive aspergillosis [52]. Specifically, while macrophages from gp91phox knockout mice display fungicidial activity, neutrophils from mice lacking gp91phox are unable to inhibit the growth of the organism in vitro, highlighting the crucial role of neutrophil-derived ROS [53]. Moreover, neutrophils isolated from chronic granulomatous patients also show impaired killing [53]. In addition to ROS, myeloperoxidase is an important neutrophil-derived effector molecule. An enzyme stored within neutrophilic granules, myeloperoxidase catalyzes the reaction of hydrogen peroxide and chloride anion to form

hydrocholorous acid [54]. Similar to gp91phox-deficient mice, immunocompetent myeloperoxidase knockout mice are susceptible to infection [53].

Neutrophil-derived cytokines also have an essential role during host defense. Neutrophils are a source of IL-17A in a non-immunosuppression model of invasive aspergillosis [45]. Werner *et al* demonstrated that neutrophil-derived IL-17A, produced in a dectin-1 dependent manner, is required for host defense against *A. fumigatus* in a mouse model of IPA [45]. Mice lacking dectin-1 had elevated fungal burden levels that correlated with reduced lung IL-17A levels. Moreover, neutralization of IL-17A in infected wildtype recapitulated the phenotype observed in the dectin-1 knockout mice, suggesting a crucial role for the cytokine in a mouse model of pulmonary invasive aspergillosis. Furthermore, patients with defects in IL-17A have been shown to have increased susceptibility to fungal invasion, supporting the data generated from mouse models [55].

***Dendritic Cells*.** Although dendritic cells are largely documented to play a role in shaping the adaptive response, during a mouse model of pulmonary IA, dendritic cells modulate the course of infection through promotion of Th17-related cytokines. Dectin-1 engagement on dendritic cells promotes IL-23 production in the lungs while neutralization of IL-23 in vitro leads to impaired IL-17A production [56]. Thus, dendritic cell-derived IL-23 is crucial in order to promote IL-17A responses during infection. In the event that the infection is not cleared in a few days, dendritic cells play a pivotal role in shaping the adaptive T-cell response.

Epithelial cells and Eosinophils. Epithelial cells are the first cells to encounter the invading conidia yet their role during an *Aspergillus* infection has received

considerably less attention compared to classically-defined immune cells. In mice, histological staining reveals uptake of fungal elements by ciliated airway epithelial cells [reviewed in [57]]. Moreover, human alveolar epithelial cell line A549 has been shown to bind both conidia and hyphae and ingest conidia [58, 59]. Recognition and binding of conidia and hyphae by epithelial cells resulted in IL-6 and CXCL8 expression. Eosinophils, a cell type commonly associated with allergy including allergic bronchopulmonary aspergillosis (ABPA), may also play a role in controlling an invasive fungal infection. In vitro, purified eosinophils from allergic mice have been shown to inhibit fungal growth of *Aspergillus niger* [60]. Moreover, eosinophil-deficient dlbGATA1$^{-/-}$ mice show a delay in clearance of *A. fumigatus* from the lungs at 24hr and 48hr post-infection in a mouse model of IPA. [manuscript submitted]. In the same study, the increased susceptibility observed in dlbGATA1 knockout mice correlated with a reduction in gene expression of eosinophil-specific genes *Epx* and *Prg2*.

***Allergic Bronchopulmonary Aspergillosis*.**

Allergic Bronchopulmonary Aspergillosis (ABPA) is an allergic disorder characterized by a hypersensitivity reaction to *Aspergillus.* The most common species associated with ABPA is *A. fumigatus*. ABPA typically only occurs in individuals with a pre-existing lung disorder, usually in asthmatic and cystic fibrosis patients. While it is estimated that 2% of asthmatics and up to 15% of cystic fibrosis patients are afflicted with ABPA, ABPA remains under-diagnosed [61].

Clinical Presentation. Sensitization to *Aspergillus* antigens is a common feature of asthmatics with 28% displaying sensitization [62]. In those individuals that were sensitized to *Aspergillus* antigens, increased severity of asthmatic symptoms was

observed [63]. However, sensitization alone is not sufficient for diagnosis. The criteria for ABPA are shown in Table 3. Patients suffering from ABPA are colonized (chronically or sporadically) with *A. fumigatus*, experience exacerbations of asthma, have elevated total IgE as well as fungus-specific IgE levels. Thus, diagnosis of ABPA is typically confirmed through both radiological and serological testing. At early stages of the disorder, chest radiographs may appear normal however as the disease progresses, pulmonary infiltrates within the upper and central lobes appears. Total IgE levels elevate (>1000 UL/ml) and positive sputum cultures may occur; bronchial washes, in addition to increased IgE, may also show elevated eosinophils counts [64]. In early stages of the disorder, pulmonary function tests show reversible lung obstruction that can become irreversible at later stages. Moreover, central bronchiectasis and pulmonary fibrosis can also develop at late stages. ABPA can be divided in to subgroups: ABPA with central bronchiectasis (ABPA-CB) and ABPA without central bronchietasis (ABPA-seropositive).

Pathology. It not clearly understood what predisposes certain individuals to develop ABPA. In asthmatic and cystic fibrosis patients, increased susceptibility to ABPA is thought to be due to the thick mucus layer often present in such patients; inhaled conidia are not cleared but germinate and grow in to hyphae. This germination leads to a high level of antigen load in the lung, recruiting cellular influxes into the airways. Genetic factors linked to the pathogenesis of ABPA are shown in Table 4.

Treatment. Currently, oral corticosteroids are the main course of treatment. Corticosteriods suppress the inflammatory and allergic response due to the organisms without promoting clearance. For most patients with ABPA, long-term, low-dose

treatments will be required to controls symptoms [65, 66]. In addition to corticosteroids, treatment with antifungals has also shown promise. Antifungal itraconazole has been effective as improving symptoms while reducing *Aspergillus* burden [64]. Additionally, voriconazole treatment has also shown favorable outcome with both clinical and serological improvements [67].

Receptors Involved In Recognition

Soluble and Toll-like Receptors

Both soluble and cell-bound receptors are involved in host defense against *A. fumigatus*. The complement cascade, both alternative and lectin pathways, are activated during infection. C3 has been shown to bind both conidia and hyphae, activating the alternative pathway while mannan-binding lectin promotes the lectin pathway through C4bC2a [68, 69]. Moreover, in vivo models have shown that administration of exogenous mannan-binding lectin is beneficial during an immunocompromised model of IPA.

Pentraxin-3 is another soluble receptor involved in the response to *Aspergillus*. Resting conidia elicits rapid expression of pentraxin-3 by phagocytic cells which ultimately binds galactomannan on *A. fumigatus* conidia [70]. In vitro studies have shown that alveolar macrophages and neutrophils lacking pentraxin-3 have decreased phagocytic and killing capabilities. Moreover, pentraxin-3-deficient dendritic cells show decreased IL-12, MHC class II, and co-stimulatory marker CD86 expression after *A. fumigatus* stimulation [71]. In vivo, immunocompetent pentraxin-3 knockout mice displayed increased susceptibility with elevated IL-4 and decreased IFN- γ during

invasive aspergillosis [72]. Similarly, treatment with exogenous pentraxin-3 or adoptive transfer of pentraxin-3-expressing neutrophils improved outcome.

Toll-like receptors are a major group of receptors responsible for pathogen-associated molecular patterns with the main adaptor molecule being MyD88. While various in vitro studies have been done examining the role TLRs during an aspergillosis infection, the collective data is conflicting. In immunocompetent mice, TLR2, TLR4, IL-1R1 or MyD88 do not appear critical for resistance [44]. Conversely, cyclophosphamide-treated mice with TLR4 or MyD88 deficiencies suffered from higher fungal loads that translated to lower rates of survival. In regards to TLR2, while TLR2-deficiency in cyclophosphamide-treated mice alone did not have an effect on survival, TLR2 knockout mice displayed higher levels of fungal burden. Conversely, in an immunosuppressed model of invasive aspergillosis using vinblastine, TLR2-deficeint mice had significantly lower rates of survival [43]. Thus, the importance of TLR2 during infection may only be observed under conditions inducing neutropenia.

The role of TLR9 in response to *A. fumigatus* is dependent on whether exposure leads to an invasive or chronic disorder. An in vitro study by Bellocchio et al, the authors demonstrated that TLR9-deficient neutrophils had increased killing ability against both conidia and hyphae [73]. Moreover, in a model of invasive aspergillosis, immunosuppressed TLR9-deficient mice had lower fungal burden and decreased mortality rates. Thus, the absence of TLR9 leads to a beneficial outcome. Conversely, during an allergic, hypersensitivity model against *Aspergillus*, $TLR9^{-/-}$ mice, despite having lower levels of methacholine-induced airway resistance, displayed increased fungal growth [74].

C-type Lectin Receptor Dectin-1

Structure. Dectin-1 is a type II transmembrane C-type lectin receptor expressed in a variety of tissues, including the heart, liver, lung, stomach, and thymus by primarily myeloid cells including dendritic cells, macrophages, and neutrophils (Figure 5) [75]. Dectin-1 is encoded by gene *CLEC-7* located in the telomeric region of the chromosome 6f3 and chromosome 12q13.2 of mouse and human, respectively. With carbohydrate recognition domains (CRDs) in its extracellular carboxy-terminal domains, dectin-1 recognizes carbohydrate structures on invading microbes.

Murine dectin-1 (mDectin-1) is a glycoprotein with a weight of 28 kDa. Due to alternative splicing, two isoforms exist: isoform A and isoform B. Isoform A has one transmembrane region, an extracellular CRD domain with two N-glycosylation sites, a short stalk, and an immunoreceptor tyrosine-based activation motif-like region (ITAM) within its cytoplasmic domain. Isoform B is structurally similar but lacks the stalk region found in form A.

Human dectin-1 (hDectin-1), at 33kDa, is homologous and structurally similar to mDectin-1. Like mDectin-1, hDectin-1 has two main isoforms, isoforms A and B. hDectin-1 isoform A has one N-glycosylation site within its stalk domain while isoform B is deficient of a stalk region and has no N-glycosylation sites. In addition to predominant isoforms A and B, six other hDectin-1 isoforms (isoforms C-H) have been described. While isoforms A and B are functionally similarly, isoforms C-H appear to have distinct functions. Isoforms A and B, in both mouse and man, are receptors of beta-glucans; the ligands for human isoforms C-H are currently unknown [76].

Dectin-1 Signaling Pathway. Dectin-1 is arguably the most important receptor responsible for recognition of beta-glucan in mice [77]. The engagement of dectin-1 leads to intracellular activation through recruitment of spleen-tyrosine kinase (Syk). Binding of Syk to the ITAM-like regions of dectin-1 is thought to lead to dimerization and activation of the NF- κB pathway via CARD9 recruitment and activation (Figure 6). Other Syk-dependent pathways have been documented including PLCγ2, NLRP3, and NIK-pathways. A Syk-independent pathway, through Raf1 recruitment, has also been characterized and leads to enhanced phosporylation of p65. Engagement of dectin-1 leads to generation of reactive oxygen species, phagocytosis and lipid mediators [78, 79]. Inflammatory cytokines produced by ligation of Dectin-1 induced TNF-α, IL-1β, IL-6, IL-10, IL-17, IL-22, IL-23, CCL2 and CCL3 [45, 56].

Dectin-1 in Candida albicans and Pneumocystis carinii infections. *C. albicans* is a common human commensal fungi present in the human mucosal tract. Pathology occurs in response to *C. albicans* when the immune system is compromised and impaired. The relevance of dectin-1 against *C. albicans* has been demonstrated in both mouse and man. In vitro, dectin-1has been shown to trigger ROS production, phagocytosis, arachdonic acid metabolite production and cytokine generation on mouse macrophages after stimulation with *C. albicans* [78]. Importantly, this effect was only seen with the yeast form of the organism, mostly likely due to the enhanced exposure of surface beta-glucans [80]. In vivo, in a strain-dependent manner, dectin-1-deficient mice have been shown to have increased mortality, impaired cytokine production and reduced neutrophil-mediated killing during a systemic infection [77]. Recently, the requirement of dectin-1 dependent type I interferons (IFN), IFN-α and IFN-β, in the antifungal

response have been shown to be critical during candidiasis. Dectin-1 has been shown to promote IFN-β production by dendritic cells in response to *C.albicans* through the Syk-CARD9 pathway. [81-83].

The finding of polymorphism Y238X in hDectin-1 has further revealed the importance of the receptor in antifungal immunity [84]. Individuals with the Y238X polymorphism have impaired dectin-1 function due to an early stop codon. While homozygous expression of the Y238X polymorphism was not associated with increased risk of systemic infection, homozygous patients demonstrated increased susceptibility to mucocutaneous candidiasis as well as decreased production of TNF-α, IL-1β, IL-6 and IL-17A.

Pneumocystis carinii is an opportunistic fungal pathogen and a common cause of pneumonia in HIV patients. Through exposed beta-glucans in the cells wall of *P. carinii*, dectin-1 is engaged and aids in host defense during infection. In vitro, dectin-1-deficient macrophages had impaired internalization and killing of *P. carinii* cysts [85]. Additionally, while cytokine production was normal, dectin-1 knockout mice had abrogated ROS generation and elevated fungal burden in the lungs early during infection [86]. Interestingly, treatment with a dectin-1 fusion protein (Dectin:Fc) lead to enhanced macrophage killing in vitro yet reduced inflammation in *Pneumocystis murina* infected $Rag^{-/-}$ mice [87].

Dectin-1 in A.fumigatus infections. Dectin-1 has been shown to play a role in both invasive and chronic exposures to *A. fumigatus*. Depending on the morphological state of the organism, dectin-1 stimulates cellular functions in response to *A. fumigatus*. As ingested conidia swell and germinate, more beta-glucans are exposed, allowing for

detection by dectin-1 [88, 89]. Recognition of beta-glucans by dectin-1 provokes phagocytosis, fungal killing, as well as expression of pro-inflammatory mediators important for the immune response. Inflammatory mediators induced downstream of dectin-1 ligation during an *A. fumigatus* infection include IL-1β, IL-6, IL-17, IL-22, CXCL1 (KC) and TNF-α. In vitro studies employing use of dectin-1-deficient macrophages and neutrophils illustrate a requirement for dectin-1-mediated killing. Moreover, in vivo studies exemplify that dectin-$1^{-/-\ mice}$ have reduced cytokine response, higher levels of fungal burden as well increased mortality rate [45].

While the significance of dectin-1 in humans remains controversial, the majority of studies suggest a requirement of dectin-1 during IPA. In one study, the Y238X polymorphism was linked with increased risk of IPA in hematopoietic stem cell transplant patients; this risk was increased if both the donor and recipient possessed the polymorphism [55]. Additionally, a recent study revealed that dectin-1 is expressed on human bronchial epithelial cells and was important for the up-regulation of cytokines and antimicrobial peptides [90]. In contrast to the studies above, another study questioned the importance of the Y238X polymorphism in the development of IPA, showing no significant association between the polymorphism and risk of IPA. Moreover, the authors show that while peripheral blood monocytic cells from homozygous patients had reduced responses to *A. fumigatus*, macrophages responded normally. The study ultimately suggested the Y238X polymorphism had no clinical importance in the development of IPA [91].

Dectin-1 and fungal asthma. The link between fungi and asthma has long been observed. While the majority of asthma patients have mild, controllable symptoms by

use of bronchodilator therapy, a portion of asthmatics have severe, difficult-to-treat asthma. Multiple studies suggest that atopy to fungal antigens may be link to asthma severity [reviewed in [92]]. Mouse models of fungal asthma have elucidated many of the mechanisms involved in fungal asthma development. Notably, receptors know to play a role during innate fungal infections have been shown to be involved in the pathogenesis of mouse models of fungal asthma. Ramaprakash et al showed that the absence of TLR9 in immunocompromised mice resulted in a delay in mortality during an invasive infection of *A. fumigatus* [74]. In allergic mice, while TLR9-deficient mice displayed increased fungal growth compared to wildtype counterparts, $TLR9^{-/-}$ mice had markedly reduced airway hypersensitivity compared to wildtype mice. Interestedly, TLR9 knockout mice had reduced dectin-1 expression and reduced IL-17A in whole lung suggesting that the absence of dectin-1 signaling may be beneficial for fungal asthma via decreased expression of pro-inflammatory IL-17A. However, the role of dectin-1 during fungal-induced asthma has produced conflicting data among groups. Fei el al demonstrated that dectin-1 knockout mice had reduced cell recruitment to the bronchoalveolar lavage fluid and reduced IL-17A during chronic exposure to resting *A. fumigatus* conidia [93]. Indeed, neutralization of IL-17A in allergic mice led to improved lung function. Similarly, using the same exposure model of the study previously mentioned, Lilly et al demonstrated that dectin-1-deficient mice, driven by the absence of IL-22, displayed reduced pro-inflammatory and allergic mediators. Moreover, compared to wildtype cohorts, dectin-1 knockout mice as well as IL-22-deficient mice had improved lung function response to methacholine challenge. Conversely, other studies show that the presence of dectin-1 has a beneficial role in fungal-related asthma. A study by Mintz-

Cole et al demonstrated that the ability of *A. versicolor* and *Cladosporium cladosporioides* to promote pathogenesis was related to the level of beta-glucan exposed on the cell surface. The authors of this study concluded that *A. versicolor* included a strong IL-17A, neutrophilic response during challenge; neutralization of IL-17A or challenge in Dectin-1-deficient mice lead to enhanced eosinophilic inflammation and robust airway hypersensitivity [94]. Similarly, another showed that TLR6 plays a protective role via decreased dectin-1 expression and reduced levels of IL-17A and IL-23 [95]. Differences in models, antigens, and strains of mice used in the above studies may explain the conflicting results observed.

Cytokines Important for Host Defense

Description of IL-17A

IL-17A is the first discovered member of the IL-17 cytokine family which includes IL-17B, IL-17C, IL-17D, IL-17E (IL-25), and IL-17F. The gene of IL-17A is located in mouse chromosome 1 and human chromosome 6. First discovered to be produced by T-helper 17 cells (Th17), IL-17A is also produced by a variety of innate and adaptive cells including γδ T cells, NKT cells, NK cells, neutrophils, and eosinophils [96-98]. Through its widely-expressed receptor, IL-17RA, IL-17A elicits its effects in both mice and man; IL-17RA is highly expressed on hematopoietic cells as well as osteoblasts, fibroblasts, endothelial cells, and epithelial cells, albeit at a lower levels [99]. Thus, IL-17A has influence on both structural and immune cells. As first discovered to be produced by T cells, the signals that drive IL-17A expression has best been characterized in Th17 cells. The development of Th17 cells can be broken down to stages. In early development of murine Th17 polarization, TGF-β and IL-6 acting in synergy, signaling

through STAT3 to induce preliminary development [100]. In humans, in addition to IL-6, IL-1β in combination with TGF-β may also drive initial development and ultimately expression of lineage-specific transcription factor, retinoic acid receptor-related orphan nuclear receptor (RORγt) [101]. During the amplification stage, IL-21 stimulation leads to expansion through additional STAT3 signaling, promoting expression of the IL-23 receptor (IL-23R). Through expression of the IL-23R, the stabilization stage occurs; IL-23 signaling leads to the development of effector functions in Th17 cells.

IL-17A in asthma. In mouse models of asthma, the role of IL-17A in the development and pathology of asthma remains controversial. The discrepancy between studies may in part be due to strains of mice used, the presence or absence adjuvant, and type of allergen employed in the asthma model. Moreover, the differences in cell source of IL-17A, which is dependent on the model used, could potentially may a crucial role in pathogenesis.

In an OVA/alum model, neutralization of IL-17A, derived from alveolar macrophages, ameliorated asthmatic features in Balb/c mice [102]. Moreover, in another OVA/alum study, Th17 cells induced asthmatic symptoms independent of Th2 cells [103]. Additionally, administration of recombinant IL-17A abrogated the protective role of regulatory T cells (Tregs) during challenge. Conversely, in an *A. fumigatus* antigen/Freund adjuvant model of asthma, IL-17A has been shown to have a protective role. In Moreira et al, $TLR6^{-/-}$ mice had increased airway hyper-responsiveness and lung inflammation [95]. This increase in asthma severity was linked to decreased levels of IL-23 and IL-17A. Treatment with IL-23 restored IL-17A levels and improved lung function.

In humans, IL-17A is associated with severe asthma with IL-17A levels elevated in the airways, sputum, and bronchoalveolar lavage fluid of asthmatic patients [104]. While most asthmatics have controllable asthma, patients with difficult-to-treat asthma are often classified as severe asthmatic or corticosteroid resistant. A recent study showed that stimulation of PMBCs with IL-17A and IL-23 held to an upregulation of glucocorticoid receptor-β (GR-β), a receptor associated with corticosteroid resistance [105]. Furthermore, stimulation of human smooth muscle airway cells in vitro with recombinant IL-17A has been linked to CCL11 (eotaxin-1) expression, a potent eosinophil-recruiting chemokine [106].

IL-17A in infections. The pro-inflammatory and beneficial role of IL-17A has been characterized in a broad range of infections. IL-17A is involved in the clearance of bacterial infections including *Klebsiella pneumonia*, *Mycobacterium tuberculosis*, and *Mycobacterium bovis* [107-109]. In vivo studies have also illustrated a crucial role for IL-17A in fungal infections; IL-17A is important for host defense against opportunistic fungi like *P. carinii*, *C. albicans*, and *A. fumigatus* [45, 110, 111]. The importance of IL-17A in host defense is likely contributed to its potent neutrophil-recruiting properties; IL-17A in vitro and in vivo can stimulate CXCL8 (IL-8) and GM-CSF expression in humans and CXCL1 (KC) in mice [112].

Description of IL-22

IL-22 is a cytokine of the IL-10 family which includes IL-10, IL-19, IL-20, IL-24, IL-26, IL-28, and IL-29. IL-22 is on chromosome 12 and chromosome 10 on human and mouse chromosomes, respectively and promotes its effector functions through a heterodimeric receptor of IL-10R2 and IL-22R1. While IL-10R2 is somewhat widely

expressed, IL-22R1 expression is more restricted. To date, IL-22R1 is only expressed on structural cells such as intestinal and respiratory epithelial cells, keratinocytes, and hepatocytes [113]. IL-22R1 is not known to be expressed on traditional cells of the immune system. While typically considered a Th17-derived cytokine, IL-22 is also produced by CD8 T cells, γδ T cells, NKT cells, neutrophils, NK cells, and lymphoid tissue inducer cells. While often co-expressed with IL-17A, expression of IL-22 is driven by STAT3, RORγt in addition to aryl hydrocarbon receptor [114].

IL-22 in asthma. The data regarding the role of IL-22 in asthma has been conflicting. In various murine models of asthma, IL-22 is induced in the airways of sensitized mice [115-118]. The first study investigating IL-22 in an OVA-induced model of asthma, Besnard et al showed that IL-22 is required during the sensitization phase for development of asthma [115]. The same study showed that neutralization of IL-22 during the challenge phase led to an exacerbation of asthmatic symptoms. Thus, the study concluded that IL-22 is necessary for the development of asthma but plays a protective role during the challenge or late phase of allergy. In support of a protective role of IL-22, multiple studies have demonstrated that antibody-mediated neutralization of IL-22 led to enhanced infiltration on eosinophils, elevated Th2 cytokine levels, and airway hyperresponsiveness in mice [118, 119]. In support of those studies, intranasal administration of exogenous IL-22 resulted in to reduced eosinophil recruitment to airways [117]. Moreover, over-expression of IL-22 through a gene delivery-system led to reduced eosinophil recruitment as well as Th2 production. Collectively, the data from mouse models suggest a complex role for IL-22 in asthma pathogenesis, which may be

dependent on multiple factors such as the timing of asthmatic development, antigen used, stage of allergic development, and IL-22 cell source.

IL-22 infections. As a key cytokine in the initiation of the innate response, IL-22 is essential for host defense against extracellular pathogens. Broadly, IL-22 promotes host defense by promoting epithelial integrity through epithelial proliferation, synergistically working with other cytokines to induce expression of antimicrobials (S100A and β-defensins in the skin, RegIIIβ and RegIIIγ in intestinal epithelial, and mucus production by goblet cells), and promoting expression of pro-inflammatory mediators. The crucial role of IL-22 in host defense has been studied in multiple bacterial infections including *Klebsiella pneumonia*, *Mycobacterium tuberculosis*, *C. rodentium*, and *Salmonella typhimurium* [116, 120-122]. Moreover, the beneficial role of IL-22 has been investigated in fungal infections including *A. fumigatus* and *C. albicans*. Gessner et al demonstrated that IL-22-deficient mice had increased lung fungal burden during *A. fumigatus* infection. Similarly, neutralization of IL-22 mimicked the phenotype observed in IL-$22^{-/-}$ mice [123]. During an intravenous administration of *C. albicans* yeasts, IL-22 knockout mice, while did not experiencing increased mortality, had higher fungal loads in both the kidney and stomach during infection [124]. Thus, IL-22 provides protection during infection, most likely through its ability to promote tissue repair at mucosal sites.

Aims of this Dissertation

This dissertation will explore the mechanisms of immunoprotection as well as immunopathology during *A. fumigatus* infections. In IPA, numerous studies examining the role of classical effector cells like macrophages and neutrophils during infection have

been examined. This dissertation seeks to investigate the possibility that an additional cell type, the eosinophil, may also confer protection during an invasive infection. Moreover, while asthma is a widely-studied allergic disorder, fungal-induced asthma has garnered significantly less attention. The second aim of this dissertation is to examine the role of beta-glucan receptor dectin-1 and related cytokines during a chronic fungal infection (Figure 7).

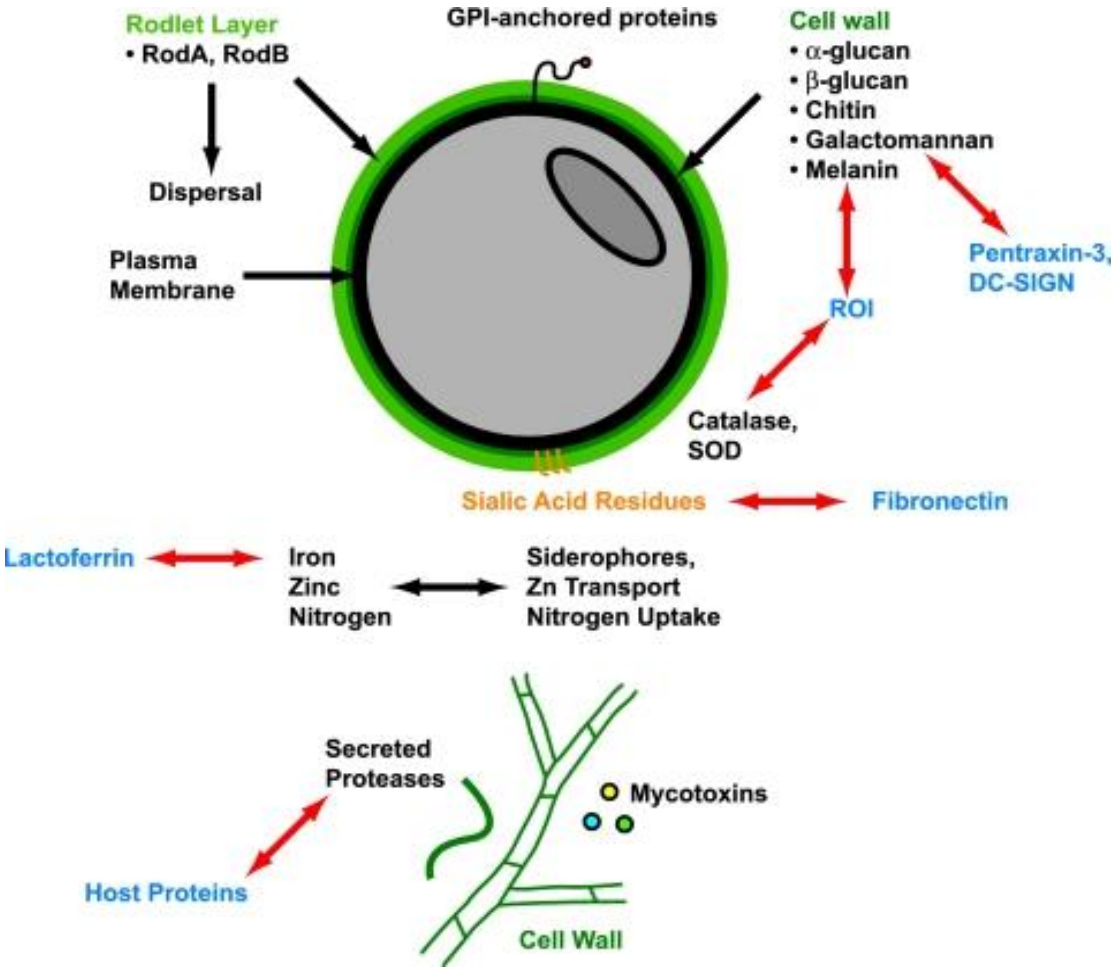

Figure 1. Molecular features of *A. fumigatus* conidia and hyphae. Schematic representation of resting conidia (top) and hyphae (bottom). The organization of the conidial cell wall is depicted, together with specific conidial and hyphal cell wall and secreted components. Host receptors and products that interact with fungal molecules are listed in blue. Note: Adapted from "Aspergillus fumigatus: principles of pathogenesis and host defense." by Hothl et. al., 2007, Eukaryot Cell. 2007 Nov;6(11):1953-63. Copyright 2007, American Society for Microbiology. Adapted with permission.

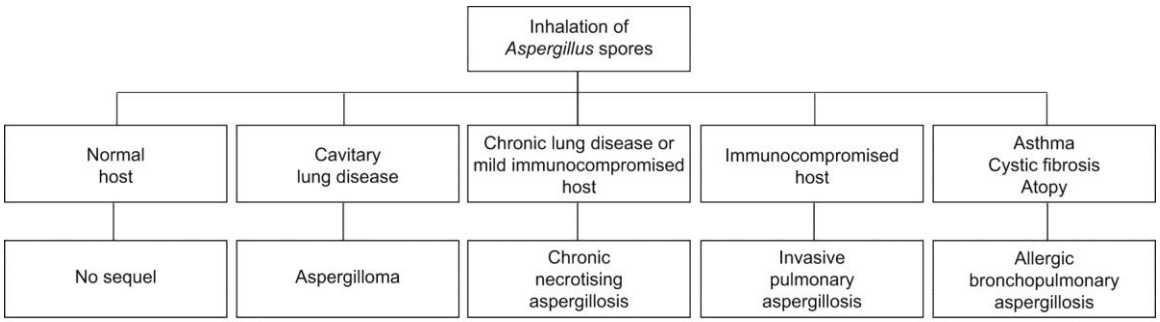

Figure 2. The spectrum of pulmonary aspergillosis. Note: Adapted from "Pulmonary aspergillosis: a clinical review." by Kousha et. al., 2011, Eur Respir Rev. 2011 Sep 1;20(121):156-74. Copyright 2013 by the European Respiratory Society. Adapted with permission.

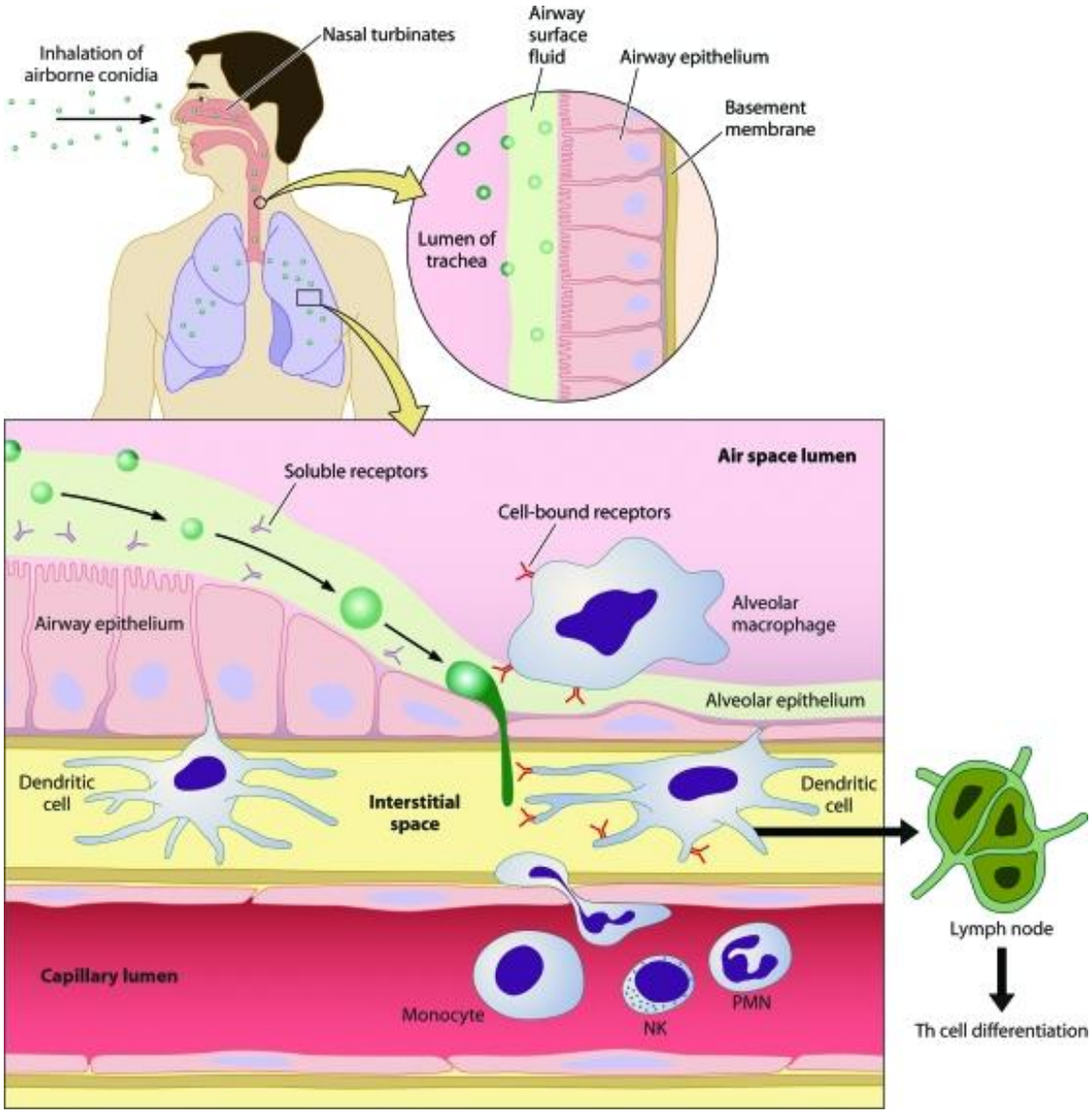

Figure 3. Schematic representation of components of the host response to inhaled *Aspergillus* conidia. Note: Adapted from "Innate immunity to Aspergillus species." by Park et. al., 2009, Clin Microbiol Rev. 2009 Oct;22(4):535-51. Copyright 2009, American Society for Microbiology. Adapted with permission.

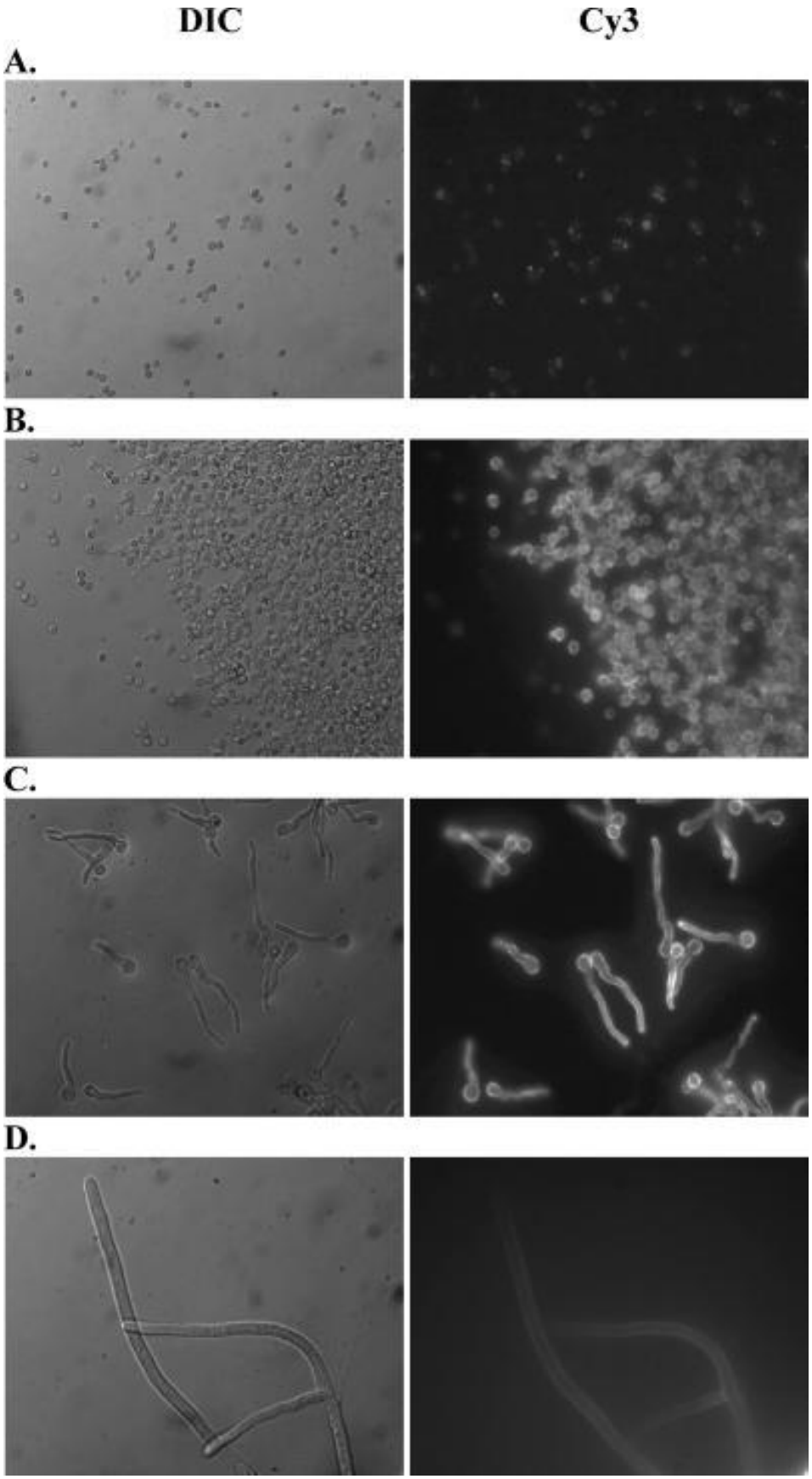
DIC
Cy3
A.
B.
C.
D.

Figure 4. Heightened Binding of Dectin-1 to A. fumigatus SC.
A soluble fusion protein consisting of the extracellular carbohydrate recognition domain of dectin-1 fused with the Fc portion of murine IgG1 (s-dectin-mFc) was constructed and incubated with live *A. fumigatus* RC and SC. Binding of s-dectin-mFc was detected by Cy3-conjugated, goat anti-mouse IgG antibody followed by imaging with a Zeiss Axioplan 2 upright fluorescent deconvolution microscope (Zeiss), and images were captured using 3i Slidebook Version 4.0 software (Optical Analysis). Representative micrographs show s-dectin-mFc binding to *A. fumigatus* grown for 2 h (A), 6 h (B), 10 h (C), and 24 h (D). Left lane images are differential interference contrast (DIC) images, and right lane images are Cy3 staining. Magnification is 630 × oil emersion for all frames.

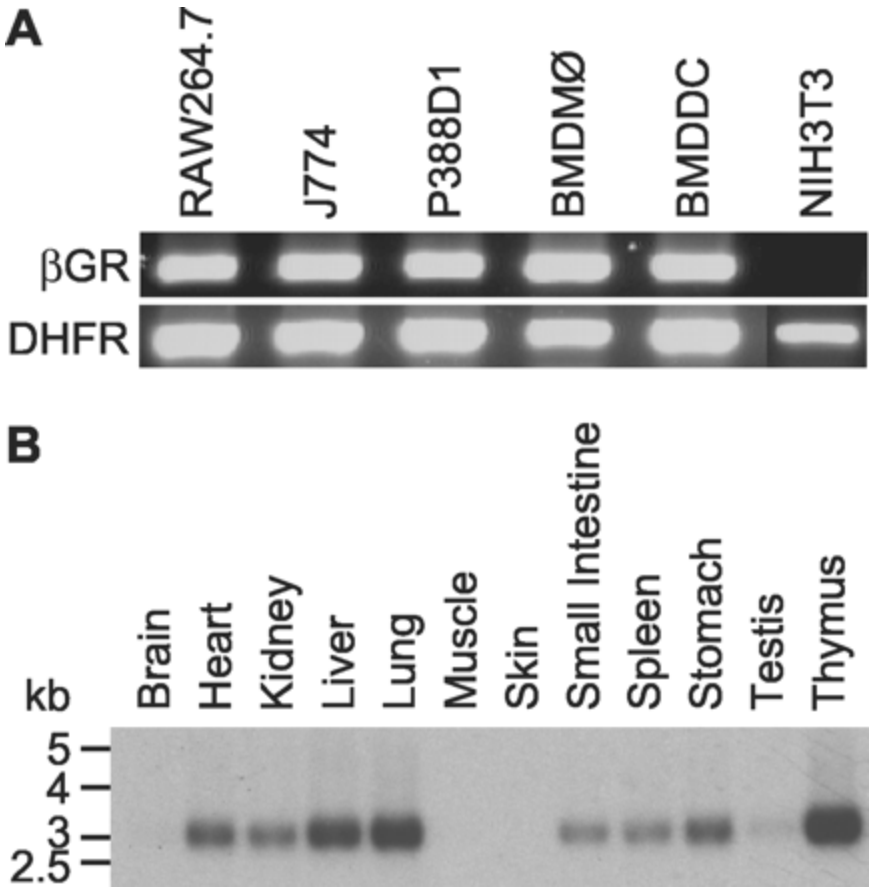

Figure 5. Expression of GR by primary macrophages and in selected tissues. A, RT-PCR analysis showed the presence of GR transcript in Mφ cell lines (RAW264.7, J774, and P388D1) and primary Mφ (BMDMφ) and DC (BMDDC), but not in a mouse fibroblast cell line (NIH-3T3). The housekeeping gene dihydrofolate reductase (DHFR) was used as a PCR control. B, A multiple mouse tissue Northern blot probed with GR cDNA showed widespread expression of GR in the mouse. Control probing with β-actin confirmed equivalent loading between lanes (data not shown). Note: adapted from ―"The β-glucan receptor, Dectin-1, is predominantly expressed on the surface of cells of the monocyte/macrophage and neutrophil lineage" by Philip Taylor et. at., 2002, The Journal of Immunology, 2002, 169:3876-3882. Copyright 2002 The American Association of Immunologists. Adapted with permission.

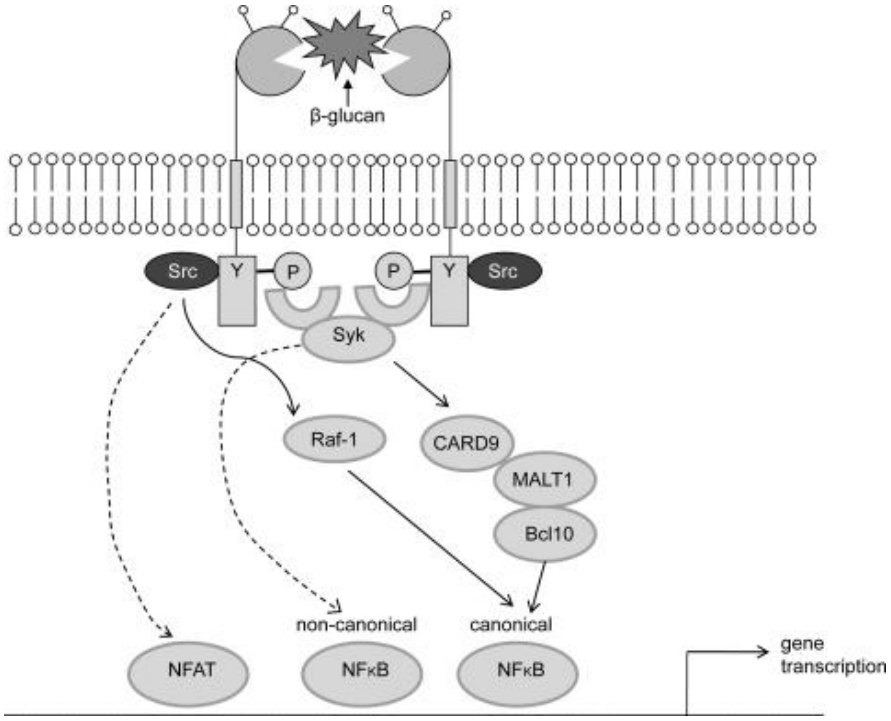

Figure 6. Signaling pathway induced by Dectin-1. Upon ligand binding, Dectin-1 becomes tyrosine-phosphorylated by Src kinases, thereby providing a docking site for Syk which initiates downstream signaling. The downstream signaling is effected by molecules such as CARD9, Bcl10, and MALT1, which lead to NF-κB activation and cytokine production. Dectin-1 can also activate NFAT and noncanonical NF-κB in a CARD9-Bcl10-MALT1-independent manner. Stimulation of Dectin-1 with β-glucans can also induce a second Syk-independent signaling pathway mediated by the serine-threonine kinase Raf-1. Note: Adapted from ―"Dectin-1: a role in antifungal defense and consequences of genetic polymorphisms in humans" by Mohlopheni Marakalala et. al., 2011, Mamm Genome 22:55-65. Copyright 2010 The Authors, published with open access at Springerlink.com. Adapted with permission.

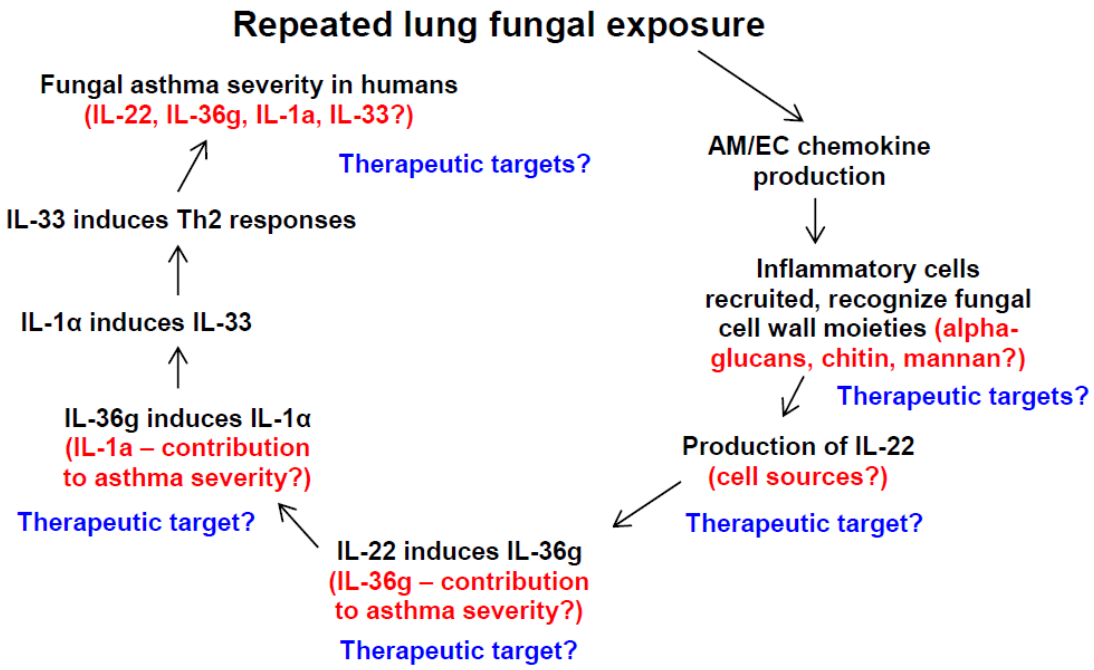

Figure 7. A schematic representation of the immune response to chronic A. fumigatus exposure.

Table 1—Major Risk Factors for IPA

1. Prolonged neutropenia (> 3 wk) or neutrophil dysfunction (chronic granulomatous disease)
2. Corticosteroid therapy (especially prolonged, high-dose therapy)
3. Transplantation (highest risk is with lung and bone marrow)
4. Hematologic malignancy (risk is higher with leukemia)
5. Cytotoxic therapy
6. AIDS (risk increases with lower CD4 count)

Table 2—Diagnostic Criteria for ABPA

1. Asthma
2. Immediate skin reactivity to Aspergillus
3. Serum precipitins to *A fumigatus*
4. Increased serum IgE and IgG to *A fumigatus*
5. Total serum IgE > 1,000 ng/mL
6. Current or previous pulmonary infiltrates
7. Central bronchiectasis
8. Peripheral eosinophilia (1,000 cells/μL)

Note: Adapted from ―The Clinical Spectrum of Pulmonary Aspergillosis by Ayman O. Soubani and Pranatharthi H. Chandrasekar, 2002, CHEST, 121:1988-1999.

Table 3- Genetic Factors Involved in the Pathogenesis of ABPA

HLA associations: presence of HLA DR-2 and absence of HLA-DQ2 sequences[42,44,45]
IL-10 promoter polymorphisms[49]
Polymorphism at position 1,082 produces higher levels of IL-10 if 1082G allele is present and lower levels of IL-10 if the 1082A allele is present
In patients with CF there is a relationship between the 1082GG genotype with both Aspergillus colonization and ABPA
Surfactant protein A gene polymorphisms[48,53]
A significantly higher frequency of the AGA allele (A1660G) of SP-A2 found in patients with ABPA vs control subjects. Coexistence of A1660G polymorphism with SP-A2 G1649C (Ala91Pro) found with 10-fold higher odds in patients with ABPA. Patients with ABPA with GCT and AGG alleles showed significantly higher levels of total IgE and percentage eosinophilia vs patients with ABPA with CCT and AGA alleles[48]
The T allele at T1492C and G allele at G1649C of SP-A2 observed at higher frequencies in ABPA patients than in controls. Also there is a higher frequency of the TT genotype at position1492 of SP-A2 than controls[53]
There were no polymorphisms found in SP-A1 gene[53]
CFTR gene mutation:[43,46,47] increased frequency of CFTR mutations in patients with ABPA vs skin-prick test positive or negative patients with bronchial asthma
IL-15 polymorphisms:[52] higher frequency of IL-15 + 13689*A allele and A/A genotype
TNF-α polymorphisms:[52] lower frequency of the TNF-α 308 * A/A genotype
Mannose-binding lectins:[53] the intronic single nucleotide polymorphism G1011A of mannose-binding lection seen with increased frequency in patients with ABPA
IL-4 receptor polymorphisms:[51] single nucleotide polymorphism of the extracellular IL-4Rα ile75val observed in 80% of ABPA patients
IL-13 polymorphisms:[50] the arg110gln polymorphism found with increased frequency in ABPA and the combination of IL-4Rα ile75val/IL-13 arg110gln polymorphism found with an even higher frequency
Toll-like receptor gene polymorphisms:[54] susceptibility to ABPA was associated with allele C on T1237C (TLR9)

*HLA = human leukocyte antigen; TNF = tumor necrosis factor; CFTR = CF transmembrane conductance regulator.

Note: Adapted from ―Allergic Brochopulmonary Aspergillosis by Ritesh Agarwal, 2009, CHEST, 135(3):805-26.

THE BETA-GLUCAN RECEPTOR DECTIN-1 PROMOTES LUNG IMMUNOPATHOLOGY DURING FUNGAL ALLERGY VIA IL-22

by

LAUREN M. LILLY, MELISSA A. GESSNER, CHAD W. DUNAWAY, ALLISON E. METZ, LISA SCHWEIBERT, CASEY T. WEAVER, GORDON D. BROWN AND CHAD STEELE

The Journal of Immunology, 2012 Oct 1; 189(7):3653-60.

Abstract

Sensitization to fungi, such as the mold *Aspergillus fumigatus*, is increasingly becoming linked with asthma severity. We have previously shown that lung responses generated via the beta-glucan receptor Dectin-1 are required for lung defense during acute, invasive *A. fumigatus* infection. Unexpectedly, in an allergic model of chronic lung exposure to live *A. fumigatus* conidia, beta-glucan recognition via Dectin-1 led to the induction of multiple proallergic (*Muc5ac*, *Clca3*, CCL17, CCL22 and IL-33) and proinflammatory (IL-1β and CXCL1) mediators that compromised lung function. Attenuated proallergic and proinflammatory responses in the absence of Dectin-1 was not associated with changes in *Ido* (indoleamine 2,3-dioxygenase), *Il12p35/Ebi3* (IL-35), IL-10 or TGF-β levels. Assessment of T helper responses demonstrated that purified lung CD4+ T cells produced IL-4, IL-13, IFN-γ and IL-17A, but not IL-22, in a Dectin-1 dependent manner. In contrast, we observed robust, Dectin-1 dependent IL-22 production by unfractionated lung digest cells. Intriguingly, the absence of IL-22 alone mimicked the attenuated proallergic and proinflammatory responses observed in the absence of Dectin-1, suggesting that Dectin-1 mediated IL-22 production potentiated responses that led to decrements in lung function. To this end, neutralization of IL-22 improved lung function in normal mice. Collectively, these results indicate that the beta-glucan receptor Dectin-1 contributes to lung inflammation and immunopathology associated with persistent fungal exposure via the production of IL-22.

Introduction

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

Severe forms of asthma are often associated with neutrophilic, rather than eosinophilic, infiltration (7) (8). IL-17A is a proinflammatory cytokine that upregulates a number of cytokines and chemokines leading to the recruitment of neutrophils to sites of inflammation (9). IL-17A gained prominence when it was discovered to be produced by CD4 T cells, a lineage now termed T helper IL-17 or Th17 cells (10). With respect to human asthma, elevated IL-17A mRNA and protein expression has been observed in the

lungs of asthmatics (11) (12). Early insight into a role for IL-17A in asthma-like changes came from experimental lung-overexpression studies, which demonstrated hypertrophic bronchial epithelium, mucus production, neutrophilic and eosinophilic infiltration and asthma-associated chemokine induction (13). Moreover, stimulation of murine lung epithelial cells with IL-17A leads to the induction of multiple genes that may contribute to asthmatic phenotypes (14). Experimental models of asthma, including OVA-challenge (15), house dust mite (16), RSV (17) and cockroach allergen (18), have each demonstrated the induction of Th17 responses. In the widely-utilized OVA model, some studies report beneficial effects when IL-17A is neutralized (15), although some studies argue for a protective role for IL-17A (19). However, in a striking finding, steroid-resistant allergic airway inflammation was found to be driven by Th17, but not Th2, responses (20). The explosion in IL-17A research over the last 15 years has also identified IL-22 as a Th17-associated cytokine (21). IL-22 is an intriguing cytokine in that it can act in both pro- and anti-inflammatory responses (22) (21) (23). Moreover, the limited cellular expression of the IL-22 receptor, predominantly epithelial cells to date (24), makes it an attractive mediator to investigate in asthma pathogenesis. Like IL-17A, IL-22 is also reported to be elevated in asthmatics (25) (26). Experimental investigation in an OVA model has shown that IL-22 is required for the onset of allergic asthma, however neutralization of IL-22 during antigen challenge enhanced allergic lung inflammation (25). In turn, administration of IL-22 has been shown to dampen allergic lung inflammation in mice (25) (27). We have recently shown a comprehensive role for the Dectin-1/IL-17A/IL-22 axis in protection against acute, invasive infection with *A. fumigatus* (28) (29) (30). In the current report, we investigated this axis during chronic *A.*

fumigatus exposure, specifically in a live *A. fumigatus* conidia repetitive challenge model of fungal allergy. Despite being required for the elimination of *A. fumigatus* from the lungs during acute exposure, our data here support an immunopathogenic role for Dectin-1 and IL-22 during chronic fungal allergy.

Materials and Methods

Mice

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

A. fumigatus chronic exposure model

A. fumigatus isolate 13073 (ATCC, Manassas, VA) was maintained on potato dextrose agar for 5-7 days at 37°C. Conidia were harvested by washing the culture flask with 50 ml of sterile phosphate buffered saline supplemented with 0.1% Tween 20. The conidia were then passed through a sterile 40 μm nylon membrane to remove hyphal fragments and enumerated on a hemacytometer. *The repeated A. fumigatus exposure model was employed as previously described (33). Briefly, mice were lightly anesthetized with isoflurane and administered 1 x 10^7 live A. fumigatus conidia in a volume of 50 μl of PBS intratracheally (i.t.). Starting at day 7, mice were challenged i.t. with 1 × 10^6 conidia live A. fumigatus in 50 μL of PBS daily for 5 days, rested for 2 days, and*

challenged daily for another 3 days. At 24 h after the final challenge, immune measures were assessed as described below.

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

Lungs were collected, homogenized in TRIzol reagent (Invitrogen) and total RNA isolated as per the manufacturer's instructions. Thereafter, RNA was transcribed to cDNA (iScript cDNA synthesis kit, Bio-Rad), and real-time PCR for *Muc5ac* (Mm01276735_m1; Applied Biosystems), *Clca3* (Mm00489959_m1, Applied Biosystems), *Saa3* (Mm00441203_m1, Applied Biosystems) *Ido1* (Mm00492586_m1, Applied Biosystems) and *Il12p35/Ebi3* (Mm00434165_m1/Mm00469294_m1, Applied Biosystems) and was performed (iQ Supermix, Bio-Rad). mRNA levels were normalized to *Gapdh* mRNA levels (primers/probe from Applied Biosystems) using the $2^{-(\Delta\Delta Ct)}$ method (29) (30).

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

The left lung was homogenized in PBS supplemented with Complete Mini protease inhibitor tablets (Roche), clarified by centrifugation and stored at -80°C. Supernatants from lung homogenates were analyzed for protein levels of 23 cytokines and chemokines using Bio-Plex multiplex suspension cytokine array (Bio-Rad Laboratories) according to the manufacturer's instructions. The data were analyzed using Bio-Plex Manager software (Bio-Rad Laboratories). IL-23, IL-33, CCL17 and CCL22 levels were quantified by ELISA (R&D Systems). For lung cell isolation, the lungs were collected and minced in IMDM media (Sigma, St. Louis, MO) supplemented with 1% pen-strep-glut (Mediatech, Herndon, VA), 10% heat inactivated FBS (Invitrogen, Carlsbad, CA) and 0.4 mg/ml polymyxin B (Thermo Fisher), followed by incubation for

60 min with tissue culture-grade type IV collagenase (1 mg/ml; Sigma, St. Louis, MO) in a 37°C orbital shaker at 100 rpm. The cell suspension was filtered through sterile 70 μm and 40 μm nylon filters and red blood cells lysed with ACK buffer (Lonza, Walkersville, MD) to create single cell preparations. One million cells in a volume of 200 μl were cultured overnight followed by assessment of T helper cytokine production by ELISA or Bio-Plex. For CD4 T cell purification, lung cells were stained for CD4 followed by FACS sorting. Purified CD4 T cells were stimulated with 5 μg/ml of anti-CD3 (clone 145-11) and 2.5 μg/ml of anti-CD28 for 24 h. ELISA or Bio-Plex was employed to assess T helper cytokine levels in supernatants.

Lung cell surface marker flow cytometry

Lung cells were isolated via bronchoalveolar lavage as previously described (34). Cells were washed and Fc receptors were blocked with Mouse BD Fc Block™ (BD Biosciences, San Diego, CA) at 4°C for 20 min. Thereafter, cells were stained with a single-color LIVE/DEAD® Fixable Dead Cell Stain (Invitrogen) followed by labeling with specific immune cell surface markers. The following staining parameters were employed: eosinophils as CD11b+ Siglec F+ Ly-6G andLy-6Clo/neg, neutrophils as CD11b+ Ly6G+ (1A8), dendritic cells as CD11b+ CD11c+, natural killer cells as CD11b+ DX5+, and T cells as CD3+ CD4+ (all antibodies purchased from eBiosciences and BD Biosciences). Samples were acquired using a four laser, 20-parameter analytic BD^{TM} LSR II and data was analyzed using FlowJo software (Tree Star, Ashland, OR). Unstained cells served as a control for background fluorescence and gating. Samples were acquired using BD^{TM} LSR II cytometer (BD Biosciences) and data was analyzed using FlowJo software (Tree Star, Ashland, OR).

Pulmonary function assessment

A tracheostomy was performed on individual anesthetized *A. fumigatus*-exposed mice. Each animal was then attached to a computer controlled volume ventilator (flexiVent, SCIREQ Montreal, PQ, Canada). Regular breathing was set at 150 bpm, with volume and pressure controlled by the flexiVent system based on individual animal weights. Positive end-expiratory pressure (PEEP) was set to 2 cm H_2O and measured during each breath stroke. Respiratory input impedance (Zrs) was measured using the Forced Oscillation Technique controlled by the flexiVent system. The Single-Compartment Model was used to describe dynamic lung resistance. All measurements were collected at baseline and after a linear dose response with methacholine challenge (10 – 40 mg/ml) as previously described (28).

IL-22 neutralization

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

Statistics

Data were analyzed using GraphPad Prism Version 5.0 statistical software. Comparisons between groups when data were normally distributed were made with the Student's t-test. Significance was accepted at a value of $P < 0.05$.

Results

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

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

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

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

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

As the results in Figure 1 indicated that CCL17, CCL22 and IL-33 production were attenuated in Dectin-1 deficient mice chronically exposed to *A. fumigatus*, we next assessed whether Th2 responses were modulated by Dectin-1 deficiency. Stimulation of lung cells isolated via enzymatic digestion with *A. fumigatus* for 24 h *in vitro*, which allows for quantification of the total amount of cytokine produced irrespective of the cell source (29) (30), revealed low production of IL-4 (Figure 3A), IL-13 (Figure 3B) and IFN-γ (Figure 3C) by WT lung cells. In contrast, we observed robust production of IL-17A (Figure 3D) and IL-22 (Figure 3E). Although produced at very low levels, IL-4 (Figure 3A) and IFN-γ (Figure 3C), but not IL-13 (Figure 3B) production were lower in the absence of Dectin-1. Intriguingly, attenuated lung inflammation and better lung function during chronic *A. fumigatus* exposure in the absence of Dectin-1 correlated with significant reductions in lung cell IL-17A (Figure 3D) and IL-22 (Figure 3E) production. Anti-CD3/CD28 stimulation of CD4 T cells sorted by flow cytometry demonstrated Dectin-1 dependent production of IL-4 (Figure 3A), IL-13 (Figure 3B), IFN-γ (Figure 3C) and IL-17A (Figure 3D), but not IL-22 (Figure 3E). Overall, although modulations in Th1 and Th2 responses were observed, the most apparent effect of Dectin-1 deficiency during chronic *A. fumigatus* exposure was attenuated IL-17A and IL-22 responses. Therefore, while our previous work indicated that acute exposure to *A. fumigatus* requires Dectin-1 mediated IL-17A and IL-22 for protection against infection, our data here suggests that Dectin-1 mediated immunopathology during fungal allergy may be due to IL-17A and/or IL-22 mediated responses.

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

To determine whether Dectin-1 deficiency was associated with a compensatory upregulation in regulatory mechanisms, we assessed the expression of indoleamine 2,3-dioxygenase (39) and IL-35 (40), both of which can negatively regulate IL-17A responses. However, neither *Ido* (Figure 3A) nor *Il12p35/Ebi3* (IL-35) (Figure 4B) mRNA expression were augmented in the lungs of Dectin-1 deficient mice nor were the lung levels of IL-10 (Figure 4C) and TGF-β (Figure 4D). Therefore, lower lung IL-17A and IL-22 production in the absence of Dectin-1 was not a result of enhanced expression/function of immunoregulatory factors. In contrast, *Saa3* (serum amyloid A 3) mRNA levels, which can promote IL-17A responses, possibly via IL-23 induction (41), were significantly lower in the lungs of Dectin-1 deficient mice (Figure 4E), as was IL-23 levels (Figure 4F) in lung homogenates. These results suggest that lower IL-23 levels, possibly as a result of impaired *Saa3* induction, contributes to lower IL-17A and IL-22 production.

Lung inflammation during chronic fungal exposure requires IL-22.

During acute *A. fumigatus* exposure, we have shown that IL-22 is produced rapidly in the lungs at levels that are much higher than, and more Dectin-1 dependent than, IL-17A (29) (30). In addition, IL-22 was produced by lung cells in higher amounts than IL-17A in chronically exposed and once again demonstrated more Dectin-1 dependency than IL-17A (Figure 2). We therefore questioned the role of IL-22 in lung proallergic and inflammatory responses after repetitive *A. fumigatus* challenge. C57BL/6 and IL-22 deficient mice were repetitively exposed to *A. fumigatus* as in Figure 1A and

parameters of lung inflammation were assessed. Results show that deficiency in IL-22 resulted in significant reductions in *Muc5ac* and *Clca3* (Gob5) mRNA expression (Figure 5A), lung CCL17 and CCL22 levels (Figure 5B), lung IL-33 levels (Figure 5C) and IL-1β and CXCL1 levels (Figure 5D). Therefore, during chronic *A. fumigatus* exposure, Dectin-1 mediated IL-22 may be a central contributor to lung inflammation during fungal allergy.

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

Cytokine modulation is currently undergoing clinical trials for efficacy in improving asthma control (35). Our data thusfar implicates Dectin-1 mediated responses, particularly the induction of IL-22, as a contributor to lung inflammation leading to decrements in lung function during chronic *A. fumigatus* exposure. To this end, we assessed the therapeutic efficacy of blocking IL-22 on improving lung function during fungal allergy. For this, mice were exposed to *A. fumigatus* as in Figure 1A and randomized to receive IL-22 neutralizing antibodies or rat IgG isotype control antibodies on days 7, 10, 13 and 16. Results show that following methacholine challenge, mice receiving anti-IL-22 neutralizing antibodies maintained lower dynamic resistance (Figure 6), indicating that IL-22 drives lung inflammatory responses that have a negative impact on lung function during fungal allergy.

Discussion

We have previously reported that mice deficient in Dectin-1 acutely exposed to *A. fumigatus* had significantly lower levels of IL-17A in the lungs (28). In an extension of this work, we have recently reported that during acute *A. fumigatus* exposure, neutrophils produce IL-17A in a Dectin-1 and IL-23 dependent manner (29). We have now extended the antifungal contribution of Dectin-1 to the induction of IL-22, as Dectin-1 deficiency resulted in a near total loss of lung IL-22 production after acute *A. fumigatus* exposure (30). Importantly, neutralization of either IL-17A (28) or IL-22 (30) resulted in impaired clearance of *A. fumigatus*, illustrating a critical role for IL-17A and IL-22 in pathogen elimination during acute infection. In the current study, we examined the contribution of the Dectin-1/IL-17A/IL-22 axis to the development of fungal allergy. Although required for host defense in an invasive infection model, our data shows that Dectin-1, via IL-22, contributes to immunopathogenesis in a model of fungal allergy.

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

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

Our data indicates that mice deficient in Dectin-1 exposed to repetitive *A. fumigatus* conidia have improved lung function which correlated with reductions in multiple proallergic and inflammatory mediators. Improved lung function in the absence of Dectin-1 also correlated with changes in CD4 T cell responses, specifically reductions in Th1, Th2 and Th17 responses. However, it is worth noting that in contrast to IL-4, IL-13 and IFN-γ, which were primarily produced by CD4+ T cells, IL-17A was produced by both unfractionated lung digest cells as well as purified CD4+ T cells. Moreover, we observed the most robust production of IL-22 in unfractionated lung digest cell cultures rather than purified CD4+ T cells, indicating that additional cell types were contributing to the levels of IL-17A and IL-22 during chronic fungal exposure. Indeed, we have recently reported that neutrophils produce Dectin-1 dependent IL-17A during acute *A. fumigatus* exposure (29). Ongoing studies seek to identify the source of both IL-17A and IL-22 over the development of fungal allergy. The role of IL-17A in chronic fungal exposure has been recently investigated (33). In a similar repetitive *A. fumigatus* exposure model, although the effects of IL-17A on pro-allergic and pro-inflammatory

responses as well as lung function were not investigated, neutralization of IL-17A or deficiency in IL-17RA resulted in attenuated neutrophil recruitment (33). A more recent study has shown that weekly *A. fumigatus* challenges in IL-17A deficient mice over four weeks resulted in attenuated lung inflammation, particularly in eosinophil numbers (46). In this report, it was hypothesized that during fungal allergy, IL-17A may function to promote/mimic what is often associated with or attributed to Th2-mediated responses. How IL-17A may drive Th2-like inflammation is not currently known and the subject of future studies.

We posit here that IL-22 may also be involved in Th2-associated inflammation during fungal allergy. Our data indicates that Dectin-1 deficiency resulted in attenuated production of IL-22 in the presence of lower mucus gene expression, pro-allergic chemokine levels and improved lung function. In turn, IL-22 deficiency also resulted in lower mucus gene expression and pro-allergic chemokine levels and neutralization of IL-22 improved lung function. Common between Dectin-1 and IL-22 deficiency was attenuated production of IL-33. Therefore, one explanation for IL-17A/IL-22 mimicking Th2-like inflammation during fungal allergy may be due to an association with IL-33. Whether IL-17A or IL-22 has the ability to induce IL-33 directly is the subject of current studies. IL-33 is a potent inducer of Th2 associated mediators from multiple cell types (reviewed in (47)). In a fungal allergy model employing i.p./s.c. sensitization with a commercial *Aspergillus* antigen in Incomplete Freund's Adjuvant followed by three weekly i.n. antigen administrations and then a final challenge with live *A. fumigatus* conidia, ST2 (IL-33R) blockade ameliorated many pathological features of fungal asthma (48). However, this study focused solely on the Th2 aspects of fungal allergy. As ST2

forms a receptor complex with the commonly utilized subunit IL-1R accessory protein (IL-1RAcP) (49), IL-33 may also induce a number of proinflammatory mediators, such as TNF-α, IL-6 and IL-8 (reviewed in (47)). Furthermore, neutrophil-derived elastase and cathepsin G can cleave IL-33 into a more active form resulting in higher elicitation of inflammatory mediators such as IL-6 (50). Murine models of collagen-induced arthritis have shown that ST2 deficiency (51) and ST2 blockade (52) lead to reduced IL-17A levels, implicating a surprising role for IL-33 in IL-17A generation. IL-33 has also been shown to be overexpressed in clinical ulcerative colitis and in experimental enteritis, where IL-33 induced IL-17A production from mesenteric lymph node cultures (53). Finally, a new study employing an OVA allergic airway model has shown that eosinophil deficient mice have augmented Th17 development and mast cells stimulated with IL-33 enhance Th17 development (54). Moreover, mast cells stimulated with IL-33 may themselves produce IL-17A (reviewed in (47)). Collectively, the role of IL-33 is enigmatic and may function in any number of responses during fungal allergy, including promotion of Th2-associated inflammation (*Muc5ac*, CCL17 etc.), proinflammatory responses (IL-1β, IL-6) or even IL-17A/IL-22 production in a feed-forward mechanism that promotes some of the inflammatory aspects of severe fungal asthma/allergy. Future studies will address the specific contribution of IL-33 in each of these responses.

In the commercial *Aspergillus* antigen/IFA fungal allergy model mentioned previously, mice deficient in TLR9 demonstrated higher serum IgE, but lower AHR (55). In this study, IL-17A levels trended lower, but were not significantly reduced, although Dectin-1 mRNA expression was significantly lower in the lung (55). In a subsequent report from the same group, IL-23 levels, Dectin-1 expression and Th17 development

was found to be significantly lower in TLR6 deficient mice, yet these mice demonstrated higher AHR and elevated expression of allergy biomarkers (56). These observations contradict the observations in TLR9 deficient mice, despite the fact that both studies reported lower Dectin-1 expression and IL-17A levels. IL-23 administration to TLR6 deficient mice augmented IL-17A and IL-22 levels and decreased AHR (56). In WT mice, neutralization of IL-17A increased *Muc5ac* and *Clca3* expression while paradoxically decreasing IL-33 levels, whereas neutralization of IL-22 decreased *Muc5ac* and *Clca3* expression while paradoxically increasing IL-33 levels (56). Therefore, although the TLR9 study implicates a role for IL-17A and Dectin-1 contributing to the development of fungal allergy, studies with TLR6 deficient mice suggest a protective role for IL-17A and IL-22 in fungal allergy. However, IL-17A and IL-22 neutralizations in the latter study complicate interpretations and indicate a complex role for both IL-17A and IL-22. IL-22 is elevated in asthmatics (25) and experimental investigation in an OVA model has shown that IL-22 contributes to the development of allergic responses (25). However neutralization of IL-22 during antigen challenge enhanced allergic lung inflammation (25). These results are in contrast to our data in the fungal allergy model, which suggests that neutralization of IL-22 during challenge is effective at improving lung function. In the commercial *Aspergillus* antigen/IFA fungal allergy model, neutralization of IL-22 increased histological evidence of lung inflammation and lung IL-33 levels while paradoxically decreasing *Muc5ac* and *Clca3* expression (56). However, no significant effect on pulmonary function was observed after IL-22 neutralization (56). As both of these reports utilized adjuvant-associated sensitization models, they may not replicate allergen sensitization in humans. Nevertheless, these collective results suggest

that IL-22 may function in different capacities over the development of allergic responses.

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

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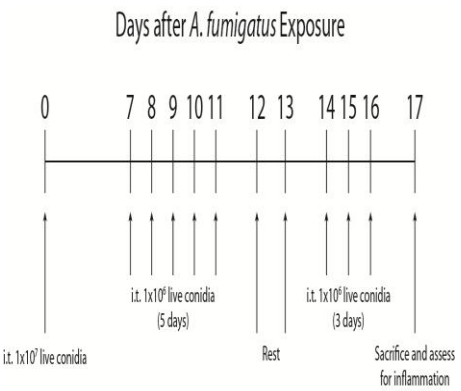
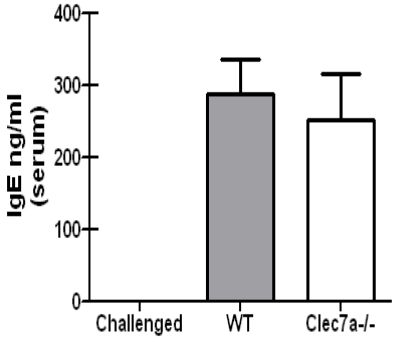
Figure 1. The absence of Dectin-1 results in improved lung function after chronic fungal exposure. (A) Diagram of the chronic *A. fumigatus* exposure model. **(B)** Total IgE levels in the serum of C57BL/6 (WT) and Dectin-1 deficient (Clec7a-/-) mice after chronic fungal exposure. Challenged represents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

A.


Days after A. fumigatus Exposure
0 7 8 9 10 11 12 13 14 15 16 17
i.t. 1x10⁸ live conidia (5 days)
i.t. 1x10⁸ live conidia (3 days)
i.t. 1x10⁷ live conidia
Rest
Sacrifice and assess for inflammation

B.


IgE ng/ml (serum)
400
300
200
100
0
Challenged
WT
Clec7a-/-

C.

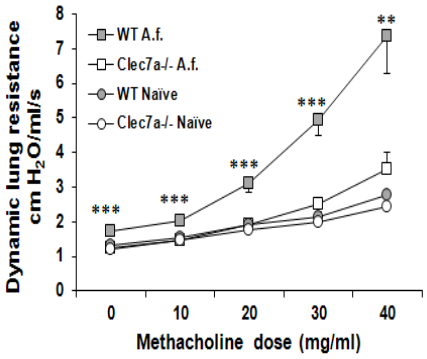
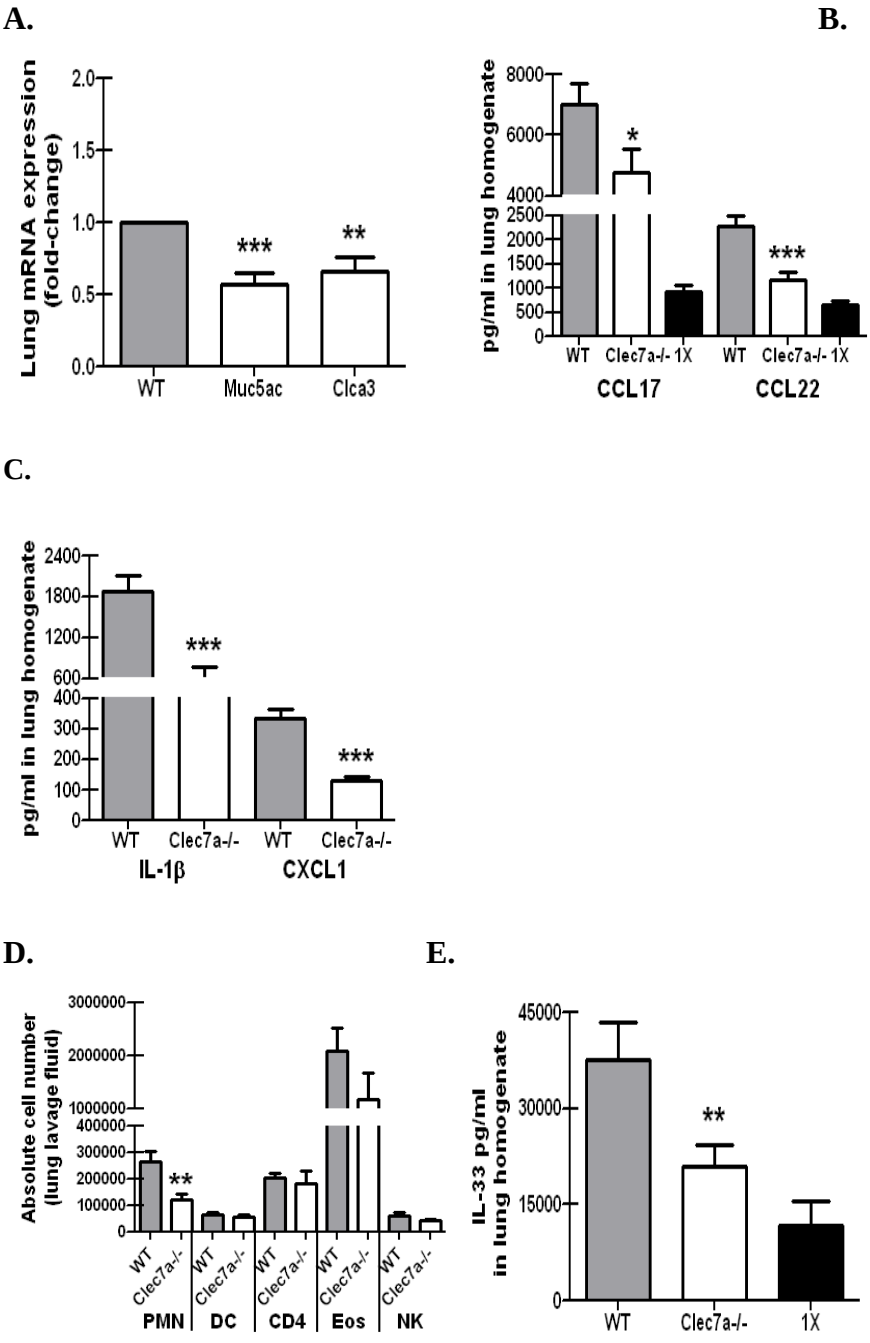
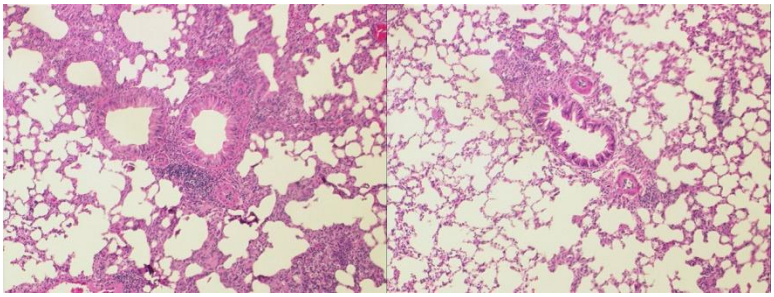

Dynamic lung resistance cm H2O/ml/s
WT A.f.
Clec7a-/- A.f.
WT Naïve
Clec7a-/- Naïve
***
***
***
***
**
0 10 20 30 40
Methacholine dose (mg/ml)

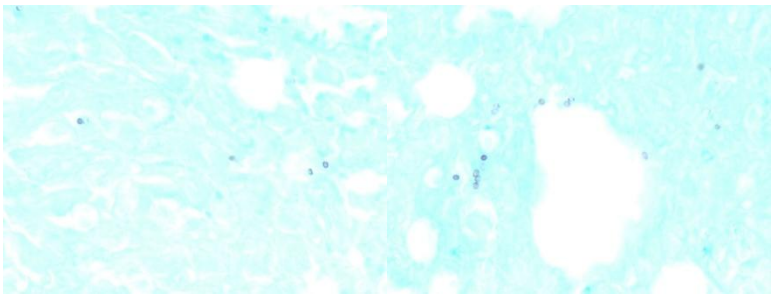
Figure 2. The absence of Dectin-1 results in attenuated proallergic and proinflammatory responses after chronic fungal exposure. (A) C57BL/6 wild-type (WT) and Dectin-1 deficient (Clec7a-/-) mice were exposed to *A. fumigatus* as described. Twenty-four hours after the last challenge, the left lungs were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


A.
B.
C.
D.
E.
Lung mRNA expression (fold-change)
2.0
1.5
1.0
0.5
0.0
***
**
WT
Muc5ac
Clca3
pg/ml in lung homogenate
8000
6000
4000
2500
2000
1500
1000
500
0
*
***
WT
Clec7a-/-
1X
WT
Clec7a-/-
1X
CCL17
CCL22
pg/ml in lung homogenate
2400
1800
1200
600
400
300
200
100
0
***
***
WT
Clec7a-/-
WT
Clec7a-/-
IL-1β
CXCL1
Absolute cell number (lung lavage fluid)
3000000
2000000
1000000
400000
300000
200000
100000
0
**
WT
Clec7a-/-
PMN
DC
CD4
Eos
NK
IL-33 pg/ml in lung homogenate
45000
30000
15000
0
**
WT
Clec7a-/-
1X

F.



G.



H.

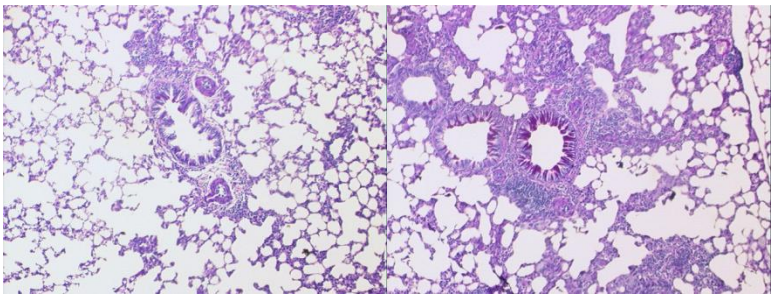
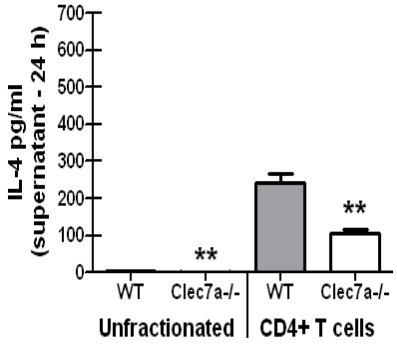
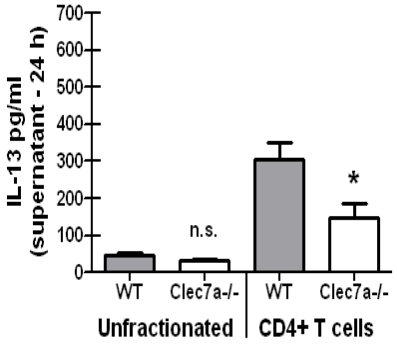


Figure 3. Dectin-1 deficiency is associated with attenuated lung cell IL-17A and IL-22 production during chronic fungal exposure. (A – E) C57BL/6 wild-type (WT) and Dectin-1 deficient (Clec7a-/-) mice were exposed to *A. fumigatus* as described. Twenty-four hours after the last challenge, the right lungs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

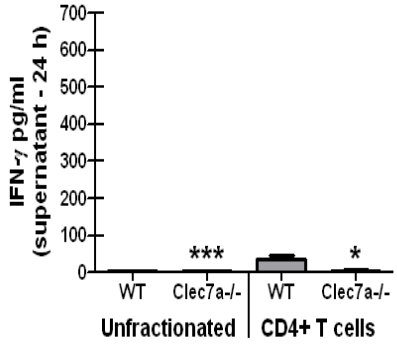
A.


IL-4 pg/ml (supernatant - 24 h)
700
600
500
400
300
200
100
0
**
**
WT
Clec7a-/-
WT
Clec7a-/-
Unfractionated
CD4+ T cells

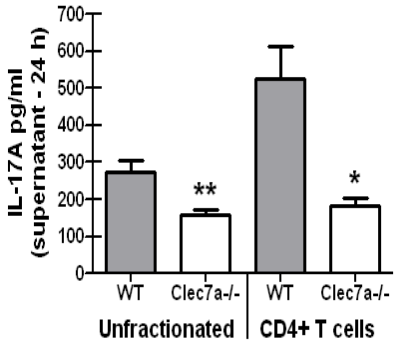
B.


IL-13 pg/ml (supernatant - 24 h)
700
600
500
400
300
200
100
0
n.s.
*
WT
Clec7a-/-
WT
Clec7a-/-
Unfractionated
CD4+ T cells

C.


IFN-γ pg/ml (supernatant - 24 h)
700
600
500
400
300
200
100
0
***
*
WT
Clec7a-/-
WT
Clec7a-/-
Unfractionated
CD4+ T cells

D.


IL-17A pg/ml (supernatant - 24 h)
700
600
500
400
300
200
100
0
**
*
WT
Clec7a-/-
WT
Clec7a-/-
Unfractionated
CD4+ T cells

E.

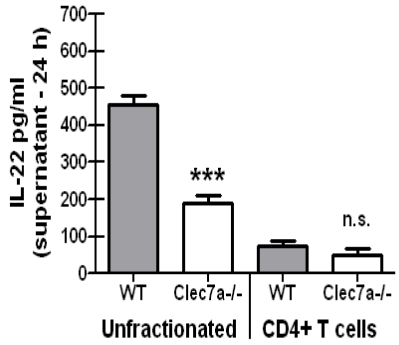
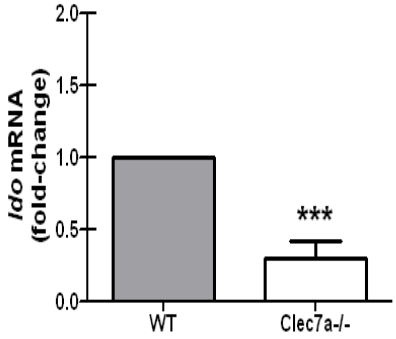
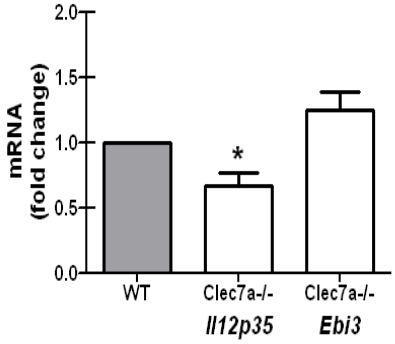

IL-22 pg/ml (supernatant - 24 h)
700
600
500
400
300
200
100
0
***
n.s.
WT
Clec7a-/-
WT
Clec7a-/-
Unfractionated
CD4+ T cells

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

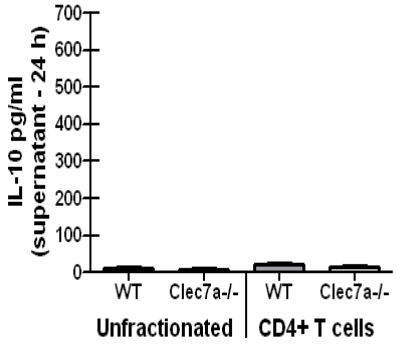
A.


Ido mRNA (fold-change)
2.0
1.5
1.0
0.5
0.0
***
WT
Clec7a-/-

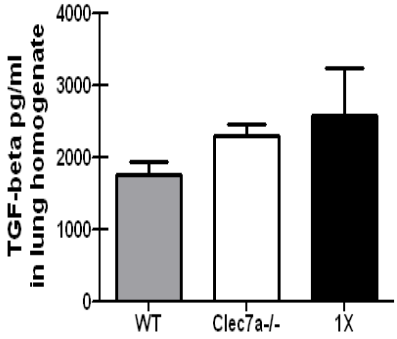
B.


mRNA (fold change)
2.0
1.5
1.0
0.5
0.0
*
WT
Clec7a-/-
Clec7a-/-
Il12p35
Ebi3

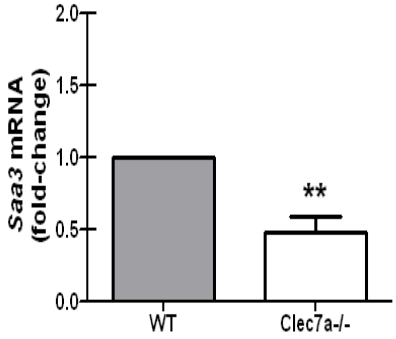
C.


IL-10 pg/ml (supernatant - 24 h)
700
600
500
400
300
200
100
0
WT
Clec7a-/-
WT
Clec7a-/-
Unfractionated
CD4+ T cells

D.


TGF-beta pg/ml
in lung homogenate
4000
3000
2000
1000
0
WT
Clec7a-/-
1X

F.


Saa3 mRNA
(fold-change)
2.0
1.5
1.0
0.5
0.0
**
WT
Clec7a-/-

E.

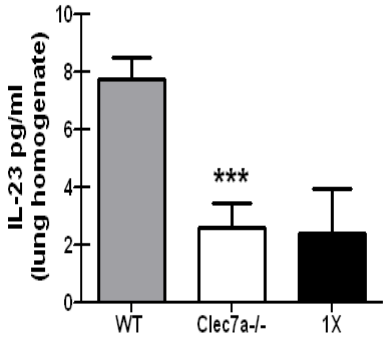

IL-23 pg/ml
(lung homogenate)
10
8
6
4
2
0
***
WT
Clec7a-/-
1X

Figure 5. Lung inflammation requires IL-22 during chronic fungal exposure. (A) C57BL/6 wild-type (WT) and IL-22 deficient (IL22-/-) mice were exposed to *A. fumigatus* as described. *Muc5ac* and *Clca3* gene expression was quantified by real-time PCR and normalized to *Gapdh*. Fold changes between WT (set at 1) and IL22-/- mice were determined using the $2^{-\Delta\Delta Ct}$ method. The Figure illustrates cumulative data from three independent studies. **(B, C, D)** CCL17 and CCL22 (C) IL-33 and (D) IL-1β and CXCL1 were quantified in clarified lung homogenates by ELISA or Bio-Plex. The Figure illustrates cumulative data from four independent studies. For all graphs, ** and *** represent P values of < 0.01 and < 0.001, respectively, and n = 4-5 mice/group for each study.

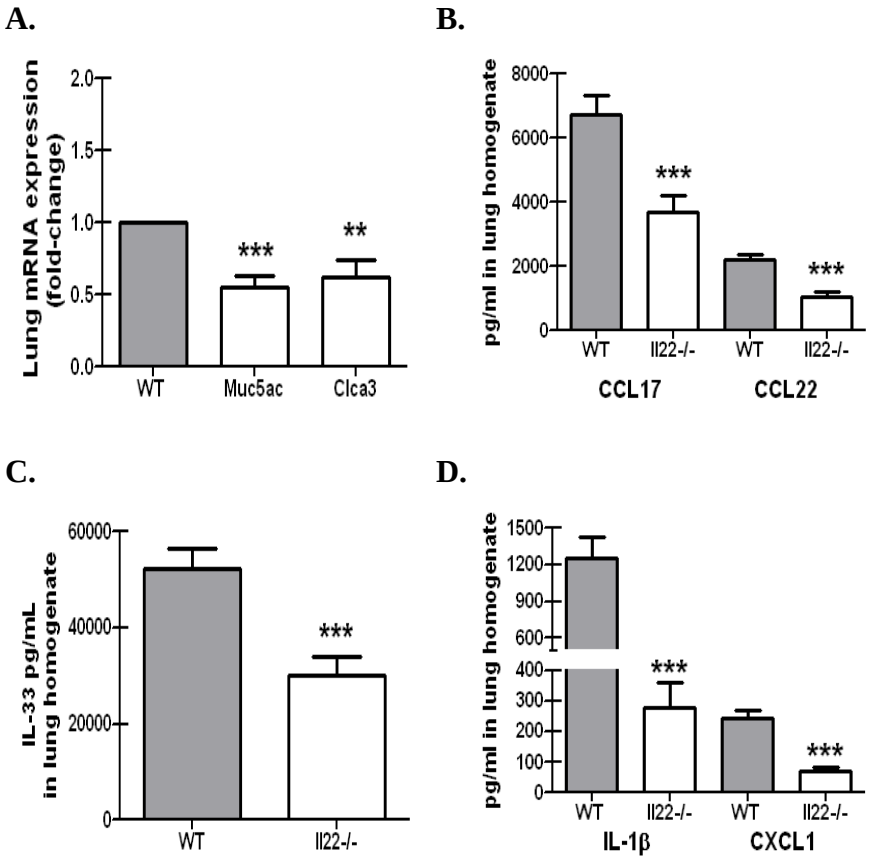
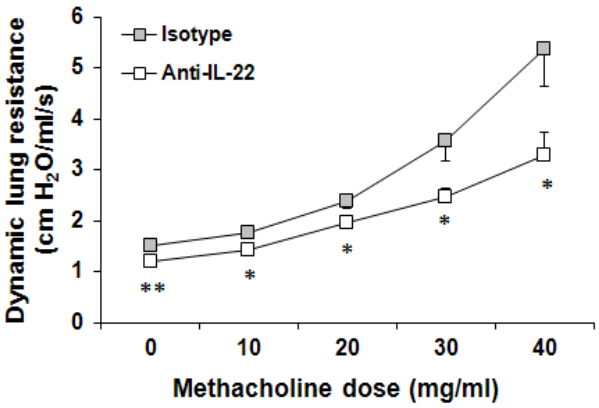

A.
Lung mRNA expression (fold-change)
2.0
1.5
1.0
0.5
0.0
***
**
WT
Muc5ac
Clca3
B.
pg/ml in lung homogenate
8000
6000
4000
2000
0
***
***
WT
Il22-/-
WT
Il22-/-
CCL17
CCL22
C.
IL-33 pg/mL in lung homogenate
60000
40000
20000
0
***
WT
Il22-/-
D.
pg/ml in lung homogenate
1500
1200
900
600
400
300
200
100
0
***
***
WT
Il22-/-
WT
Il22-/-
IL-1β
CXCL1

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


Isotype
Anti-IL-22
Dynamic lung resistance (cm H2O/ml/s)
Methacholine dose (mg/ml)
0
10
20
30
40
1
2
3
4
5
6
**
*
*
*
*

EOSINOPHIL DEFICIENCY COMPROMISES LUNG DEFENSE AGAINST *ASPERGILLUS FUMIGATUS*

LAUREN M. LILLY, MICHAELLA SCOPEL, MICHAEL P. NELSON, ASHLEY R. BURG, CHAD W. DUNAWAY AND CHAD STEELE

Submitted to Infection & Immunity
Format adapted for dissertation

Abstract

Invasive aspergillosis (IA), caused by the opportunistic mold *Aspergillus fumigatus*, is a severe, life-threatening infection commonly observed in immunocompromised individuals. Neutrophil deficiency or dysfunction is the predominant risk factor for the development of IA. Here, we demonstrate that in addition to neutrophils, eosinophils are also an important contributor to lung host defense during IA. Acute *A. fumigatus* challenge in normal mice induced the recruitment of $CD11b^{hi}$ Siglec F^{hi} Ly-$6G^{lo}$ Ly-$6C^{neg}$ eosinophils to the lungs, which was accompanied by an increase in lung *Epx* (eosinophil peroxidase) mRNA levels. Employing mice deficient in the transcription factor dblGATA1, which exhibit a selective deficiency in eosinophils, we show a requirement for eosinophils in lung clearance of *A. fumigatus*. Impaired *A. fumigatus* clearance in dblGATA1 deficient mice correlated with lower mRNA expression of *Epx* (eosinophil peroxidase) and *Prg2* (major basic protein) as well as changes in the levels of IL-1β, IL-6, IL-17A, G-CSF, GM-CSF and CXCL1. However, examination of inflammatory cell populations failed to demonstrate defects in neutrophil recruitment in dblGATA1-deficient mice, suggesting that eosinophil depletion in dlbGATA1-deficient mice was the sole cause for reduced lung clearance. In turn, we show that eosinophils generated from bone marrow have potent killing activity against *A. fumigtaus in vitro* and produce proinflammatory cytokines and chemokines as well as Th2 cytokines. Collectively, our data supports a role for eosinophils in the host response during invasive aspergillosis.

Introduction

Invasive fungal infections (IFIs) caused by *Aspergillus fumigatus* remain one of the most lethal human infectious diseases. Development of invasive aspergillosis (IA) may be a result of multiple predisposing factors, yet immunosuppression leading to neutropenia remains the predominant risk factor [25] [11]. Between 2002 and 2011, there were more than 270,000 solid organ transplants (www.srtr.org) and more than 42,000 bone marrow transplants (http://marrow.org/Home.aspx) performed in the US alone. In turn, more than 48,000 children and adults are diagnosed with leukemia annually (www.cancer.gov). Collectively, these are the most susceptible populations for the development of IA [2]. The most recent data from the Transplant Associated Infections Surveillance Network (March 2001 – March 2006) has reported incidence rates for IFIs of ~7% in both solid organ ([46], 19% due to IA) and HSC transplants ([31], 43% due to IA). Although IA is a known infectious complication of these conditions, there is a growing concern for the development of nosocomial IA in the ICU. One study has indicated that 7% of ICU patients were diagnosed with IA at autopsy, 70% of which did not have a hematological malignancy [41]. Of the 73 patients (66%) who died in this study, 61 (84%) were found to have proven IA (n = 26), probable IA (n = 8) or possible IA (n = 27) upon post-mortem examination [41]. The admitting diagnosis in those with proven IA that were non-neutropenic included patients on high dose steroids (for COPD), cirrhosis and solid cancers. Subsequent studies have identified "new" non-neutropenic conditions associated with the development of IA as COPD, chronic steroid use and liver failure [58] [61] [20]. Data from studies assessing IA in COPD is particularly intriguing. A recent study identified 173 patients with COPD that were not neutropenic, yet 70 (40.5%) patients were

classified as colonized with *A. fumigatus* and 48 (27.7%) as probable/proven IA [3]. These recent clinical reports provide the foundation for a better understanding of the role additional cell types may play in host defense against *A. fumigatus*.

Allergic bronchopulmonary aspergillosis (ABPA) is a hypersensitivity disorder caused by *A. fumigatus* (and other *Aspergillus* species) and is often marked by an eosinophilic immune response in humans [47]. Moreover, in experimental repetitive exposure models of *A. fumigatus*, we [37] and others [17 42] have demonstrated that chronic exposure to *A. fumigatus* resulted in substantial recruitment of eosinophils to the airways. Indeed, cell wall preparations of *Aspergillus* isolated from house dust can induce robust recruitment of eosinophils after administration to mice, an effect that was attenuated by enzymatic degradation of cell wall chitin and beta-glucans [60]. However, some studies suggest a different requirement for eotaxin/CCL11 and eosinophils in acute vs. chronic fungal asthma [54]. In another study that sought to determine the role of house dust associated fungal-derived proteases (many from *Aspergillus niger*) in asthma, results showed that proteases administration alone did not result in an asthmatic phenotype, but were required for *A. niger* mediated asthma [51]. This study further investigated the role of allergic inflammatory responses on fungal clearance in mice that were pretreated with *A. oryzae* protease followed by challenge with *A. niger*, which showed that *Il13*-/- mice as well as IL-5 neutralization resulted in attenuated lung clearance of *A. niger*. In turn, eosinophils at an effector to target ration of 25:1 and greater inhibited the growth of *A. niger* in vitro. Collectively, these results unexpectedly suggest that Th2 responses and eosinophils play a role in immune-mediated clearance of *Aspergillus* spp. from the lungs. To this end, the goal of the current study was to further evaluate the role of eosinophils

during invasive lung infection with *A. fumigatus*. Employing mice deficient in the eosinophil lineage and in vitro eosinophil cultures, we found that this cell type is a potent lung effector cell against *A. fumigatus*.

Materials and Methods

Mice

WT Balb/c and dblGATA1 deficient mice, 6 to 8 weeks of age, were obtained from The Jackson Laboratory (Bangor, ME) and bred at the University of Alabama at Birmingham (UAB). All animals were housed in a specific pathogen-free, Association for Assessment and Accreditation of Laboratory Animal Care-certified facility and handled according to Public Health Service Office of Laboratory Animal Welfare policies after review by the UAB Institutional Animal Care and Use Committee.

Preparation of *A. fumigatus*, *in vivo* challenge and lung fungal burden assessment

A. fumigatus isolate 13073 (ATCC, Manassas, VA) was maintained on potato dextrose agar for 5-7 days at 37°C. Conidia were harvested by washing the culture flask with 50 ml of sterile phosphate buffered saline supplemented with 0.1% Tween 20. The conidia were then passed through a sterile 40 μm nylon membrane to remove hyphal fragments and enumerated on a hemacytometer. For challenge, mice were lightly anesthetized with isoflurane and administered 7×10^7 *A. fumigatus* conidia in a volume of 50 μl intratracheally as previously described [63] [22].. Briefly, mice are held in a vertical, upright position and the tongue is withdrawn from the mouth using forceps. A pipette is used to deliver the 50 μl inoculum to the caudal oropharynx in which normal breathing results in fluid aspiration into the lungs [45]. For lung fungal burden analysis, the left lungs were collected at 24 or 48 h post-exposure and homogenized in 1 ml of PBS. Total RNA was extracted from 0.1 ml of unclarified lung homogenate using the MasterPure™ yeast RNA purification kit (Epicentre Biotechnologies, Madison, WI), which includes a DNAse treatment step to eliminate genomic DNA as previously reported [40]. Total RNA

was also extracted from serial 1:10 dilutions of live *A. fumigatus* conidia (10^1 – 10^9) and DNAse treated to form a standard curve. Lung *A. fumigatus* burden was analyzed with real time PCR measurement of the *A. fumigatus* 18S rRNA (GenBank accession number AB008401 [5]) and quantified using a standard curve of *A. fumigatus* conidia as previously described [40]. As a validation of the real-time PCR method, heat-killed *A. fumigatus* did not yield a signal by real-time PCR and were unable to grow on potato dextrose agar plates [40]. In addition, no amplification controls (i.e. no reverse transcriptase included in the cDNA reaction) yielded a signal of <0.001% by real-time PCR, indicating that the DNAse treatment step was efficient at eliminating contaminating *A. fumigatus* DNA (as DNA is not predicative of organism viability [28]).

Bronchoalveolar lavage cell surface marker flow cytometry

Bronchoalveolar lavage cell isolations were performed as previously described [56] [44]. Cells were washed and Fc receptors blocked with Mouse BD Fc Block (BD Biosciences, San Diego, CA) at 4°C for 20 min. Thereafter, cells were stained with a single-color Live/Dead fixable dead cell stain (Invitrogen), followed by labeling with specific immune cell surface markers. The following staining parameters were employed: macrophages were identified as $CD11b^{lo/neg}$ $CD11c^{+}$, eosinophils as $CD11b^{+}$ Siglec F^{+} Ly-$6G^{lo}$Ly-$6C^{neg}$, neutrophils as $CD11b^{+}$ Ly-$6G^{+}$, and dendritic cells as $CD11b^{+}$ $CD11c^{+}$ (all antibodies purchased from eBiosciences or BD Biosciences). Samples were acquired using a four-laser, 20-parameter analytic BD LSR II flow cytometer, and data were analyzed using the FlowJo software program (Tree Star, Ashland, OR).

Real-time PCR analysis for *Epx, Prg2, Ear1,* and *Ear2* expression in lung tissue

WT Balb/c and dblGATA1 deficient mice were challenged with *A. fumigatus* as described and 18 h after exposure, lungs were collected and homogenized in TRIzol reagent (Invitrogen), and total RNA was isolated as per the manufacturer's instructions. Thereafter, RNA was transcribed to cDNA (iScript cDNA synthesis kit; Bio-Rad), and real-time PCR for *Epx* (Mm00514768_m1; Applied Biosystems), *Prg2* (Mm00435905_m1; Applied Biosystems), *Ear1* (Mm03059811_g10; Applied Biosystems) and *Ear2* (Mm04207376_gH; Applied Biosystems) was performed (iQ Supermix; Bio-Rad). mRNA levels were normalized to *Gapdh* mRNA levels (primers/probe from Applied Biosystems) using the $2^{-(\Delta\Delta Ct)}$ method.

Whole lung cytokine and chemokine analysis

WT Balb/c and dblGATA1 deficient mice were challenged with *A. fumigatus* as described and 24 or 48 h after exposure, the right lungs were isolated and homogenized in PBS supplemented with Complete Mini protease inhibitor tablets (Roche), clarified by centrifugation, and stored at −80°C. Supernatants from lung homogenates were analyzed for protein levels of 23 cytokines and chemokines using Bio-Plex multiplex suspension cytokine array (Bio-Rad Laboratories), according to the manufacturer's instructions [63] [22]. The data were analyzed using Bio-Plex Manager software (Bio-Rad Laboratories).

Derivation of eosinophils from bone marrow and assessment of *A. fumigatus* killing and cytokine production

Bone marrow-derived eosinophils were generated using a previously described protocol [15]. Briefly, bone marrow was isolated from naïve Balb/c mice and cells plated at 1 X 10^6 cells/ml in RPMI 1640 containing 20% FBS (Irvine Scientific, Santa Ana, CA), 2 mM Glutamine, 25 mM HEPES, 1X MEM nonessential amino acids, 1 mM sodium

pyruvate (all from Life Technologies BRL, Rockville, MD), 50 μM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), 100 ng/ml stem cell factor and 100 ng/ml FLT3-L (both from Peprotech). After 4 days, cells were replated in the above media supplemented with 10 ng/ml IL-5. After 10 days, bone marrow cells were fully differentiated into eosinophils. Samples of 1 x 10^5 cells were taken for RNA analysis each time media was changed for real time PCR analysis of *Epx* (Mm00514768_m1; Applied Biosystems) for eosinophil development and *Mpo* (Mm01298424_m1; Applied Biosystems) for neutrophil development. On the tenth day the bone marrow-derived eosinophils were enumerated and utilized in experiments. To assess eosinophil-mediated killing of *A. fumigatus*, 1 x 10^5 eosinophils in a volume of 50 μl were cultured in triplicate with 3 x 10^5 *A. fumigatus* conidia in a volume of 50 μl for 24 h at 37°C, 5% CO_2. Controls included 3 x 10^5 *A. fumigatus* conidia cultured in triplicate in a volume of 100 μl for 24 h. Thereafter, total RNA was extracted from each well using the MasterPure™ yeast RNA purification kit (Epicentre Biotechnologies, Madison, WI) and real time PCR performed as described above. To assess eosinophil cytokine and chemokine production, eosinophils and *A. fumigatus* were cultured at the ratio above (except 1 x 10^6 eosinophils and 3 x 10^6 conidia were employed). Twenty-four h after culture, the contents of each well were collected, centrifuged and clarified supernatants were assessed using the Bio-Plex multiplex suspension cytokine array (Bio-Rad Laboratories) as detailed above.

Statistics

Data were analyzed using GraphPad Prism® Verion 5.0 statistical software (GraphPad Software, San Diego, CA). Comparisons between groups when data were normally distributed were made with the two-tailed unpaired Student's t test and the two-

tailed Mann-Whitney U test when not normally distributed. In real-time PCR analyses, the two-tailed paired Student's t test was employed. Significance was accepted at a value of $p < 0.05$.

Results

Eosinophils are recruited to lung after *A. fumigatus* challenge

Eosinophils are well-documented to play a role in allergic disorders, including the allergic form of aspergillosis, allergic bronchopulmonary aspergillosis [47]. In contrast, a previous study employing chemically immunosuppressed mice challenged with a low dose of *A. fumigatus* suggested that eosinophils likely played a minimal role in defense [7]. Therefore, we sought to clarify their role in a model of invasive aspergillosis in immunocompetent mice. We first show that in normal Balb/c mice challenged with *A. fumigatus*, there is a significant increase in lung expression of the eosinophil marker *Epx* (eosinophil peroxidase) 24 h after exposure (Figure 1A). We subsequently verified the presence of eosinophils by detecting $CD11b^{+}$ Siglec F^{+} Ly-$6G^{lo}$Ly-$6C^{neg}$ cells in lung lavage fluid 24 and 48 h after *A. fumigatus* exposure (Figure 1B). The absolute eosinophil numbers are roughly a log less than what we have previously reported for neutrophils in lung lavage fluid after *A. fumigatus* challenge in C57BL/6 mice [63] [22]. Thus, gene expression and flow cytometric analysis reveal that eosinophils are recruited to the lungs during an *A. fumigatus* infection.

Enhanced susceptibility of eosinophil deficient mice after challenge with *A. fumigatus*

As eosinophils are recruited to the lungs during infection, we questioned whether they played a role in clearance an *A. fumigatus* challenge from the lung. For this, we challenged WT Balb/c and dblGATA1 deficient mice with *A. fumigatus* conidia and assessed fungal burden 24 h and 48 h post-infection. dblGATA1 deficient mice are deficient in a high-affinity GATA-binding site in the GATA-1 promoter, which have a

selective depletion of eosinophils, but not mast cells or platelets [67]. Assessment of *A. fumigatus* fungal burden demonstrated nearly a 2-log higher burden in dblGATA1 deficient mice 24 h post-challenge (Figure 2A). Although there was some level of fungal clearance in dblGATA1 deficient mice between 24 and 48 h post-challenge, these mice nevertheless maintained a 2-log higher burden at 48 h compared to that in *A. fumigatus* challenged WT Balb/c mice (Figure 2A). Neutrophils are widely acknowledged to be the most important effector cell for clearing *A. fumigatus* from the lungs [4 18 57]. Assessing cell recruitment via flow cytometry of lung lavage cells after *A. fumigatus* challeng did not demonstrate a difference in neutrophil recruitment between WT Balb/c and dblGATA1 deficient mice (Figure 2B). Moreover, we verified that thioglycollate-elicited neutrophils from dblGATA1 stimulated with *A. fumigatus* conidia did not demonstrate impaired ROS production (data not shown). Thus, mice deficient in eosinophils demonstrate impaired clearance of *A. fumigatus* from the lungs, which is not a result of defects in neutrophil recruitment or function.

Eosinophil deficient mice challenged with *A. fumigatus* demonstrate reductions in specific eosinophil antimicrobial factors

Like neutrophils, eosinophils are equipped with potent granule proteins that possess antimicrobial activity, including basic protein (MBP), MBP2, eosinophil cationic protein (ECP), eosinophil peroxidase (EPX) and eosinophil-derived neurotoxin (EDN) [30]. However, although highly expressed in eosinophils, some eosinophil associated factors, such as various ribonucleases, may be expressed by additional cell types, such as macrophages and epithelial cells [8]. To this end, we sought to determine which of these eosinophil antimicrobial components were differentially expressed in the lungs between

WT Balb/c and dblGATA1 deficient mice after *A. fumigatus* challenge. Results show that despite demonstrating significantly higher *A. fumigatus* lung burden at 24 and 48 h post-challenge (Figure 2), mRNA levels of eosinophil peroxidase, *Epx,* were significantly lower in dblGATA1 deficient mice (Figure 3A). Similar results were observed for major basic protein, *Prg2* (Figure 3B). In contrast, mRNA levels of eosinophil-associated ribonuclease-1, *Ear1,* was significantly elevated in *A. fumigatus* exposed dblGATA1 deficient mice at 24 h, but significantly reduced at 48 h (Figure 3C). mRNA levels of eosinophil-associated ribonuclease-2, *Ear2,* followed the same trend (Figure 3D). Thus, lower eosinophil peroxidase and major basic protein expression levels, but not eosinophil-associated ribonuclease-1 and -2, correlate with impaired *A. fumigatus* lung clearance in dblGATA1 deficient mice and thus may contribute to antifungal host defense during infection.

Eosinophil deficient mice challenged with *A. fumigatus* demonstrate differences in lung proinflammatory cytokine and chemokine levels

Despite observing no defects in neutrophil recruitment to the lungs of dblGATA1 deficient mice (Figure 2), we nevertheless determined whether the absence of eosinophils during *A. fumigatus* exposure affected the magnitude of the lung inflammatory response. We analyzed the protein levels of 23 cytokines and chemokines in clarified whole lung homogenates from WT Balb/c and dblGATA1 deficient mice 24 and 48 h post-infection. Results in Figure 4 show that despite having significantly higher *A. fumigatus* lung burden at 24 h and 48 h post-challenge (Figure 2), the proinflammatory cytokines IL-1β (Figure 4A), IL-6 (Figure 4B) and IL-17A (Figure 4B) were significantly lower in dblGATA1 deficient mice at 24 h, 48 h and 48 h post-challenge, respectively. Likewise,

the growth factors G-CSF (Figure 4D) and GM-CSF (Figure 4E) and the chemokine CXCL1/KC (Figure 4F) were significantly lower in dblGATA1 deficient mice at 24 h, 48 h and 48 h post-challenge, respectively. Thus, attenuated lung cytokine and chemokine production in the absence of eosinophils correlates with impaired *A. fumigatus* lung clearance in dblGATA1 deficient mice.

Eosinophils recognize and respond to *A. fumigatus*

Data thus far has shown that mice deficient in the generation of eosinophils have a profound defect in clearing *A. fumigatus* from the lungs, and despite impairments in proinflammatory responses, there were no defects in neutrophil recruitment or function in the absence of eosinophils. These results suggest that eosinophils themselves are active inhibitors of *A. fumigatus* growth in the lungs. To address this, we generated eosinophils from the bone marrow of WT Balb/c mice and determined whether they had the ability to affect *A. fumigatus* viability in vitro. We first show that initial culturing of bone marrow cells in the presence of stem cell factor and FLT3-L for 4 days followed by the addition of IL-5 for an additional 6 days resulted in a significant enhancement of *Epx* mRNA expression with a > 300-fold increase by Day 10 of the culture (Figure 5A). In contrast, throughout the 10 day culture period, we saw a parallel decrease in mRNA expression of *Mpo* (myeloperoxidase), a widely employed biomarker of neutrophils (Figure 5B). We next determined whether eosinophils limited the growth of *A. fumigatus* in vitro. The previously mentioned *A. niger* study employed eosinophil to conidia ratios of 6:1 up to 100:1 which inhibited *A. niger* growth ~25% and 100%, respectively [51]. We assessed killing by employing an opposite ratio, as the low numbers of eosinophils detected in lung lavage fluid (Figure 2) suggested a scenario whereby the organism burden

outnumbered the eosinophils. Employing an eosinophil to conidia ratio of 1:3, results showed that culturing *A. fumigatus* in the presence of eosinophils decreased viability by more than 2 logs after 24 h of culture (Figure 5C), a percent killing of > 99%. As eosinophils were capable of recognizing and killing *A. fumigatus* in vitro, we next determined whether this interaction also resulted in cytokine and chemokine production. Employing an identical culture design, we observed significant production of the chemokine CCL4/MIP-1β (Figure 5D) and the Th2 cytokine IL-4 (Figure 5E). Eosinophils cultured in the presence of *A. fumigatus* also produced IL-1β and CCL2/MCP-1 (Figure 5D), as well as the Th2 cytokines IL-13 and IL-9 and the well-recognized eosinophil chemoattractant CCL11/eotaxin (Figure 5E). Thus, eosinophils respond to *A. fumigatus* with the production of proinflammatory cytokines and chemokines as well as Th2 cytokines, an event that also leads to destruction of the organism.

Discussion

Eosinophils are recognized to play important roles in two main immune responses, antiparasitic responses and proallergic/asthmatic responses. However, the development of unique eosinophil deficient mouse strains [67] [33] and additional mouse strains in which eosinophil recruitment or function is diminished [32] [50] [27] have provided novel insights in the role of eosinophils in T helper responses, interactions with immune cells such as macrophages and DCs and antimicrobial defense [53]. With respect to the latter, the identification of TLR expression and responsiveness by human eosinophils [13] [65] has renewed interest in their host defense capabilities, which is supported by findings such as a role for eosinophils in host defense against the Gram negative bacterium *Pseudomonas aeruginosa* [38] and RSV [12] [48]. To this end, the goal of the current study was to determine whether the eosinophil antimicrobial activity extended to fungi, specifically *A. fumigatus*.

We have reported that eosinophils are the predominant cell type observed in the lungs of mice repetitively challenged with *A. fumigatus* in an experimental model of fungal asthma [37]. However, during acute *A. fumigatus* challenge, we have reported that neutrophils represent the dominant lung cell type, albeit eosinophils were present as well [63]. Curiously, eosinophils were significantly higher, and neutrophils significantly lower, in mice deficient in the beta-glucan receptor Dectin-1, which are highly susceptible to *A. fumigatus* [63]. This observation suggests the possibility that the ratio of neutrophils to eosinophils could determine the outcome of *A. fumigatus* infection, i.e. that eosinophils may actually be harmful for host defense. We first confirmed that eosinophils were recruited to the lungs of normal Balb/c mice after acute *A. fumigatus* challenge (as

opposed to our previous report with normal 129/SvEv mice [63]). We assessed this via the expression of the gene for eosinophil peroxidase, *Epx*, which showed significant upregulation in the lungs after *A. fumigatus* challenge. Eosinophils are sometimes difficult to identify via flow cytometry, yet we corroborated the *Epx* analysis by employing a thorough staining procedure that identified eosinophils in lung lavage fluid via the absence of CD11c expression and moderate Gr-1 expression, which were subsequently identified by high expression of CD11b and Siglec F [55].

Having established that eosinophils are a component of the acute inflammatory response after *A. fumigatus* exposure, we next determined their relevance in *A. fumigatus* lung defense. As mentioned earlier, the development of mice with specific deficiencies in eosinophils has afforded the ability to better understand the roles these cells play in immune responses. We chose mice deficient in the dblGATA1 transcription factor, as they have been extensively employed for assessing eosinophil-mediated responses in models of asthma and infectious diseases [33 6 29 21]. Although bone marrow progenitors from dblGATA1 deficient mice may be forced to differentiate into eosinophils in vitro [14] (via various cytokine stimulations and differential promoter usage), this does not occur in dblGATA1 deficient mice in vivo during homoestatic [67] or disease/pathological [26] settings. Moreover, chronic *A. fumigatus* exposure, employed as a model of chronic experimental allergic airway inflammation, shows a >99% reduction in Siglec F+/CCR3+ cells in lung lavage fluid from dblGATA1 deficient mice [19]. In turn, employing dblGATA1 deficient mice on the Balb/c background, we observed a profound, yet somewhat unexpected impairment in their ability to clear *A. fumigatus* from the lungs. In fact, the difference between WT and eosinophil deficient mice at 24 and 48 h was more

than 2 logs, much greater that the observed differences we have previously reported for *Clec7a*-/- (Dectin-1) deficient mice [63] and *Il22*-/- deficient mice [22] (both approximately 5-6 fold higher lung burden).

The susceptibility of dblGATA1 deficient mice to *A. fumigatus* lung infection appeared to be solely a result of eosinophil deficiency as, despite reductions in proinflammatory cytokines such as IL-1β and IL-6 and pro-neutrophil mediators such as G-CSF and CXCL1/KC, we did not observe reductions in lung neutrophil recruitment nor function. We therefore concentrated our efforts on identifying specific eosinophilic inflammatory mediators. Whole lung mRNA analysis revealed significantly reduced expression of *Epx* and *Prg2* at 24 h and 48 h post-infection in dblGATA1 deficient mice. The eosinophil-associated ribonucleases *Ear1* and *Ear2* were unexpectedly observed to be significantly higher in dblGATA1 deficient mice 24 h post-*A. fumigatus* exposure, yet the opposite was true at 48 h. This data suggests that during a lung infection with *A. fumigatus, Epx* (EPO) and *Prg2* (MBP) may play a role in host defense whereas *Ear1* and *Ear2* are dispensable. Multiple mechanisms for killing by eosinophils have been proposed, including antibody-mediated release and/or complement-mediated release of eosinophil granule proteins, such as EPO, MBP and ECP [10 24 23 36]. Eosinophils may also produce nitric oxide (NO) and *Ear2* as antiviral factors, as demonstrated for RSV [48] [12][125, 126][125, 126][125, 126][125, 126][125, 126][125, 126]. LPS stimulation of IL-5 or IFN-γ primed eosinophils [66] or with PAF in the presence of IL-5 or GM-CSF [59] promote the release of eosinophil extracellular traps (EETs) which contain DNA of mitochondrial origin as well as granule proteins. *Epx* encodes for EPO, a heme-containing cationic protein which comprises the bulk of protein found within eosinophilic

granules [34] and has been shown to selectively kill bacteria, parasites and viruses [39 62]. To the best of our knowledge, a potential requirement of EPO in fungal clearance has not been previously reported and the substrate that drives fungal clearance by EPO remains to be identified. Although very little is known regarding EPO and eosinophil-mediated anti-fungal responses, a previous study reported that exposing macrophages to EPO enhanced their respiratory burst leading to enhanced killing of the opportunistic yeast *Candida albicans* [35]. This study suggests that a function of eosinophils and EPO may be to activate the antimicrobial activity of other innate cell types. Another study reported that stimulating human eosinophils with GM-CSF, IL-1, IL-3 and IL-5 augmented phagocytosis of *C. albicans*, yet this paradoxically did not result in increased killing of the organism [16]. In contrast, while these studies suggest a possible role for eosinophils in anti-*Candida* defense, studies with the pathogenic yeast *Cryptococcus neformans* are decidedly opposite. Mice deficient in scavenger receptor A demonstrate increased resistance to *C. neoformans* lung infection, which correlated with reduced Th2 responses and significantly lower eosinophils numbers in the lung [52]. In fact, *C. neoformans* infection in dblGATA deficient mice is associated with lower lung fungal burden in the presence of enhanced Th1 and Th17 responses [49].

Our data suggests that the susceptibility of dblGATA1 deficient mice to *A. fumigatus* is directly due to the specific loss of eosinophils, thus we felt it was imperative to demonstrate that eosinophils are capable of recognizing and responding to *A. fumigatus* conidia. Indeed, eosinophils derived from bone marrow cells with SCF, FLT3-L and IL-5 were adept at inhibiting *A. fumigatus* viability in vitro, an interaction that also led to the production of proinflammatory mediators, chemokines and Th2 cytokines.

These observations indicate the use of pattern recognition receptors in eosinophil anti-*Aspergillus* responses. Studies with human eosinophils have identified expression of numerous TLRs, including TLR1, TLR4, TLR7, TLR9 and TLR10 [43], whereas murine eosinophils have been documented to express TLR2, TLR4 and TLR7 [48 9]. With respect to fungal-associated PRRs, the beta-glucan receptor Dectin-1 has been observed in human eosinophils [64 1]. Identifying which PRR is utilized for *A. fumigatus* recognition is the subject of current investigations.

In summary, we show here that eosinophils are recruited to the lung after *A. fumigatus* exposure and play a necessary role in the elimination of this pathogenic mold from the lungs. Our data further suggests that the eosinophil antimicrobial factors eosinophil peroxidase and major basic protein likely mediate lung clearance. Moreover, we show that eosinophils possess potent antifungal activity against *A. fumigatus* in vitro and serve as a source of numerous cytokines and chemokines. Future studies targeted at understanding which PRRs play a role in eosinophil mediated recognition of *A. fumigatus* as well as specific mechanism(s) of antifungal activity will further define the importance of eosinophils in host defense against diseases such as invasive aspergillosis and allergic bronchopulmonary aspergillosis.

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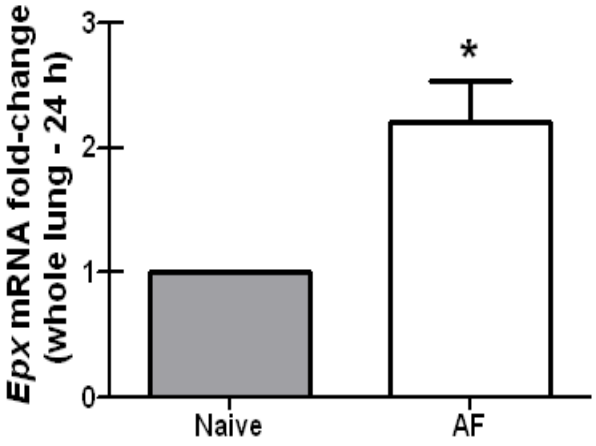
Figure 1. Eosinophils are recruited to lung after *A. fumigatus* challenge. (A) Balb/c wild-type mice were challenged intratracheally with 7 x 10^7 *A. fumigatus* conidia (AF) and 24 h post-exposure, whole lungs were collected, total RNA was isolated, transcribed to cDNA and quantitative real-time PCR was performed for *Epx*. Gene expression was normalized to *Gapdh* and fold changes between naïve mice (set at 1) and *A. fumigatus*-exposed Balb/c mice were determined using the $2^{-\Delta\Delta Ct}$ method. The Figure illustrates cumulative data from three independent studies (n = 3-5 mice per group, per study). * represents a P value of < 0.05 (Paired two-tailed Student's t test). **(B)** Balb/c wild-type mice were challenged intratracheally with 7 x 10^7 *A. fumigatus* conidia (AF) and 24 or 48 h post-exposure, lung cells were isolated via bronchoalveolar lavage, Fc-blocked, stained with a live/dead staining kit and thereafter stained with fluorochrome-conjugated CD11c, CD11b, Gr-1 and Siglec F. The Figure illustrates cumulative data from three independent studies (n = 2-3 mice per group, per time point, per study). Data are expressed as absolute number of live cells in lung lavage fluid. * and *** represent P values of < 0.05 and < 0.0001, respectively (Unpaired two-tailed Student's t test).

A.


Epx mRNA fold-change
(whole lung - 24 h)
3
2
1
0
*
Naive
AF

B.

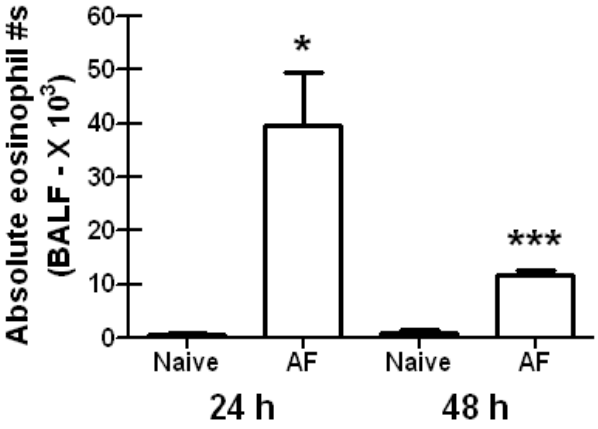
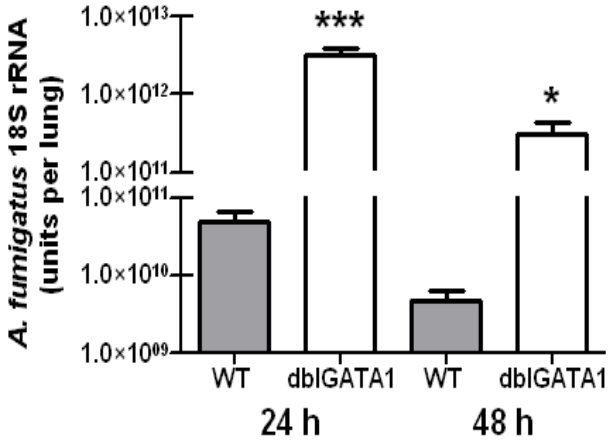

Absolute eosinophil #s
(BALF - X 10^3)
60
50
40
30
20
10
0
*
***
Naive
AF
Naive
AF
24 h
48 h

Figure 2. Enhanced susceptibility of eosinophil deficient mice after challenge with *A. fumigatus*. (A) Balb/c wild-type (WT) and dblGATA1 deficient (dblGATA1) mice were challenged intratracheally with 7 x 10^7 *A. fumigatus* conidia and 24 and 48 h after exposure, lung fungal burden was assessed by real-time PCR analysis of *A. fumigatus* 18S rRNA levels. The Figure illustrates cumulative data from two independent studies (n = 4-5 mice per group, per time point, per study). Data are expressed as mean *A. fumigatus* 18S rRNA + SEM. * and *** represent P values of < 0.05 and < 0.0001, respectively (Unpaired two-tailed Student's t test). **(B)** WT and dblGATA1 mice were challenged intratracheally with 7 x 10^7 *A. fumigatus* conidia (AF) and 24 h post-exposure, lung cells were isolated via bronchoalveolar lavage, Fc-blocked, stained with a live/dead staining kit and thereafter stained with fluorochrome-conjugated CD11b and Ly-6G. The Figure illustrates cumulative data from two independent studies (n = 3-4 mice per group, per study). Data are expressed as absolute number of live cells in lung lavage fluid.

A.


A. fumigatus 18S rRNA
(units per lung)
1.0×10^13
1.0×10^12
1.0×10^11
1.0×10^11
1.0×10^10
1.0×10^09
***
*
WT
dblGATA1
WT
dblGATA1
24 h
48 h

B.

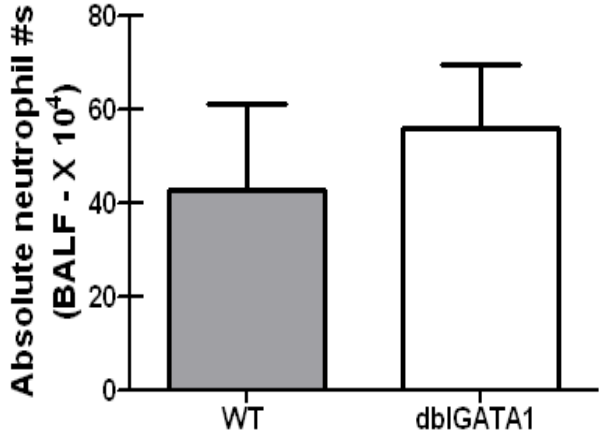
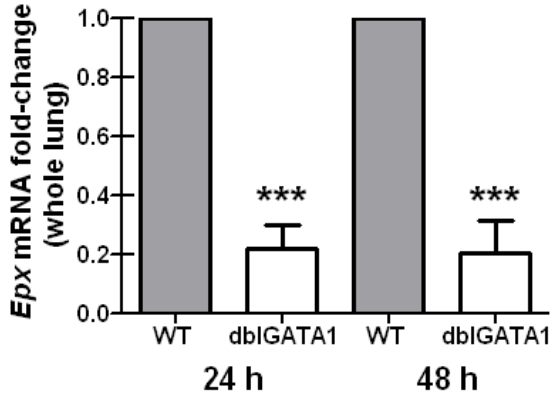
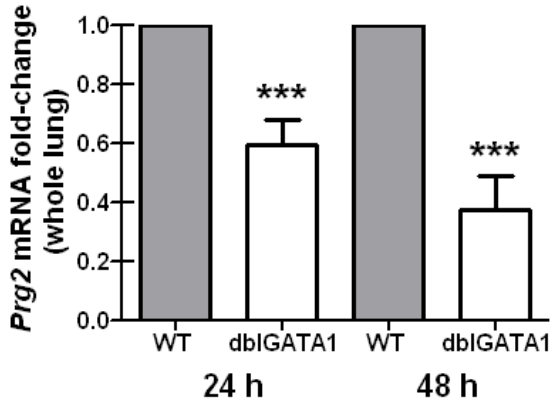

Absolute neutrophil #s
(BALF - X 10^4)
80
60
40
20
0
WT
dblGATA1

Figure 3. Eosinophil deficient mice challenged with *A. fumigatus* demonstrate reductions in specific eosinophil antimicrobial factors. Balb/c wild-type (WT) and dblGATA1 deficient (dblGATA1) mice were challenged intratracheally with 7 x 10^7 *A. fumigatus* conidia and 24 and 48 h after exposure, whole lungs were collected, total RNA was isolated, transcribed to cDNA and quantitative real-time PCR was performed for **(A)** *Epx*, **(B)** *Prg2*, **(C)** *Ear1* and **(D)** *Ear2*. Gene expression was normalized to *Gapdh* and fold changes between WT mice (set at 1) and dblGATA1 mice were determined using the $2^{-\Delta\Delta Ct}$ method. The Figure illustrates cumulative data from two independent studies (n = 4-5 mice per group, per time point, per study,). *, ** and *** represent P values of < 0.05, < 0.01 and < 0.0001, respectively (Paired two-tailed Student's t test).

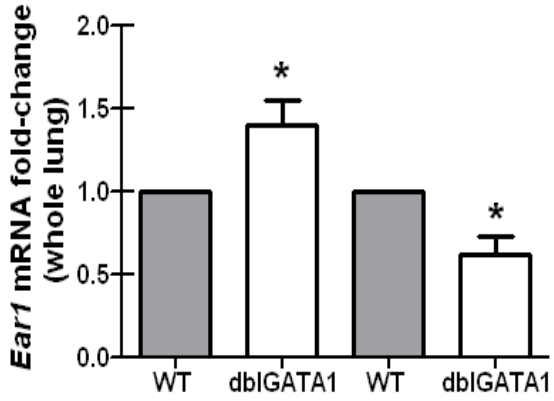
A.


Epx mRNA fold-change (whole lung)
1.0
0.8
0.6
0.4
0.2
0.0
***
***
WT
dblGATA1
WT
dblGATA1
24 h
48 h

B.


Prg2 mRNA fold-change (whole lung)
1.0
0.8
0.6
0.4
0.2
0.0
***
***
WT
dblGATA1
WT
dblGATA1
24 h
48 h

C.


Ear1 mRNA fold-change (whole lung)
2.0
1.5
1.0
0.5
0.0
*
*
WT
dblGATA1
WT
dblGATA1

D.

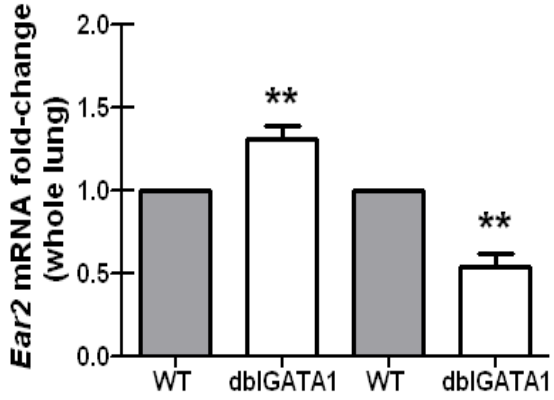
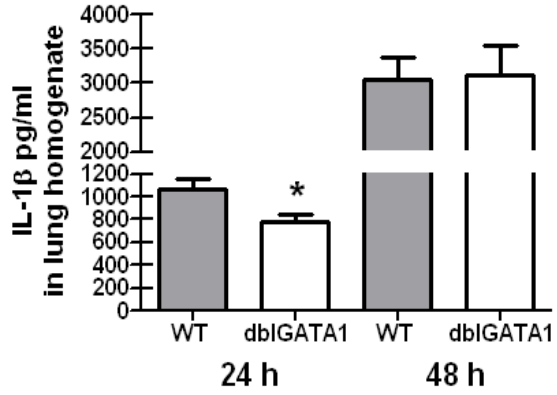
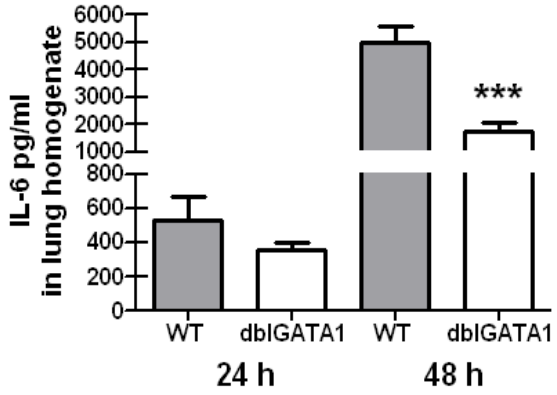

Ear2 mRNA fold-change (whole lung)
2.0
1.5
1.0
0.5
0.0
**
**
WT
dblGATA1
WT
dblGATA1

Figure 4. Eosinophil deficient mice challenged with *A. fumigatus* demonstrate differences in lung proinflammatory cytokine and chemokine levels. Balb/c wild-type (WT) and dblGATA1 deficient (dblGATA1) mice were challenged intratracheally with 7 x 10^7 *A. fumigatus* conidia and 24 and 48 h after exposure. Levels of **(A)** IL-1β, **(B)** IL-6, **(C)** IL-17A, **(D)** G-CSF, **(E)** GM-CSF and **(F)** CXCL1 and were quantified in lung homogenates by Bio-Plex. The Figure illustrates cumulative data from two or three independent studies (n = 4-6 mice per group, per time point, per study). Data are expressed as mean pg/ml + SEM. * and *** represent P values of < 0.05 and 0.001, respectively (Unpaired two-tailed Student's t test).

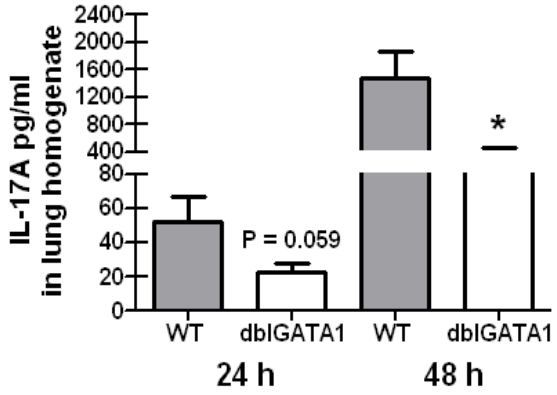
A.


IL-1β pg/ml
in lung homogenate
4000
3500
3000
2500
2000
1200
1000
800
600
400
200
0
*
WT
dblGATA1
WT
dblGATA1
24 h
48 h

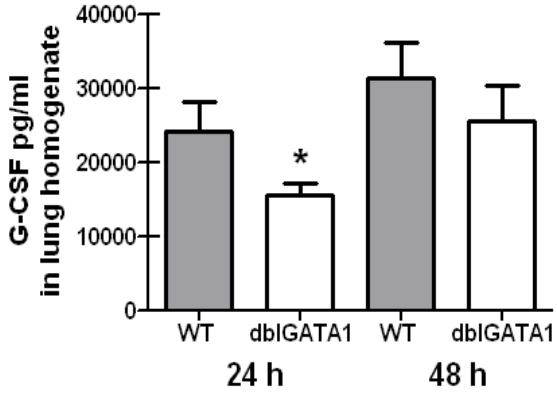
B.


IL-6 pg/ml
in lung homogenate
6000
5000
4000
3000
2000
1000
800
600
400
200
0
***
WT
dblGATA1
WT
dblGATA1
24 h
48 h

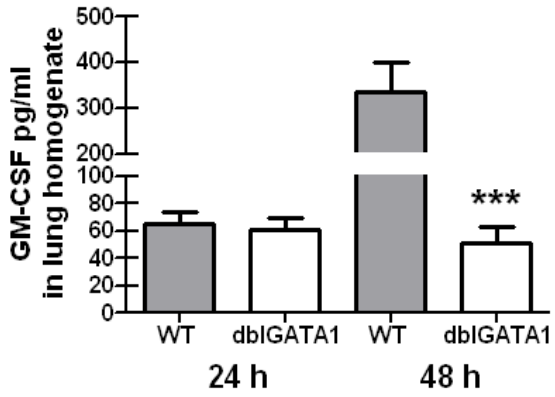
C.


IL-17A pg/ml
in lung homogenate
2400
2000
1600
1200
800
400
80
60
40
20
0
P = 0.059
*
WT
dblGATA1
WT
dblGATA1
24 h
48 h

D.


G-CSF pg/ml
in lung homogenate
40000
30000
20000
10000
0
*
WT
dblGATA1
WT
dblGATA1
24 h
48 h

E.


GM-CSF pg/ml
in lung homogenate
500
400
300
200
100
80
60
40
20
0
***
WT
dbIGATA1
WT
dbIGATA1
24 h
48 h

F.

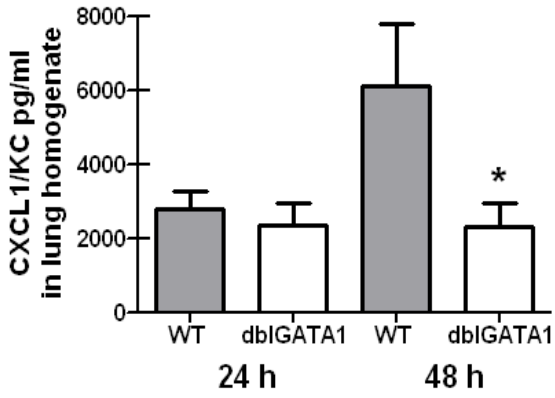
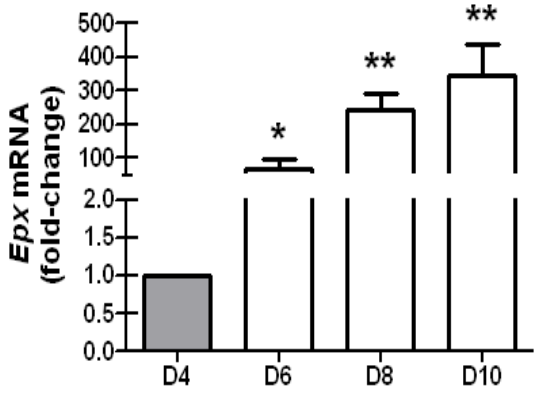
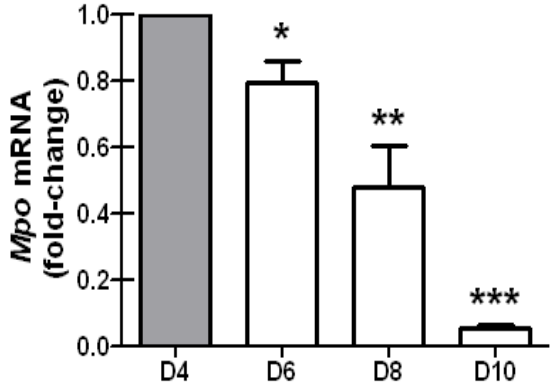

CXCL1/KC pg/ml
in lung homogenate
8000
6000
4000
2000
0
*
WT
dbIGATA1
WT
dbIGATA1
24 h
48 h

Figure 5. Eosinophils recognize and respond to *A. fumigatus*. Bone marrow cells were isolated from naïve Balb/c mice and cultured with 100 ng/ml stem cell factor and 100 ng/ml FLT3-L. After 4 d, cells were replated in medium supplemented with 10 ng/ml IL-5 for an additional 6 d. At 4, 6, 8 and 10 d, an aliquot of cells was collected, total RNA was isolated, transcribed to cDNA and quantitative real-time PCR was performed for **(A)** *Epx* and **(B)** *Mpo*. Gene expression was normalized to *Gapdh* and fold changes between Day 4 (set at 1) and each subsequent time point was determined using the $2^{-\Delta\Delta Ct}$ method. The Figure illustrates cumulative data from three independent studies. *, ** and *** represent P values of < 0.05, < 0.01 and < 0.0001, respectively (Paired two-tailed Student's t test). **(C)** Bone marrow-derived eosinophils (1×10^6 cells) were cultured in the presence of *A. fumigatus* conidia (3×10^6) in a volume of 100 µl for 24 h (AF + Eos). Controls included *A. fumigatus* conidia (3×10^6) cultured alone in a volume of 100 µl for 24 h (AF alone). The Figure illustrates cumulative data from four independent studies. Each dot represents a single well. *** represents a P value of < 0.0001 (Unpaired two-tailed Student's t test). **(D)** Bone marrow-derived eosinophils were cultured as in (C) and supernatants collected after 24 h and clarified by centrifugation. IL-1β, CCL2 and CCL4 and **(E)** IL-4, IL-13, IL-9 and CCL11 were quantified by Bio-Plex. Controls included bone marrow-derived eosinophils cultured in medium alone (gray columns). The Figure illustrates cumulative data from two independent studies. ** and *** represent P values of < 0.01 and < 0.0001, respectively (Unpaired two-tailed Student's t test).

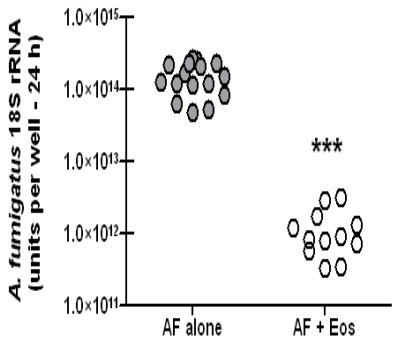
A.


Epx mRNA
(fold-change)
500
400
300
200
100
2.0
1.5
1.0
0.5
0.0
*
**
**
D4
D6
D8
D10

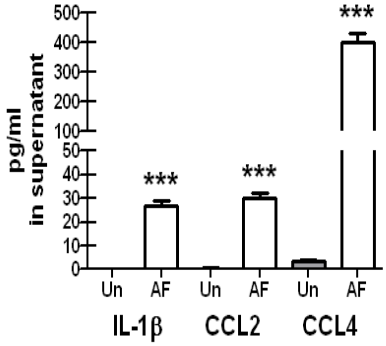
B.


Mpo mRNA
(fold-change)
1.0
0.8
0.6
0.4
0.2
0.0
*
**
***
D4
D6
D8
D10

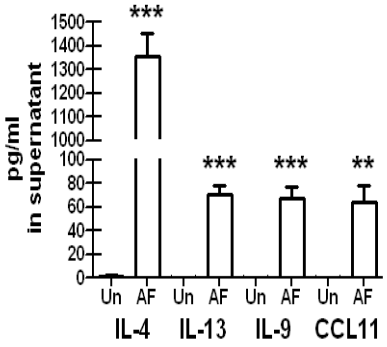
C.


A. fumigatus 18S rRNA
(units per well - 24 h)
1.0×10^15
1.0×10^14
1.0×10^13
1.0×10^12
1.0×10^11
***
AF alone
AF + Eos

D.


pg/ml
in supernatant
500
400
300
200
100
50
40
30
20
10
0
***
***
***
Un AF Un AF Un AF
IL-1β CCL2 CCL4

E.


pg/ml
in supernatant
1500
1400
1300
1200
1100
1000
100
80
60
40
20
0
***
***
***
**
Un AF Un AF Un AF Un AF
IL-4 IL-13 IL-9 CCL11

DISCUSSION

A. fumigatus is a leading cause of pulmonary infections in immunocompromised patients with incidence of disease on the rise. The observed increase in *A. fumigatus*-related cases is largely due to the increase in the immunosuppressed patients. In order for successful treatment, early detection and aggressive treatment must occur. However, with the growing resistance to antifungal azole treatments, the mortality rate associated with *Aspergillus* infections remains high [127] . Thus, better understanding of the mechanisms and factors of both host defense and susceptibility remains vital for optimal outcome during infection. This dissertation seeks to define contributors of protection in a model of chronic as well as an invasive *A. fumigatus* infection.

Fungal allergy

The beta-glucan receptor dectin-1 has been well characterized in mouse models of IPA [45, 56, 123] . Our lab has shown that dectin-1 is required for a robust pro-inflammatory and cellular recruitment response and is thus essential during IPA. Dectin-1 expression, in particular on neutrophils, is responsible for production of IL-17A and IL-22, both of which are required for clearance. However, the role of dectin-1 during a chronic, allergic *Aspergillus* infection remained undefined. Our data suggests that although dectin-1 is required for a protective host defense response during an invasive infection, during fungal allergy dectin-1 drives immunopathology via Th17-related cytokines.

Through use of a sensitization and repetitive challenge model, we discovered the absence of dectin-1 in mice led to significantly better lung function compared to wildtype mice. To better understand the mechanism behind these findings, we further analyzed

wildtype and dectin-1-deficient mice for pro-inflammatory and pro-allergic markers. Assessment of whole lung RNA revealed that mucin genes commonly up-regulated during allergy, *MUC5AC* and *Clca3,* were expressed at lower levels in the dectin-$1^{-/-}$ mice. Moreover, quantification of total IgE in the serum revealed lower, albeit not significant, levels in dectin-1 knockout mice (data not shown). In addition to decreased antibody levels, Th2-related cytokines, IL-33, CCL17 (TARC), and CCL22 (MDC), were also lower in challenged dectin-1-knockout mice compared to wildtype mice.

Besides CCL17 and CCL20, pro-inflammatory mediators IL-17A and CXCL1 were also lower in whole lung tissue of dectin-1-deficient mice. Both cytokines have known neutrophil-recruiting capabilities, thus we investigated whether dectin-1 knockout mice have a reduction in neutrophil recruitment to the airways. Flow cytometry analysis on cellular recruitment to the bronchoalveolar lavage fluid revealed neutrophils were significantly lower in dectin-$1^{-/-}$ compared to wildtype post-challenge. Interestingly, eosinophils were also trending down. This data mimics previously published data from our lab; during an invasive *A. fumigatus* infection, neutrophils are present in lower numbers in mice lacking the beta-glucan receptor [56]. Moreover, the cellular profile also mirrors the data shown in Fei et al with dectin-1 knockout mice having a reduction in cellular recruitment to the lungs [93]. A recent study revealed that IL-17A knockout mice, after a weekly *A. fumigatus* challenge, have attenuated cellular infiltration to the lungs with the greatest decrease observed in eosinophil recruitment [128]. Moreover, IL-17A-deficient mice also had a decrease in goblet cell hyperplasia. Collectively, the above study suggests the possibility of IL-17A promoting allergic features that are often

attributed to Th2-related cytokines. Thus, the protection observed in dectin-1-deficient mice may, in part, be due to a decrease in IL-17A levels.

In addition to IL-17A, we also propose that IL-22 contributes to the allergic phenotype. Like dectin-1-deficient mice, mice with neutralized IL-22 or lacking the *il-22* gene had better lung function that correlated with lower *MUC5AC* and *Clca3* expression levels in the lung. Interestingly, when accessing the cytokine and chemokine profile of the IL-22-deficient mice, IL-1α protein levels in lung homogenate were strikingly low. This phenomenon was also observed in IL-22-knockout mice during the invasive aspergillosis model [123] . This observation is the foundation for future studies. Like IL-17A, we hypothesize that IL-22 drives a Th2-like phenotype through a cytokine cascade that ultimately up-regulates IL-33. Specifically, we anticipate that IL-22 promotes production of pro-inflammatory cytokine, IL-36γ. IL-36γ is produced by a variety of cells, including IL-22R-expressing epithelial cells. A recent paper by Gresnigt et al revealed that IL-36γ is produced during an *A. fumigatus* infection in a dectin-1 and TLR4 dependent manner [129]. Moreover, our group has supporting unpublished data showing that IL-36γ is produced in an IL-22 dependent manner during fungal asthma.

In another study regarding IL-36, the authors show that the related cytokine IL-36α can up-regulate IL-1α gene expression in splenic $CD11C^{+}$ cells [130]. In turn, a paper investigating the role of IL-1α in a house dust mite model of asthma demonstrated that neutralized IL-1α, and to a lesser extent IL-1β, led to a reduction of pro-allergic cytokine, IL-33 [131]. The neutralization of IL-1α and reduction in IL-33 correlated with an improvement of lung function. Furthermore, in support of the above study, our group has observed that administration of recombinant IL-1α to naïve mice lead to increased

IL-33 expression in lung homogenate compared to mice receiving PBS (unpublished data). Ultimately, we anticipate that IL-22 drives IL-36γ production by lung epithelial cells, leading to IL-1α production that ultimately stimulates IL-33 upregulation in the lung (Figure 1). Through use of IL-36 receptor (IL-36R) knockout mice and a neutralizing IL-1α antibody, future studies will investigate the contribution of IL-36γ and IL-1α in the development of fungal allergy and their roles in driving Th2 responses.

The cellular source of IL-17A and IL-22 during fungal allergy still remains unknown. Purified $CD4^+$ cells in addition to unfractionated lung digest cultures were able to produce both cytokines. In the invasive model, neutrophils have been shown to make IL-17A in a dectin-1 dependent manner [56]. Moreover, unpublished data from our lab also suggest that thioglycolated-isolated neutrophils respond to *A. fumigatus* through IL-22 production. Furthermore, a recent paper by Zindl et al showed that during a mouse model of colitis, neutrophils are responsible for the bulk of IL-22 present in the colon [132]. Thus, we hypothesize that during fungal allergy, neutrophils are a contributing cell source of both IL-17A and IL-22 in the lungs. Future studies utilizing intracellular staining in addition to IL-22 reporter mice will investigate the cellular sources of IL-17A and IL-22 during a chronic *A. fumigatus* infection.

Although we have shown that beta-glucan engagement of dectin-1leads to IL-17A and IL-22 production in the lungs, the contribution of other fungal cell wall moieties remains largely undefined. The majority of studies focus of the role of chitin in allergy with chitin being linked to asthma development and severity in both mice and humans [133-135]. Chitin-containing dust collected from homes was shown to induce eosinophilic infiltration in the mouse lung. Moreover, mice expressing acidic

mammalian chitinase displayed reduced inflammation after challenge with an *Aspergillus* extract. Lastly, chitin, in a size-dependent manner, has been showed to induce IL-17A production by macrophages during a chitin-induced model of allergy [136]. Thus, we anticipate that during challenge with live *A. fumigatus* conidia, chitin, in concert with beta-glucans, promotes IL-17A and IL-22 in the lungs. To investigate the role of chitin, in addition to other cell wall components, in fungal asthma, we will use mutant *A. fumigatus* strains. A list of mutant strains to be used in future studies are shown in Table 1.

To further define the function of various cell moieties in fungal asthma, in addition to mutant strains we will also employ the use of antifungal drugs known to modulate the structure of the fungal cell wall. Recent clinical studies have shown that antifungal treatment may be beneficial in individuals suffering from ABPA as well as patients that demonstrate elevated fungal sensitivity [137]. The therapeutic effect is thought to be due to the reduction in antigen load via the elimination of *A. fumigatus*. However, although effective in modulating the severity of asthma, chronic treatment with intraconazole is not a desireable treatment due to adverse effects. In future studies, chronic use of static antifungals will be performed on challenged mice; the goal being not to simply lower fungal burden but rather to manipulate the *A. fumigatus* cell wall *in vivo.* Indeed, preliminary data from our lab shows that static antifungals targeting the cell wall may manipulate the levels of some cell wall moieties (data not shown). Thus, future studies will investigate the effects of this modulation on the development and severity of fungal asthma.

While the contribution of Th2 in allergy/ABPA has been documented, the role of Th-17 related cytokines has yet to be studied [138]. Although shown to have a neutrophillic/IL-8 component, the cytokines produced by the elevated neutrophils observed in the sputum have not been investigated. As our data suggest that IL-17A and IL-22 are produced by neutrophils after an *A. fumigatus* exposure, we question where these same cytokines are elevated in patients with fungal hyper-sensitivity. Similarly, polymorphisms in Th17-related cytokines and their involvement in asthma have garnered attention. A SNP in the *IL-23* gene has recently been reported to be associated with asthma incidence [139]. Furthermore, several SNPs in IL-17A and IL-17F as well as various IL-17 receptors have been reported in asthmatics, predominantly in Asian populations [140, 141]. However, whether the protein levels of these cytokines are modulated still needs to be investigated. Therefore, future studies will determine the magnitude by which IL-23, IL-17A and IL-22 protein levels are modulated in fungal skin-test (+) vs. skin-test (-) asthmatics with various SNPs and further correlate these levels with the various asthma traits. Additionally, we will also correlate the levels of IL-23, IL-17A and IL-22 with the levels of IL-36γ, IL-1α and IL-33 to better understand the interactions of these immunopathogenic pathways during fungal asthma. The SNPs and traits to be studied are detailed in Table 2.

Eosinophils are Protective during an A. fumigatus Infection

Eosinophils are historically associated with immune responses that are antiparasitic or allergic in nature. However with the discovery of toll-like receptors on eosinophils, the possibility of a more classical host defense role for eosinophils have been raised. We have previously shown above that during a chronic infection, eosinophils are

present in the BALF. Moreover, we previously have published that neutrophils and neutrophil-derived IL-17A are critical for host defense against lung infection with *A. fumigatus*. As it widely known that neutrophils, in addition to macrophages, play a protective role during infection, we questioned whether other cell are required for host defense during an *A. fumigatus* infection. Data from our lab show that eosinophils may also serve as effector cells against *A. fumigatus.*

We have shown that during an invasive *A. fumigatus* infection, eosinophils were observed in the lungs of infected mice 24 hours and 48 hours post-challenge compared to naïve controls. To further assess the role of eosinophils during an acute *A. fumigatus* infection, dblGATA-1 knockout mouse were utilized. Exposure of the dlbGATA-1 knockout mice with *A. fumigatus* resulted in an elevated fungal burden at 24 hours and 48 hours post-infection. As the presence of other cell infiltrates was not altered, we questioned where eosinophils were capable of killing *A. fumigatus* in vitro. To directly access the ability of eosinophils to kill, BMDEos were plated in the presence of conidia for 24 hours and killing was assessed by PCR. After 24 hours, wells plated with *A. fumigatus* conidia in addition to BMDEos displayed significantly less fungal growth compared to wells with *A. fumigatus* alone. Thus, BMDEos have the ability to kill *A. fumigatus* conidia in vitro.

Further assessment of dblGATA-1 mice revealed that eosinophil-related genes, *Epx* and *Prg2* were significantly reduced in whole lungs of dblGATA-1 mice. In contrast, ribonucleases *Ear1* and *Ear2* were elevated in dblGATA-1 knockout mice at 24 hours post-infection. Indeed, expression of *Ear1* and *Ear2* has been observed in other cell types besides eosinophils, including non-leukocytes such as airway epithelial cells

[142]. Collectively, the data suggests that the host response during an *A. fumigatus* infection is specific; *Epx* and *Prg2* are required during an invasive fungal infection whereas *Ear1* and *Ear2* are dispensable.

The killing mechanisms of eosinophils against *A. fumigatus* remain to be defined. However, the reduction of *Exp* and *Prg2*, coupled with elevated burden, in the dblGATA-1-deficient mice is the groundwork for future studies. As cytokine and chemokine protein levels were not greatly reduced in dblGATA-$1^{-/-}$ mice, the data suggests that eosinophilic mediators may be the driving factor of host defense. Candidate proteins include catanionic proteins eosinophil peroxide (EPO) and major basic protein (MBP). Various studies have shown the ability of recombinant eosinophil granular proteins to kill bacteria and viruses in vitro [143-145]. Through use of recombinant proteins, future studies will assess the ability of EPO and MBP to kill *A. fumigatus* conidia. Specifically, like the BMDEos assay, recombinant EPO and MBP will be plated with resting *A.fumigatus* condia and fungal viability will be assessed by PCR. To complement in vitro studies, EPO- and MBP-deficient mice will also be utilized to assess the role of the proteins in vivo during infection.

In the above study, we show that stimulation of BMDEos with resting *Aspergillus* conidia for 24 hours resulted in a pro-inflammatory and Th2 response, suggesting that BMDEos are capable of recognizing fungal moieties present on *A. fumigatus*. Indeed previous reports have demonstrated that murine eosinophils express multiple TLRs including TLR4. Similarly, human eosinophils have been shown to activate in response to various fungi after stimulation [146-148]. Human eosinophils have been shown to constitutively express TLR1, TLR4, TLR7, TLR9, TLR10 as well as beta-glucan receptor

dectin-1 [149, 150]. Future studies will seek to investigate the receptors expressed by eosinophils that mediate recognition of *A. fumigatus*. Therefore, BMDEos will be generated and receptors TLR2, TLR4, TLR9 and dectin-1 will be analyzed by flow cytometry. To further access the contribution of each receptor, BMDEos will be isolated from TLR- and dectin-1 knockout mice and stimulated with *A. fumigatus* conidia. Cytokine and chemokine levels will be quantified and fungal viability determined by PCR.

In ABPA patients and asthmatics with fungal sensitivity, eosinophilia is a commonly observed feature. However, in IA, while neutropenia is a well-recognized risk factor, the modulation of other cell types has yet to be fully investigated. Therefore, to better understand the role of eosinophils in IA, in future studies we will assess the levels of eosinophils in the blood and BALF of invasive aspergillosis patients. Moreover, the levels of eosinophilic granular proteins will also be quantified in various biological samples including blood, sputum, BALF and lung tissue.

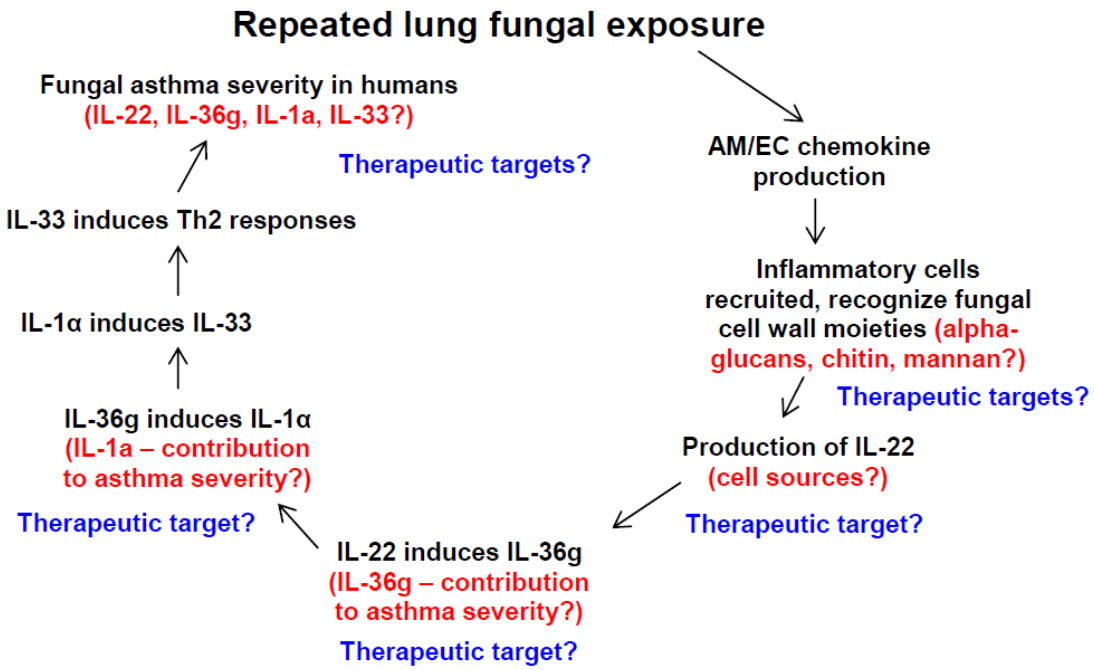


Figure 1. A schematic representation of the immune response to chronic A. fumigatus exposure.

Table 1. *A. fumigatus* cell wall mutants.

Mutant strain	**Affected cell wall moiety**	**WT strain**
gel1,2	Beta-glucan	CBS144-89
ags1,2,3	Alpha-glucan	Ku-80
chsA-G	Chitin	Ku-80
och1,2,3,4	Galactomannan	Ku-80
ugm1	Mannan	Ku-80
rodA	Rodlet	CBS144-89
alb1	Melanin	B-5233

Table 2—IL-23/IL-17A/IL-22 SNPs.

CAUCASIANS

Trait	Chromosome	SNP	Gene	Type	P value
Max Reversibility	1	rs16829204	*Il22ra1*	Missense	0.03164
FEV/FVC ratio[2]	1	**rs11209026**	*Il23r*	Intronic	0.00177
	1	**rs10889665**	*Il23r*	Intronic	0.01812
	1	**rs4655690**	*Il23r*	Intronic	0.02224
	1	rs12751814	*Il23r*	Intronic	0.02630
FEV1 (% predicted)	1	**rs10889665**	*Il23r*	Intronic	0.01315
	1	**rs4655690**	*Il23r*	Intronic	0.02544
	1	**rs11209026**	*Il23r*	Intronic	0.02660
	12	rs2046068	*Il22*	Intronic	0.04266
	12	**rs2227491**	*Il22*	Intronic	0.04699
FVC (% predicted)	22	**rs879577**	*Il17ra*	Missense	0.04901

AFRICAN-AMERICANS

Trait	Chromosome	SNP	Gene	Type	P value
Log PC20	6	rs3819025	*Il17a*	Intronic	0.00090
	1	**rs2863212**	*Il23r*	Intronic	0.04773
Max Reversibility	22	**rs879577**	*Il17ra*	Missense	0.01517
	1	rs10489630	*Il23r*	Intronic	0.01867
	12	**rs2227491**	*Il22*	Intronic	0.03022
	1	**rs2863212**	*Il23r*	Intronic	0.04839
FEV1 (% predicted)	12	**rs2227491**	*Il22*	Intronic	0.00719
	22	rs2241046	*Il17ra*	Intronic	0.02607
FVC (% predicted)	12	**rs2227491**	*Il22*	Intronic	0.00395

SNPs observed in both Caucasians and African-Americans are in **bold red font**.
SNPs observed in multiple traits within an ethnic group are in **bold blue font**.

References

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: January 8, 2013

TO: CLAUDE HENRY STEELE III, Ph.D.
THT -422
FAX: (205) 934-1721

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Pulmonary Defense Against Aspergillus Fumigatus
Sponsor: NIH
Animal Project Number: 130109031

As of January 8, 2013, the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	A	498
Mice	C	498

Animal use must be renewed by January 7, 2014. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 130109031 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

Institutional Animal Care and Use Committee
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205.934.7692
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