FUNGAL KERATITIS: IMMUNE EVASION, HOST-PATHOGEN INTERACTIONS, AND VIRULENCE FACTORS DURING ASPERGILLUS FUMIGATUS INFECTION

by

STEVEN DE JESUS CARRION

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Thesis Advisor: Eric Pearlman, Ph.D.
Department of Pathology
CASE WESTERN RESERVE UNIVERSITY
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CASE WESTERN RESERVE UNIVERSITY
SCHOOL OF GRADUATE STUDIES

We hereby approve the thesis/dissertation of

Steven de Jesus Carrion

candidate for the degree of Pathology

Committee chair:
George R. Dubyak

Committee members:
Mahmoud Ghannoum
Amy G. Hise
Derek Abbott
Clive R. Hamlin
Eric Pearlman

(Date) 11/07/2014

*We also certify that written approval has been obtained for any proprietary material contained herein
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Fungal Keratitis: Immune Evasion, Host-Pathogen Interactions, and Virulence Factors during *Aspergillus Fumigatus* Infection

Abstract

by

STEVEN DE JESUS CARRION

Corneal infections with *Aspergillus fumigatus*, *A. flavus*, *Fusarium solani* and *F. oxysporum* are major causes of corneal ulcers worldwide, resulting in visual impairment and blindness. During infection, these pathogenic molds synthesize cell wall components that mask immune recognition or confer resistance to leukocyte anti-microbial responses, resulting in increased virulence. To better understand how these fungal pathogens survive in the presence of a pronounced host immune response, we examined the role of the RodA protein and cell wall chitin polymers in fungal pathogenesis during corneal infection.

Airborne spores (conidia) of *A. fumigatus* express the RodA protein, which forms an outer layer that coats the cell wall. We therefore determined if RodA acts as a virulence factor by masking the exposure of cell wall components that would otherwise induce a host immune response. Using a RodA deficient *A. fumigatus* mutant strain (ΔrodA), we observed increased surface exposure of β-glucan and α-mannose on ΔrodA conidia than on the parent strain. We also found that ΔrodA conidia induced more nuclear translocation of the NFκB p65
subunit and cytokine production in bone marrow macrophages from C57BL/6, but not Dectin-1−/− or Dectin-2−/− mice. Using a murine model of fungal keratitis, we found that ΔrodA conidia induced significantly higher cytokine production and neutrophil infiltration into infected corneas, resulting in more rapid fungal killing compared with the parent strain. This response was dependent on Dectin-1 and Dectin-2 activation. Overall, these results identify the RodA protein as a virulence factor in *A. fumigatus* that prevents fungal recognition by masking Dectin-1 and Dectin-2 activation, resulting in impaired fungal clearance and worse clinical disease.

Chitin is a cell wall polysaccharide present in all fungi, including *A. fumigatus*. Chitin is inert to the host immune system; however, fragmentation of chitin can lead to pro and anti-inflammatory responses. We therefore examined the role of chitin in *A. fumigatus* keratitis. *In vivo*, we found that expression of acidic mammalian chitinase (AMCase) co-localized with neutrophils in the corneal stroma. *In vitro*, mouse and human neutrophils expressed AMCase in response to *Aspergillus* hyphal extract (AspHE), and this up-regulation was dependent on Dectin-1 and spleen tyrosine kinase (Syk). AMCase expression was also up-regulated by IL-6 and IL-23 (IL-6/23). Moreover, chemical inhibition of AMCase using Bisdionin F or C resulted in impaired neutrophil killing of hyphae, suggesting that AMCase has anti-fungal activity against *A. fumigatus*. Conversely, chitin synthase mutant strains were more susceptible to neutrophil killing *in vitro* and following corneal infection. In contrast, we found that a chitin deacetylase mutant strain that has higher cell wall chitin was resistant to
neutrophil killing and was more virulent than the parent strain. Pharmacological inhibition of chitin synthesis using nikkomycin Z reduced *A. fumigatus* virulence during infection and augmented neutrophil-mediated killing *in vitro*. Finally, chemical inhibition of both chitin and β-glucan synthesis significantly increased susceptibility to neutrophil killing. These findings indicate that AMCase is an essential mediator of anti-fungal immunity, and also identifies chitin as a virulence factor that promotes resistance to neutrophil responses during *A. fumigatus* keratitis.

In conclusion, we have shown that the RodA protein in *A. fumigatus* conidia masks the exposure of surface β-glucan and α-mannose, which prevents Dectin-1 and Dectin-2 activation in resident corneal macrophages and enhances fungal survival during infection. Further, we established an anti-fungal role for neutrophil AMCase against hyphae and identified chitin as a virulence factor in *A. fumigatus* that confers resistance to neutrophil killing. These results indicate that structural cell wall components can circumvent immune recognition of *A. fumigatus* by host innate immune cells.
Chapter 1

Introduction
1.1 Pathogenesis of fungal keratitis

Fungal keratitis is the second leading cause of blindness after cataracts worldwide [12]. The predominant etiological agents are *Aspergillus* and *Fusarium* species and their distribution depends on environmental conditions [12,13]. In the USA and other industrialized countries, the use of contact lens is the predominant risk factor for fungal keratitis [14,15]. Fungal keratitis has significantly achieved notoriety in the USA after the multistate *Fusarium* outbreak in 2005/2006, where it was shown that fungi can grow in the contact lens care solution [16]. In developing countries, the predominant risk factor for fungal keratitis is trauma to the eye caused mainly by vegetable or organic matter, where injury to the corneal epithelial layer occurs and spores (conidia) present in the air or soil enter the corneal stroma and germinate into hyphae to cause disease [17-19]. After germination of conidia, hyphae penetrate the corneal stroma, which triggers a strong inflammatory response that causes corneal opacification, leading to visual acuity impairment and subsequently, vision loss. During infection, the robust cellular infiltration which is primarily neutrophils and induced by the fungal pathogen is responsible for the development of corneal opacification and “cloudiness” of the cornea (Figure 1.1). Further progression of the disease could require the patient to undergo a corneal transplant because of all the damage caused by the fungal pathogen and the inflammation, but unfortunately only a 30% of corneal transplants are successful. Corneal infections due to *Aspergillus* species lead to a corneal transplant in 60% of cases.
In contrast, infections with *Fusarium* species lead to corneal ulcers in 23-32% of cases [12].

**Figure 1.1 Clinical manifestations of fungal keratitis.** A) Representative image of an infected cornea from a patient with *Aspergillus flavus*. B) Histology section of a normal hematoxylin and eosin stained human cornea. C) GMS stained cornea of a patient with *A. flavus* keratitis [1]. Reprint permission obtained from the publisher.

### 1.2. Diagnosis and treatment of *Aspergillus* keratitis

Laboratory diagnosis is always performed when fungal keratitis is suspected. The four ways utilized to diagnose fungal infection in the cornea are by smear, fungal culture, polymerase chain reaction (PCR), and confocal microscopy [12]. Direct microscopy of stained smears is the fastest diagnostic tool for the detection of fungal hyphae in corneal scrapings. Different stains utilized in the clinic are calcofluor white, Grocott methenamine silver staining (GMS), or 10% potassium hydroxide (KOH). For fungal cultures, corneal material is inoculated into agar plates and incubated at 30-37°C. The growth of fungi is considered significant if it correlates with the clinical presentation or if the growth of the fungus is repeated in more than two separate culture media. The use of PCR and confocal microscopy are now used as a rapid way for diagnosis;
however, these tools are not always available in developing countries where the incidence of fungal keratitis is high.

The current anti-fungal therapies are not very successful in the treatment of fungal keratitis due to the poor efficiency of penetration into the corneal stroma [20,21]. Further, these agents are mostly fungistatic and not fungicidal, which means that a prolonged course of therapy is required. There are several compounds utilized for fungal infections which can be classified into three groups: polyenes, azoles, and echinocandins [9,22] (Figure 1.2). The polyenes target ergosterol in the cell

![Figure 1.2 Sites of action for antifungal drugs][9]. Reprint permission obtained from the publisher

membrane, which induces leakage of monovalent cations like K⁺ and Na⁺, leading to cell death [9]. This group comprises natamycin and amphotericin B. Natamycin is the drug of choice to treat filamentous fungal infections although it is relatively costly and in most developing countries is not available. The Azoles
inhibit the enzyme lanosterol 14 α-demethylase which is required for the conversion of lanosterol to ergosterol [23]. This leads to inhibition of fungal growth. This group includes triazole, clotrimazole, imidazoles, fluconazole, and voriconazole [24]. For these reasons, understanding the mechanisms of pathogenicity during infection will lead to the development of better therapeutic treatments to improve the outcome of disease.

1.3. Aspergillus fumigatus and human disease

*Aspergillus fumigatus* is a ubiquitous saprophytic mold, which is typically found in soil and decaying organic matter where it plays an essential role in recycling carbon and nitrogen in the environment. The life cycle of these organisms is described in Figure 1.3: 1) resting conidia in the environment swell to start a germination process, 2) polarized growth leads to the formation of hyphae, 3) which will branch and form a conidiophore that carries hundreds of conidia, 4) and subsequently these conidia will be released into the environment and spread to other places. This results in a very high concentration of conidia in the air and it is estimated that humans breathe approximately 100 conidia m\(^{-3}\) [25]. In tropical climates the concentration of conidia in the air is higher, indicating a greater risk of fungal infection.

*Aspergillus fumigatus* conidia are small, which allows them to penetrate into the alveoli in the lung once inhaled. In an immunocompromised patient, *Aspergillus fumigatus* conidia will overcome the host immune defenses and germinate to produce branched, vegetative mycelium that will invade the lung tissues [25,26]. Over the
past 10-20 years, because of the increase in immunosuppressive therapies and immunocompromised patients, *A. fumigatus* has become the most prevalent airborne fungal pathogen that causes severe and usually fatal invasive infections with a mortality of more than 50% in immunocompromised hosts [27-29]. Patients that are mostly at risk of being infected with this fungal pathogen are HIV infected individuals, cancer patients, and organ transplant recipients due to the corticosteroids that are administered during the surgical procedure. As mentioned in segment 1.2, the currently available antifungal drugs are mostly fungistatic and do not lead to complete elimination of *A. fumigatus*. Thus, the virulence of *A. fumigatus* can be caused either by the secretion of fungal proteins.

**Figure 1.3 A. fumigatus life cycle.** Reprint permission obtained from the publisher [8].
that promote mycelial growth in the lung parenchyma, molecules that down-regulate the inflammatory response, or by structural properties of the conidia that confer resistance to the host antifungal mechanisms.

1.4. Structure of the fungal cell wall

Initial recognition of fungal pathogens happens mainly through innate immune recognition of molecules present in the cell wall. The major components on the fungal cell wall are β-1,3-glucans (50-60%), α-mannose (30-40%), and chitin (5%) [30,31]. The central core of the cell wall is composed of branched β-1,3-glucans cross-linked to chitin in order to strengthen the cell wall (Figure 1.4) [6]. This complex of glucan-chitin can covalently bind to other polysaccharides

![Figure 1.4 Hypothetical model showing the putative organization of cell wall polymers in A. fumigatus cell wall](image)

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and this composition will vary depending on the species. For example, A. fumigatus has β-1,3-glucans on its cell wall whereas Candida albicans contains β-1,6-glucans [32]. There is a protein component on the fungal cell wall, which has been estimated to represent approximately 20-30% of the cell wall by mass in filamentous fungi [33]. Some of these proteins go through the secretory pathway in transit to the cell wall. These cell wall proteins are generally modified with N-linked and O-linked oligosaccharides in A. fumigatus [34]. Proteins that are required to stay at the cell wall surface are initially anchored to the plasma membrane by GPI anchor proteins.

One important feature of the fungal cell wall is its ability to adapt in response to changes in stress or environmental conditions. In A. fumigatus, the conidia are coated by a layer of the hydrophobin RodA protein and melanin [6]. The RodA protein is inert to the immune system and thus it prevents exacerbated host immune responses by innate immune cells and inflammation [4,35]. Melanin protects conidia from killing by reactive oxygen species (ROS) in the phagosomes [36]. Polymers of galactosaminogalactan (GAG) hide the presence of surface β-glucans to mask and prevent fungal recognition, resulting in increased virulence during infection [37]. Since these cell wall components except melanin are not present in mammalian cells, the fungal cell wall represents a potential therapeutic target during disease.

1.5. Expression and immune recognition of the RodA hydrophobin

Hydrophobins are proteins produced by filamentous fungi that have special properties. They are amphiphiles that have hydrophilic and hydrophobic
moeities and are located in the outer fungal cell wall [38]. Different hydrophobins are expressed at different stages of the fungal life cycle ranging from vegetative hyphae and sporulating cultures to fruiting bodies such as mushrooms. Hydrophobins were originally found when studying highly expressed genes during the emergence of fruit bodies and aerial hyphae in the fungus *Schizophyllum commune* [39]. Sequencing of these genes identified at least three of the genes belonged to a family encoding small hydrophobic proteins with signal sequences for secretion and eight cysteine residues characteristically spaced, but with quite distinct amino acid sequences. Based on the occurrence of hydrophilic and hydrophobic amino acid residues, hydrophobins can be classified into two classes, class I and class II [40]. Class II hydrophobins have been identified as abundantly excreted proteins and class I as the putative products of genes abundantly expressed at certain stages of development of the fungus. These proteins can be found as SDS-insoluble aggregates in conidial and in hyphal walls from aerial structures [41]. The SDS-insoluble aggregates can be solubilized in cold 100% formic acid, 100% trifluoroacetic acid (TFA), or 50% hydrofluoric acid (HF), and then visualized with SDS-PAGE as monomers. In *S. commune* the interface between a hydrophilic and hydrophobic moiety is sufficient to trigger the assembly of an amphipathic membrane, suggesting that hydrophobins with similar properties are well suited to establish adhesion between the hydrophilic fungal cell wall and the hydrophobic host surface [41]. Thus, the first role that was suggested for hydrophobins was the lowering of surface tension of water to allow growth of hyphae into the air. First, hyphae grow
in an environment surrounded by a film of water and hydrophobins are secreted in abundance to aggregate at the air-water interface to form an amphipathic layer. Second, assembly of the hydrophobins causes a dramatic reduction in surface tension, allowing the hyphae to escape and grow into the air. Lastly, the aerial hyphae continue to secrete hydrophobins, which form a hydrophobic rodlet layer on the outside of the cell wall [42]. Although these studies have been done in *S. commune*, the same process likely occurs with fungi that express hydrophobins with similar properties. The role of hydrophobins in growth of hyphae into the air was shown by mutating the *THN* gene that controls the expression of the Sc3 gene, which encodes for an *S. commune* hydrophobin [43]. These studies suggest that hydrophobins might have a role in fungal pathogenicity by enabling adhesion of fungi to the host surface or to the hydrophobic surfaces of plants.

RodA protein is a type I hydrophobin expressed in the conidial cell wall of filamentous fungi. This protein was first discovered in the 1990’s and it was shown to assemble into a SDS-insoluble layer in the cell wall of *A. fumigatus* (Figure 1.5) [44]. RodA is a 19kDa protein with the eight conserved cysteine residues characteristic of hydrophobins [6]. The RODA gene contains a glycosylphosphatidylinositol (GPI) sequence, suggesting that RodA protein is covalently bound to the cell wall polysaccharides [31]. The RodA protein can also coat the conidial cell wall and hide the fungus from host recognition, thus making it inert to the immune system [35]. Intact dormant conidia from *A. fumigatus* failed to activate macrophages and dendritic cells (DCs), and to induce
lymphoproliferation \textit{in vitro}. However, in the absence of RodA protein, \textit{A. fumigatus} conidia triggered strong inflammatory reactions in macrophages and DCs and was highly phagocytosed, suggesting that the RodA hydrophobin helps fungal conidia to overcome the host immune responses. Also, since hydrophobins assemble exposing their hydrophobic part to the outside, RodA protein protects the conidia from desiccation. Once conidia start the germination process (swelling), RodA protein gets cleaved by fungal proteases, making swollen conidia and hyphae highly immunoreactive to the host immune system. The RodA protein therefore confers protection to the dormant conidia by blocking the immune response until germination and formation of hyphae. The virulence of \textit{A. fumigatus} can be caused either by the secretion of fungal proteins that promote mycelial or by cell wall components of the conidial cell wall that confer resistance to the host antifungal mechanisms, and the RodA protein represents a protein that potentially confers resistance to host antifungal immunity.
1.6. Anti-fungal immune responses by the C-Type lectins, Dectin-1 and Dectin-2

C-type lectin receptors are pattern recognition receptors (PRRs) that have a C-type lectin-like domain (CTLD) in their extracellular region (Figure 1.6) [11]. Some family members can recognize multiple endogenous ligands, whereas others are specific for endogenous ligands. Similar to other PRRs like Toll like receptors (TLRs), CLRs are essential for host defense during infection against fungi through recognition of carbohydrates [45]. The major CLRs are Dectin-1, Dectin-2, and Mincle. Dectin-1 binds β-glucan, Dectin-2 binds α-mannose, and

![Figure 1.6 Signal transduction pathways of C-type lectin receptor signaling in anti-fungal immunity.](image)

Dendritic Cell

Macrophage

CARD9

MALT1

NFκB

IL-23

IL-6

Th17

IL-12

IFNγ

IL-17

Th1

Figure 1.6 Signal transduction pathways of C-type lectin receptor signaling in anti-fungal immunity. Dectin-1, Dectin-2, and Mincle all signal through Syk kinase. Syk signaling results in the formation of a CARD9, BCL10, and MALT1 complex that ultimately leads to NFκB activation and other transcription factors (not shown). These transcription factors initiate expression of key cytokines to induce Th1 or Th17 cell differentiation, which activates anti-fungal responses in innate cells such as neutrophils and macrophages [11]. Reprint permission obtained from the publisher.
Mincle binds intracellular molecules that are released after cell damage [46]. Signaling through these receptors drives inflammation and adaptive immunity to protect the host from infection with fungal pathogens. As shown in Figure 1.6, these receptors share a common signaling pathway that involves spleen tyrosine kinase (Syk), CARD9, and NFκB.

Dectin-1 is a glycosylated type II transmembrane receptor with a single extracellular C-type lectin-like domain (CTLD) and a cytoplasmic immunoreceptor tyrosine-based activation (ITAM)-like motif (also known as a hemi-ITAM) (Figure 1.7) [7]. Alternative splicing determines the presence or absence of a stalk region (links the CTLD to the transmembrane region), and it also gives rise to multiple other minor isoforms [47]. Dectin-1 was originally thought to be a dendritic cell (DC)-specific receptor, from which its name ‘dendritic-cell-associated C-type
lectin-1’ was derived, but this receptor is now known to be expressed in other cell types including monocytes/macrophages, DCs, neutrophils, and a subset of T cells, although it is not expressed on these cells in all tissues [48-50]. In humans, but not in mice this receptor is also expressed by B cells and eosinophils.

Dectin-1 recognizes β1,3-linked glucans, which are carbohydrates that are found in the cell walls of plants and fungi, and is the major receptor on myeloid cells for these molecules [51-53]. Dectin-1 can also recognize other unidentified molecules including an endogenous ligand on T cells and a ligand on mycobacteria [54]. Although pathogen recognition receptors (PRRs) can facilitate responses to pathogens through TLRs, Dectin-1 is the first example of a non-TLR PRR that can mediate its own intracellular signals. Dectin-1 signaling abilities depend on the cytoplasmic ITAM-like motif, which resembles sequences found in other activation molecules such as DAP12, Fc receptors, and lymphocyte antigen receptor complexes. The binding of Dectin-1 to β-glucan leads to phosphorylation of the cytoplasmic ITAM-like motif by Src family kinases, providing a docking site for spleen tyrosine kinase (Syk) (Figure 1.8) [55-57]. A recent study showed that soluble β-glucans inhibit Dectin-1 signaling and that it is large β-glucan polymers that can activate Dectin-1 signaling [58]. This happens because the phosphatases CD45 and CD148 constantly maintain the hemi-ITAM dephosphorylated; however, when Dectin-1 binds to large β-glucan polymers they get physically excluded from the phagocytic synapse enabling the Src family kinases to phosphorylate the hemi-ITAM of Dectin-1 leading to signaling. Despite involving both SH2 domains of Syk, only the
membrane proximal tyrosine \((Y_{XXX}I/L_{X7}Y_{X}L)\) of Dectin-1 was found to be required for signaling [57]. The requirement for Syk mediated signaling for Dectin-1 is limited and cell specific. In macrophages, Syk is required for induction of the respiratory burst but does not contribute to the phagocytic process [59]. In DCs, Syk is required for Dectin-1-mediated phagocytosis and is essential for IL-2, IL-10, TNF, IL-23, and IL-6 production [56]. Syk is also essential for mediating the production of this cytokine profile in macrophages. While components of the signaling pathway have yet to be fully elucidated, caspase recruitment domain 9 (CARD9), which assembles with BCL10 and MALT1, has been identified as an

![Figure 1.8 Dectin-1 signaling pathways.](image)

Dashed lines represent suggested interactions. Solid lines represent established interactions. Reprint obtained from the publisher.
essential downstream adaptor linking Syk-coupled receptors to the canonical NFκB pathway [11,60,61]. Dectin-1 activates PI3K through an unknown mechanism and Syk activates PLCγ2, which hydrolyzes PIP2 to generate IP3 and diacylglycerol (DAG). IP3 will bind to its receptor in the endoplasmic reticulum (ER) to induce release of calcium into the cytoplasm. Calcium will bind to the calcium sensor protein, calmodulin, which in turn activates the serine phosphatase calcineurin that will dephosphorylate cytoplasmic NFAT, inducing its translocation into the nucleus to initiate gene expression of pro-inflammatory cytokines [62-64]. It has been shown previously that Dectin-1 can lead to NFAT activation in macrophages and DCs, but its role in innate antifungal immunity remains unknown [65]. Production of IL-1β by Dectin-1 requires collaboration between Dectin-1 and the NLRP3 inflammasome (not shown in diagram). NLRP3 is activated in response to β-glucans and this is dependent on Dectin-1 and Syk [66]. The molecular details of this pathway are still largely unexplored; however, Syk-mediated ROS production and potassium efflux are required and there may be a partial dependency on TLR2 [56]. Syk can also activate the MAPK pathway, leading to activation of ERK1/2, which activates the AP-1 subunits, c-Fos and c-Jun.

Dectin-1 can also induce intracellular signaling through Syk-independent pathways. Phagocytosis in macrophages does not require Syk, although this response still involves the ITAM-like motif of the receptor [67]. Dectin-1 was recently found to induce a Syk-independent pathway involving the serine-threonine kinase Raf-1 [47]. This pathway integrates with the Syk pathway at the
level of NFκB and can also control Dectin-1 mediated cytokine production. Dectin-1 has also been shown to interact with TLR2 and TLR4 to mediate antifungal immunity; however, how Dectin-1 signaling integrates with the TLR pathway is unknown and it might involve kinase recruitment to Dectin-1 and subsequent TLR2 phosphorylation and activation [68]. Dectin-1 can also link the innate immune system with the adaptive immune system and this has been an important recent discovery. It has been shown that stimulation of DCs with purified β-glucan induces the differentiation of Th17 and Th1 CD4+ T cells in vitro and in vivo, and these responses are independent of the TLR signaling pathways [69]. It has also been shown that activation of DCs with specific Dectin-1 agonists can drive the conversion of selected populations of Treg cells into IL-17 producing T cells [70]. Both Th1 and Th17 cell responses have been shown to confer protection during *C. albicans* and *A. fumigatus* infections, although the role of Th17 cells in host protection is still somewhat controversial. A recent study showed that Dectin-1 activation in DCs induces differentiation of Th17 cells and decreases the differentiation of Th1 cells [71]. This is consistent with previous studies that show a mutual exclusive relation between Th1 and Th17 cells [29]. Activation of Dectin-1 can also drive CD8+ T cell responses and purified β-glucan was found to act as a potent adjuvant for CTL cross-priming in vivo [72]. These elicited cytotoxic responses protected mice from experimental tumor challenge. All of these studies have led us to suggest that Dectin-1 can link innate and adaptive immune responses to fungi and that this receptor represents a potential immunotherapeutic target.
Dectin-2 is also a type II glycosylated transmembrane receptor that contains a single carbohydrate recognition domain (CRD), which is highly conserved in mice and humans (mouse Dectin-2 shares a 75% homology to human Dectin-2) [73]. It was originally identified as a Langerhans cell specific C-type lectin using the murine Langerhans cell-like cell line XS52 [74]. It is expressed in macrophages, some DCs, and at low level on Langerhans cells and peripheral blood monocytes [75,76]. The CRD on this molecule contains an EPN (Glu-Pro-Asn) motif, which is a Ca\(^{2+}\)-dependent mannose binding amino acid sequence that gives the receptor the ability to bind high mannose-type carbohydrates [73,77]. Dectin-2 lacks a known signaling motif in the cytoplasmic domain. However, it was shown that Dectin-2 associates with the FcRγ chain via the arginine residue in the cytoplasmic domain (Figure 1.9) [78]. The FcRγ chain is essential for mediating the biological effects of Dectin-2, and it has been shown that cytokine production is abolished in FcRγ chain deficient mice after stimulation with α-mannan [78]. Despite the differences in structure found between Dectin-1 and Dectin-2, the downstream signaling pathways are very similar. As in Dectin-1 signaling, Src family kinases will phosphorylate the tyrosine residues on the ITAM immediately after binding of Dectin-2 with α-mannose. Following tyrosine phosphorylation, spleen tyrosine kinase (Syk) will bind to the phosphorylated tyrosine via its SH2 domains and can activate several signaling pathways. Syk can activate the caspase recruitment domain 9 (CARD9), which in concert with Bcl10 and MALT1 will lead to activation of the NFκB transcription factor. A recent study showed that the only NFκB subunits
that get activated by Dectin-2 are c-Rel and p50 [79]. Dectin-2 also leads to the activation of the MAPK pathway that activates the AP-1 subunits, c-Fos and c-Jun to drive expression of cytokine and chemokine genes. As with Dectin-1, there is controversy about the key players of the Dectin-2 signaling pathway and its role in fungal immunity has yet to be fully defined. The role of Dectin-2 in innate immunity has never been studied, but several studies have shown a role for Dectin-2 in the induction of Th17 cell differentiation during *C. albicans* infections [80,81]. Other studies have focused on trying to establish if Dectin-2 is more important than Dectin-1 for inducing Th17 cell differentiation during fungal
infections. The answer to this question remains inconclusive as some studies support the notion that Dectin-1 is more important, whereas others suggest that Dectin-2 is in fact the most essential receptor for mediating these responses [80]. Since this is the only role that has been shown for Dectin-2, it has been suggested that Dectin-2 enhances the inflammatory response mediated by Dectin-1, but it is not essential for these responses. Our group showed that Dectin-2, but not Dectin-1, contributes to the autocrine IL-17 response in neutrophils by up-regulating expression of the IL-17RC [82]. Although IL-17 has a role in host protection during fungal infections, the main receptor responsible for induction of Th17 cell differentiation still remains unidentified.

1.7. Innate immune responses to chitin

Chitin is a polymer of N-acetylglucosamine, which assembles into microfibrils in the fungal cell wall [83]. It is also the second most abundant polymer in nature after cellulose. Chitin is found in arthropods including insects, crustaceans, nematodes, and fungi [84]. After chitin synthesis, the nascent chain folds back on itself to form anti-parallel chains and then form hydrogen bonds to create a strong microfibril in the cell wall (Figure 1.10) [85]. Cell wall chitin can be de-acetylated by chitin deacetylases and converted into chitosan. The degree of deacetylation varies based on the species, but it has been shown that in *C. albicans* approximately 5% of the chitin is converted into chitosan [86]. Further, in *Cryptococcus neoformans* more than two thirds of the cell wall chitin is deacetylated into chitosan [87]. Chitosan can elicit different immune responses
than chitin and is thought that chitin deacetylation could protect the fungus from the action of host chitinases which can hydrolyze chitin. Chitin is synthesized by a family of chitin synthases (CHS), which are membrane bound proteins that catalyze the polymerization of GlcNAc from UDP-GlcNAc [85]. These enzymes can be clustered in seven classes (I to VII) and the number of chitin synthase
genes is proportional to the amount of chitin present in the cell wall. In *A. fumigatus* there are eight chitin synthase genes identified with at least one representative for each class. The role of each chitin synthase and their specific interactions in the biosynthetic pathway are not well understood. Several groups have developed fungal strains that lack a chitin synthase gene, but no role for virulence has been found, indicating possible redundancy of these enzymes. Since chitin is important to maintain cell wall integrity, it is essential for fungal survival and probably virulence.

Since chitin particles can be phagocytosed [88], it seems likely that there could be immune recognition through receptors that mediate phagocytosis. Until now, no specific receptors that can bind chitin and promote a phagocytic response have been identified. Several receptors have been shown to have affinity for chitin oligosaccharides, including: a homotetrameric type II transmembrane protein called FIBCD1 [89]; NKR-P1, which is an activating receptor on rat natural killer (NK) cells; a secreted C-type lectin, RegIIIγ [90]; and galectin-3, which is a lectin that binds β-galactosides. Although these receptors can recognize chitin fragments, it is not clear if they can recognize full-length chitin as it is present in the cell wall.

Because some fungal pathogens like *A. fumigatus* are airborne and ubiquitous in the environment, exposure either by food or inhalation occurs very often. Recently, chitin has been identified as a molecule capable of triggering an immune response associated with allergy and asthma. Intranasal administration of chitin into the lungs of mice that have a GFP gene under the control of the IL-4
promoter leads to the accumulation of IL-4 producing eosinophils and basophils [91]. Other groups have also shown that intranasal administration of chitin beads into the lungs of mice induces production of IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), which are cytokines involved in allergic responses [92].

Previous reports showed that large intact chitin polymers are immunologically inert and do not induce an immune response [93]. However, chitin fragments have the ability to trigger pro or anti-inflammatory responses [94]. Intermediate chitin fragments (40-70µm) stimulated IL-17A production and IL-17A receptor expression in macrophages, which was dependent on Toll-like receptor (TLR)-2 and MyD88 [95]. The intermediate and small (<40 µm) chitin fragments induced TNF-α production. In contrast, small chitin fragments mediated IL-10 production which was dependent on Dectin-1, Syk, and NFκB pathways [96]. Based on these studies, it has been suggested that chitin-mediated responses could depend on size, the method of preparation, and route of administration. More studies are needed to determine if chitin represents a virulence factor during fungal infection.

1.8. Neutrophil Acidic mammalian chitinase (AMCase)

Since chitin is not present in mammalian cells, the expression of chitinases serves as a defense mechanism against chitin-containing pathogens. Chitinases are part of an evolutionarily conserved 18 glycosyl hydrolase family and are expressed in organisms from prokaryotes to eukaryotes, including mammals [83]. Six proteins homologous to chitinases have been identified in
humans (Table 1.1). This group includes Chitotriosidase (CHIT-1) and Acidic mammalian chitinase (AMCase), which are the only two enzymatically active chitinases able to hydrolyze chitin. These proteins are also called true chitinases. The other four of these chitinases have amino acid substitutions in their catalytic sites that render them non-catalytic [97].

<table>
<thead>
<tr>
<th>Name</th>
<th>Alias(es)</th>
<th>Humans</th>
<th>Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic mammalian chitinase (AMCase)</td>
<td>CHIA, eosinophil chemoattractant cytokine</td>
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<td>+</td>
</tr>
<tr>
<td>Chitotriosidase</td>
<td>Chitinase I</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>YKL-40/BRP-39</td>
<td>CHI3L1, cartilage glycoprotein 1, GP-39, HcGP-39</td>
<td>+</td>
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<tr>
<td>Oviductin</td>
<td>Oviductal glycoprotein 1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chondrocyte protein 39</td>
<td>CHI3L2, YKL-39</td>
<td>+</td>
<td></td>
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<td>Stabilin-1-interacting chitinase-like protein (SI-CLP)</td>
<td>+</td>
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<td>Ym2</td>
<td>Chi334</td>
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Table 1.1 Representative members of the 18 glycosyl hydrolase family in humans and mice [3]. Reprint permission obtained from the publisher.

AMCase has the ability to hydrolyze chitin in the cell wall of *A. fumigatus* [98], but its role in fungal infection has not been established. However, there is extensive data that shows a role for AMCase in the pathogenesis of asthma. AMCase is produced by lung epithelial cells, eosinophils, and macrophages during Th2-mediated inflammation [10]. After exposure to an allergen, Th2 cells will develop and produce IL-13 which then induces AMCase expression by lung epithelial cells and alveolar macrophages (Figure 1.11). In support of this, AMCase neutralization in an aeroallergen asthma model ameliorated Th2 mediated inflammation and airway hyperresponsiveness through the inhibition of IL-13 pathway activation [99]. During asthma, AMCase also contributes to the
recruitment of eosinophils and neutrophils, which suggests that this chitinase has other biological effects besides hydrolyzing cell wall chitin. In support of these observations, AMCase inhibits apoptosis of epithelial cells and point mutations in the catalytic site of AMCase do not abrogate this anti-apoptotic effect on the epithelium [100].

1.9. Innate immunity at the ocular surface
The ocular surface – cornea and conjunctiva has direct contact with environmental pathogens and thus, it can be threatened by invasion with these organisms. The visual axis of the eye must focus light images on the retina and in order to achieve this, some tissues like the cornea, lens, vitreous humor, and subretinal space must be transparent [101]. Since immune responses can have deleterious effects with vital functions in the eye which could potentially lead to blindness, the development of ocular immune privilege is essential. In order to achieve immune privilege, the anterior chamber contains immunosuppressive cytokines such as TGF-β and neuropeptides such as α-melanocyte stimulating hormone, which inhibit the host response to minor trauma to the cornea [102]. These effector mechanisms can induce an immune response with the potential to trigger the least deleterious effects and still protect the eye against environmental pathogens.

Despite all the mechanisms that the eye has to maintain ocular immune privilege, infection with microbial pathogens leads to the breakdown of this immune privilege. Hence, the presence of immune cells is essential for protection or defense during infection. The central cornea contains a population of resident cells from the monocytic/macrophage lineage that are CD45+ and F4/80+ [103]. Most of these cells express major histocompatibility complex (MHC) class II antigens, which led to the assumption that these cells could perform antigen processing and presentation to initiate an adaptive immune response. Further, the periphery of the anterior corneal stroma contains a population of MHC class II+CD80−CD86+ that express dendritic cell (DC) markers [104]. In contrast, the
central cornea has MHC class II^CD80^-CD86^- DCs, which can serve as a major reason for corneal immune privilege. Cells from the adaptive immune system have not been identified to reside in the corneal stroma, but they are able to migrate through the limbal vessels after antigen presentation.

The first line of protection for the eye is probably eyelid closing and blinking to remove microbial pathogens from the ocular surface. The tear film is derived from two principal sources: the lacrimal glands and the secretions from the two ocular epithelial surfaces, the cornea and the conjunctiva [105]. There is an increasing number of reported antimicrobial peptides such as lipocalin, lysozyme, and β-defensins, etc. that are present in the tear film [106]. Due to their relative concentration, three secreted antimicrobial proteins are most important. First, lysozyme destroys the bacterial cell wall by cleaving the peptidoglycan [107]. Second, lactoferrin binds free iron thus reducing the availability of free iron necessary for microbial growth and survival as well as pathogenicity [108]. Besides antimicrobial properties, lactoferrin exhibits anti-inflammatory activities and this is essential for mediating protection and defense without immunopathology [109]. Lastly, tear-specific prealbumin (lipocalin) acts as a physiological scavenger of hydrophobic, potentially harmful molecules [106]. Previous studies have shown that tear lipocalin (TL) can bind to microbial siderophores with high affinities. Siderophores are low molecular weight compounds produced by bacteria, fungi, and graminaceous plants for scavenging iron from the environment to supply iron to the cell. By adding exogenous lipocalin, bacterial and fungal growth under iron limiting conditions
was inhibited [110]. Overall, these tear components play an important role in protecting the cornea from infection with microbial pathogens.

1.10. Innate immune responses to *A. fumigatus* in mouse models of corneal infection

After the integrity of the corneal epithelial layer is breached due to trauma, conidia will enter the corneal stroma which contains a dense and highly organized matrix with antiparallel layers of collagen separated by heparin sulfate proteoglycans that are essential for corneal transparency [111]. At this point, fungal conidia encounter the first phagocytic line of immune defense against the fungal pathogen, the resident corneal macrophages (Figure 1.12) [103]. This initial contact with the resident macrophages leads to direct phagocytosis of resting conidia. A recent report showed that *A.fumigatus* conidia are coated by a “rodlet layer” composed of monomers of the hydrophobic protein RodA, and that this protein is able to mask immune-triggering molecules of the conidial surface thereby avoiding innate immune responses effectively [35]. If conidia are not killed by resident macrophages, they will start germinating into hyphae. During germination of conidia, structural arrangements in the cell wall occur and the “rodlet layer” gets degraded at the hyphal tips, which are the places on swollen conidia where hyphae starts elongating and this leads to exposure of fungal cell wall components [2]. Resident macrophages express pathogen recognition receptors (PRR) that will recognize these polysaccharides and initiate an inflammatory response that will lead to the recruitment of circulating monocytes and neutrophils. Dectin-1 is the major receptor for β-1,3-glucan, although CD18
can also bind to this polysaccharide [51,112]. Previous studies in our lab have shown an essential role for Dectin-1 in mediating leukocyte recruitment into the corneal stroma upon conidial germination, and Dectin-1 deficient mice exhibited impaired cell infiltration and fungal killing during *A. fumigatus* keratitis [113]. In humans, mutations in the Dectin-1 gene lead to increased susceptibility to mucosal fungal infections, even in immunocompetent hosts [114]. In most cases resident macrophages are not able to eliminate the fungal pathogen and swollen conidia further germinate into hyphae, which is too long to be phagocytosed. Thus, infiltrating neutrophils become the most important cell type with the ability
to kill hyphae through the secretion of soluble antimicrobial peptides [115]. This is supported by the increased incidence of systemic and pulmonary fungal infections in neutropenic patients [116], and patients with defects in neutrophil proteins [117]. Neutrophils can also kill fungal hyphae by the production of ROS and GP91phox\(^{-/}\) mice are unable to clear these organisms [118]. Activated neutrophils show an abrupt increase in oxygen consumption, called the phagocyte respiratory burst [119]. The enzyme responsible for the respiratory burst is the NADPH oxidase, which is a multicomponent complex that catalyzes the conversion of molecular \(O_2\) to superoxide anion and releases them to the extracellular space [120]. In resting neutrophils, components of the NADPH oxidase complex are segregated into the membrane and cytosol (Figure 1.13).

![Figure 1.13 Assembly and activation of the NADPH oxidase complex. Reprint permission obtained from the publisher [5].](image)

In the membrane is flavocytochrome b\(_{558}\), a heterodimeric heme protein composed of gp91\(^{phox}\) and p22\(^{phox}\). In the cytosol is the complex of p47\(^{phox}\), p67\(^{phox}\), and p40\(^{phox}\), and Rac2 which is a member of the Rho family of small
GTPases. Rac2 is present in its GDP state and is associated with a GDP-dissociation inhibitor (RhoGDI). Upon stimulation, p47phox gets phosphorylated on multiple serine residues and translocates along with p67phox and p40phox to the membrane while Rac2 undergoes GDP-GTP exchange. After assembly of the complex at the membrane, electrons from cytosolic NADPH will be shuttled across the stacked heme groups in gp91phox to molecular oxygen, thereby generating superoxide anion. A previous study in our lab showed that 

\textit{A. fumigatus} hyphae activate the neutrophil NADPH oxidase through CD18 to mediate fungal killing in the cornea. Further, studies done by our lab characterized fungal infected corneas and showed that neutrophils constitute >90% of the cellular infiltrates in corneal ulcers in patients infected for <7 days, thereby underscoring the importance of neutrophils for hyphal killing during fungal keratitis [121].

During the past years it has been suggested that neutrophils have the ability of producing IL-17A, which has been shown to be protective during fungal infections with \textit{C. albicans} [122,123]. A recent study showed that neutrophils can produce IL-17 and this is dependent on Dectin-1 activation [124]. Further, a recent study from our lab showed that this neutrophil population both produces and responds to IL-17A, and this was dependent on IL-6 and IL-23 [82]. The IL-17 made acted in an autocrine fashion to further enhance the production of ROS by the neutrophils, which then made them more efficient at killing \textit{A. fumigatus} hyphae. During infection, IL-17 producing neutrophils were detected at 24h post-infection and by depleting circulating neutrophils it was shown that this population
is essential to clear fungal hyphae. This is consistent with recent data obtained by our lab in collaboration with the Aravind Eye hospital in Madurai, India, showing that patients with fungal infections in the cornea have IL-17 producing neutrophils in their blood [125]. Further understanding of the mechanisms utilized by neutrophils to kill hyphae is needed to develop more effective anti-fungal drugs.

**1.11. Host adaptive immune responses to *A. fumigatus* in mouse models of corneal infection**

Host adaptive immunity during fungal keratitis is currently not well understood and there are no published studies available establishing a role in the cornea. However, a recent study by our lab shows for the first time the presence of Th17 cells in the corneal stroma at 48h post-infection [126]. Th17 cells contributed to IL-17 production in the cornea, though most of the IL-17 being made came from infiltrating neutrophils. WT and Rag2\(^{-/-}\) primed mice were more protected against *A. fumigatus* than unprimed mice, but the protection observed in the Rag2\(^{-/-}\) primed mice was only partial when compared to the WT mice. This suggested that Th17 cells contribute to anti-fungal immunity during *A. fumigatus* corneal infection but are not as essential as neutrophils. Antibody depletion of the Th1 cytokine IFN-\(\gamma\) did not impair the anti-fungal response during infection. Future studies ought to focus on the role of Th17 cells during keratitis since protective roles for IL-17 have increasingly been shown during *A.fumigatus* and *C.albicans* systemic infections.
1.12. Host immune responses in human infected corneas

A recent study published through collaboration of our lab with the Aravind Eye hospital in southern India characterized the expression of innate and adaptive immune mediators in human infected corneas with *Aspergillus* and *Fusarium* at early and late stages of infection [127]. Individuals that were examined within the first 2 weeks of experiencing ocular trauma were considered to be at early stages of infection. RNA was extracted from patients with corneal ulcers and gene expression was measured by quantitative polymerase reaction (qPCR). Expression of Dectin-1, Toll like receptor 2 (TLR2), TLR4, TLR9, and NOD-like receptor protein (NLRP3)3 messenger RNA was elevated >1000 fold compared with uninfected donor corneas. Further, IL-1β was also elevated >1000 fold, whereas IL-1α was not elevated compared to the uninfected donor corneas. The expression of IL-8, IL-17, and TNF-α was also elevated. This is consistent with our studies done using our murine model of fungal keratitis where we observed a requirement for Dectin-1, TLR2, TLR4, and IL-1β in infected mouse corneas [113]. Lastly, >95% of the cells present from the corneal scrapings are neutrophils, indicating that these cells are essential players to mediate fungal clearance during corneal infection.

To study late stages of infection (>2 weeks), infected corneas recovered after transplant surgery (keratoplasty) were examined by pPCR and immunohistochemistry. Extensive hyphal growth with β-glucan expression was observed in these post-transplant corneas, indicating a role for Dectin-1 receptor. Examination of infiltrating cells revealed that neutrophils comprise a 65-75% of all
the infiltrating cells, with a 25-35% for macrophages, and a 3-8% was CD3+ and CD4+ T cells. Lastly, expression of IL-17 and interferon γ (IFN-γ) was elevated in the post-transplant corneas, whereas IL-4 was not elevated. This is consistent with the Th1 and Th17 cell infiltration observed in immunized mouse corneas [126].
Chapter 2

The RodA Hydrophobin on Aspergillus fumigatus Spores Masks Dectin-1 and Dectin-2 Dependent Responses and Enhances Fungal Survival In Vivo
Abstract

*Aspergillus* and *Fusarium* species are important causes of fungal infections worldwide. Airborne spores (conidia) of these filamentous fungi express a surface protein that confers hydrophobicity (hydrophobin) and covers cell wall components that would otherwise induce a host immune cell response. Using a mutant *Aspergillus fumigatus* strain (ΔrodA) that does not express the RodA hydrophobin, and *Aspergillus* and *Fusarium* conidia from clinical isolates that were treated with hydrofluoric acid (which removes the *A. fumigatus* RodA protein), we observed increased surface exposure of β1,3-glucan and α-mannose on *Aspergillus* and *Fusarium* conidia. We also found that ΔrodA and hydrofluoric acid–treated conidia stimulate significantly higher NF-kB p65 nuclear translocation and cytokine production by macrophages from C57BL/6, but not from Dectin-1−/− or Dectin-2−/− mice. Using a murine model of *A. fumigatus* corneal infection, we showed that ΔrodA conidia induced significantly higher cytokine production, neutrophil infiltration, and more rapid fungal clearance from C57BL/6 corneas compared with the parent G10 strain, which was dependent on Dectin-1 and Dectin-2. Together, these findings identify the hydrophobin RodA as a virulence factor that masks Dectin-1 and Dectin-2 recognition of conidia, resulting in impaired neutrophil recruitment to the cornea and increased fungal survival and clinical disease.
Introduction

Although *Aspergillus* spores (conidia) and those of other filamentous fungi are ubiquitous in the air we breathe and can reach high concentrations (>10^9 spores per cubic meter in some environments), they do not generally cause inflammatory disease following inhalation [128,129]. Fungal cell wall components such as β1,3-glucan and α-mannan have the potential to induce inflammation; however, conidia are coated by a hydrophobic "rodlet layer" composed of regularly arranged RodA hydrophobins, which are covalently bound to cell wall polysaccharides by glycosylphosphatidylinositol (GPI) anchor proteins [6,38]. A recent study showed that removal of *A. fumigatus* RodA by hydrofluoric acid, which cleaves the phosphodiester bonds attaching RodA to the cell wall, confers conidia recognition by human dendritic cells and murine alveolar macrophages [35]. Further, mice infected with an *A. fumigatus ΔrodA* mutant caused increased lung inflammation compared with the parent strain. As *Aspergillus* and *Fusarium* species are also major causes of corneal infection and blindness worldwide [13], the current study examined the role of hydrophobins in a murine model of fungal keratitis. We also identified cell wall components that are exposed in the absence of RodA, and the pathogen recognition molecules that are activated.

We show that β1,3-glucan and α-mannan are exposed on the cell wall of *Aspergillus* conidia in the absence of RodA, and that the c-type lectins Dectin-1 and Dectin-2 mediate the host response. Using a murine model of *Aspergillus* corneal infection, we also demonstrate that in the absence of RodA, *A. fumigatus* induces Dectin-1 and Dectin-2 dependent neutrophil recruitment to the corneal
stroma and enhanced fungal killing. Together, these data represent a novel fungal adaptation to evade early recognition by Dectin-1 and Dectin-2, enabling conidia to germinate and form hyphae prior to immune recognition, which thereby enhances fungal survival during infection.

**Materials and Methods**

**Source of mice**

All animals were treated in accordance with the guidelines provided in the Association for Research in Vision and Ophthalmology ARVO statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by Case Western Reserve University IACUC. C57Bl/6 mice (5-12 week old) were purchased from The Jackson Laboratory (Bar Harbor, ME). Dectin-1 and Dectin-2 deficient mice were a kind gift from Yoichiro Iwakura (University of Tokyo, Japan).

**Fungal strains, media, and growth conditions**

*Aspergillus* strains used in this study were cultured in Vogel’s minimal media (VMM) w/wo 4% agar +/- supplementation with 10mM uracil and 5mM uridine at 37°C/5%CO₂ unless stated otherwise. The *A. fumigatus* parent (G10) and mutant (ΔrodA) strains were provided by Jean Paul-Latge (Institut Pasteur, Paris, France) *A. fumigatus* Af-BP strain is a clinical isolate from a patient treated at Bascom Palmer Eye Institute (Miami, FL), provided by Dr. Darlene Miller. The *F. oxysporum* 8996 strain is a clinical isolate from a patient treated at the Cleveland Clinic Institute (Cleveland, Ohio). The *A. flavus* TN-302 strain is a
clinical isolate obtained from the Aravind Eye Hospital-Madurai, Tamil Nadu, India. *F. solani* B6970 was from a contact lens keratitis patient and obtained from the Centers for Disease Control.

**RodA protein extraction method**

The rodlet layer was extracted from the cell wall by incubating dry spores with 4% hydrofluoric acid (HF) for 72h at 4°C. The content was centrifuged (10,000rpm, 10min) and supernatant was discarded. Samples were fixed with 4% paraformaldehyde for 30min and washed 3X with sterile PBS.

**Detection of surface β1,3-glucan and α-mannose**

*Aspergillus* strains were cultured for 3 days in VMM+4% agar and *Fusarium* strains were cultured in SDA media. Pure conidial suspensions were prepared from the 3-day culture and fixed in 4% PFA as described before. Conidia were centrifuged (10,000rpm, 5min) and blocked with 1.5% normal rabbit serum in PBS for 1h, then incubated with a Dectin-1 Fc fusion protein) [130], which was a gift from Dr. Chad Steele, University of Alabama at Birmingham Conidia were then washed 3x with PBS and incubated with FITC-conjugated goat-anti-mouse IgG (Invitrogen) diluted to 1mg/ml in PBS for 1h at 37°C.

**Murine model of corneal infection**

*A. fumigatus* strains were cultured in VMM agar in 25cm² tissue culture flasks. Dormant conidia were disrupted with a bacterial L-loop and harvested in 5ml PBS. Pure conidial suspensions were obtained by passing the culture
suspension through PBS-soaked sterile gauges placed at the tip of a 10ml syringe. Conidia were quantified using a hemacytometer and a stock was made at a final concentration of $2.5 \times 10^4$ conidia/µl in PBS. Mice were anaesthetized by intraperitoneal (IP) injection of 0.6% Tribromoethanol, 1.2% tert-butyl alcohol, and PBS. Corneal epithelium was abraded with a 30 gauge needle. Through the abrasion was inserted a 33-gauge Hamilton needle and a 2 µl injection containing $5 \times 10^4$ conidia (Optimal inoculum to study the disease was determined by preliminary studies; data not shown) was released into the corneal stroma. Mice were examined daily under a stereomicroscope for corneal opacification, ulceration, and perforation. At set time points, animals were euthanized by CO$_2$ asphyxiation, and eyes were either placed in 10% formalin and embedded in paraffin and sectioned at 5 micron intervals, or excised and placed in 1 ml of sterile saline and homogenized for quantitative culture. All animals were bred under specific pathogen-free conditions and maintained according to institutional guidelines.

**Imaging corneal opacity**

Mice were sacrificed by CO$_2$ asphyxiation and positioned in a three-point stereotactic mouse restrainer. Corneal opacity (Brightfield) was visualized in the intact cornea using a high resolution stereo fluorescence MZFLIII microscope (Leica Microsystems) and Spot RT Slider KE camera (Diagnostics Instruments). All images were captured using SpotCam software (RT Slider KE; Diagnostics Instruments).
Quantification of colony forming units (CFUs)

For assessment of fungal viability, whole eyes were homogenized under sterile conditions in 1 ml PBS, using the Mixer Mill MM300 (Retsch, Qiagen, Valencia, CA) at 33 Hz for 4 min. Subsequently, serial log dilutions were performed and plated onto Sabouraud dextrose agar plates (Becton Dickenson). Following incubation for 24h at 37°C, the number of CFUs was determined by direct counting.

Identification of fungi and inflammatory cell recruitment

Eyes were enucleated and fixed in 10% formalin in PBS (Fisher) for 24h. Tissues were then dehydrated in graded ethanol concentrations at room temperature (65% 1x, 80% 2x, 95% 1x, 100% 3x; 1 h for each change of solution), followed by three 1h changes of xylene, and 4 changes of paraffin at 60°C under 15mm Hg vacuum to remove air bubbles. Five mm sections from the center of the cornea (as determined by noncontiguous iris morphology) were cut and stained with Periodic-Acid Schiff (PASH) for identification of fungi and inflammatory cell recruitment.

Immunohistochemistry

To detect neutrophils during live corneal infection, 5 micron paraffin sections were deparaffinized. Slides were blocked with 1.5% normal rabbit serum in PBS for 1h, then incubated with monoclonal rat-anti-mouse neutrophil IgG (NIMP-R14, Abcam, Cambridge, MA), for 1h at 37°C. The slides were then washed 3x in PBS plus 0.05% Tween20 (PBS-T; Sigma) and incubated with alexafluor-488 tagged
rabbit-anti-rat IgG (Invitrogen) for 1h at 37°C. The slides were washed 3x in PBS-T and imaged by fluorescence microscopy (magnification, 40x).

**Isolation of peritoneal and bone marrow derived macrophages (BMM)**

For peritoneal macrophages, 1.5mL of 4% thioglycolate was injected into the peritoneal cavity of naïve mice. Three days later, mice underwent euthanasia by CO₂ asphyxiation and macrophages were isolated from the peritoneal cavity in sterile PBS using a 10mL syringe (BD syringes). Red blood cells were lysed using 1X RBC lysis buffer (eBioscience) and washed in sterile PBS. Cells were then counted and harvested.

For bone marrow macrophage isolation, mice were euthanized by CO₂ asphyxiation and femurs and tibias were removed, cleaned, and centrifuged at 6000xg for 45s at 4°C. Any contaminating red blood cells were lysed in 5 ml RBC Lysis Buffer (eBioscience), and remaining bone marrow cells were cultured in bacteriologic grade petri dishes in 6 ml Macrophage Growth Medium (MGM: DMEM w/L- Glutamine, Na-Pyruvate, HEPES, 10% FBS, P/S, 30% L929 cell conditioned medium). On day 5, and every 2 days thereafter, the cell supernatant was aspirated, and fresh MGM media was added. Adherent cells were harvested between 7–14 days of culture, and counted.

**Detection of NFkB nuclear translocation and cell-associated conidia in bone marrow derived macrophages (BMDM)**

Adherent cells were harvested between 7–14 days of culture. 2.5x10⁴ cells were cultured onto sterile 18mm2 coverslips (Corning) in a 6 well-plate, and treated
with LPS (100 ng/ml; positive control), G10, and ΔrodA conidia (MOI =100) for 15, 30 and 60 min. Following activation, BMM were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized using 0.1% Triton X-100 in PBS for 1 min at RT, and incubated with rabbit anti-mouse p65 (1:100; eBioscience Ltd) in PBS containing 10% goat serum for 1h at RT. Coverslips were washed 2x with PBS and cells were incubated with Alexa Fluor 488- labeled goat anti-rabbit IgG antibody (Molecular Probes Inc.) in PBS at RT for 1h and washed 2x with PBS. The cells were mounted on glass slides using Vectashield mounting medium with DAPI (Vector Laboratories, UK), and examined by fluorescence microscopy.

For cell association studies, bone marrow macrophages incubated with naïve or hydrofluoric acid (HF) treated conidia were washed and fixed in 4% paraformaldehyde as described above, then incubated 5 min with Calcofluor white (Sigma) at a 1:1 ratio with 10% KOH. After washing 2x with PBS, cells were examined by DIC and fluorescence microscopy, and associated conidia per 100 cells, and the percent cells with associated conidia was determined after direct examination of at least 50 cells per coverslip. Two coverslips were examined, and the mean and SD were calculated.

**Cytokine Assays**

Peritoneal macrophages were incubated with naïve, HF treated, and ΔrodA dormant conidia for 6h and cell culture supernatants were obtained. LPS (10ng/ml, Invitrogen) and Curdlan (100µg/ml, Invivogen) were used as controls.
for stimulations. In Syk inhibition experiments, Piceatannol (50µM, Sigma) was added 30min prior to stimulation. Half-well cytokine assays were performed using Duoset ELISA kits (R & D Systems) according to the manufacturer’s directions.

**Image Analysis**

To quantify the NIMP stained histological sections, we obtained the images in 12 bits on a Leica DMI 6000 B inverted microscope using a 40x objective connected to a Retiga EXI camera (Q-imaging Vancouver British Columbia). Analysis was performed using Metamorph Imaging Software (Molecular Devices Downington, PA). Images were stitched together to create an entire cornea. The area of the cornea was delineated and recorded. Next the image was thresholded to recognize the NIMP staining. This resulting NIMP area within the cornea was recorded and utilized to plot a graph.

To quantify corneal opacity, brightfield images of mouse corneas were analyzed using Metamorph software (Molecular Devices) as described [113]. Briefly, a constant circular region encompassing the cornea was defined, and the pixel intensity within this region summed to yield a numerical value which corresponds to the total amount of light reflected from the cornea (i.e. opacity). Images were taken in a Spot RT Slider KE camera using Spot Advanced Software under the same magnification, exposure, and gamma parameters.

**Statistical Analysis**

Statistical analysis was performed for each experiment using an unpaired t test (Prism, GraphPad Software). A $P$ value of <0.05 was considered significant.
Results

RodA protein masks surface β1,3-glucan and α-mannose in Aspergillus and Fusarium conidia and masks cytokine production by macrophages

Fusarium species, which cause systemic disease and corneal infections [12,16,131], also express hydrophobins [132]. Therefore, we treated Aspergillus and Fusarium conidia from clinical isolates with hydrofluoric acid (HF), which unmasks the RodA hydrophobin from A. fumigatus as previously shown [35]. We also examined the A. fumigatus RodA mutant and the parent G10 strain as described [35].

Using a Dectin-1 Fc fusion protein to detect β1,3-glucan, we found increased binding to ΔrodA compared with G10 conidia (Figure 2.1A). Similarly, there was increased binding to HF treated Aspergillus and Fusarium conidia compared with untreated. Since α-mannose is also abundant in the fungal cell wall and is a ligand for Dectin-2 [11,55,81], we used the lectin Concavalin A (ConA) to detect this sugar on the cell surface. Figure 2.1B shows higher α-mannose staining on ΔrodA conidia compared with the G10 strain, and increased binding on HF treated Aspergillus and Fusarium clinical isolates compared with untreated conidia. We further confirmed this data by detecting β1,3-glucan and α-mannose on the surface of ΔrodA conidia by confocal microscopy (Supplemental figure 2.1).
Figure 2.1 Removal of RodA protein exposes surface β1,3-glucan and mannose in *Aspergillus* and *Fusarium* conidia, leading to increased cytokine production by macrophages. A,B) Dormant conidia from *Aspergillus* and *Fusarium* clinical isolates were treated with hydrofluoric acid (HF) to remove the rodlet layer. A. HF-treated and ΔrodA conidia were fixed with PFA, and β1,3-glucan was detected using a dectin-1-Fc fusion protein and a FITC-conjugated goat anti-mouse IgG secondary antibody. Images were taken at 100x magnification. B. Concanavalin A (ConA) was used to detect α-mannose, and DyLight 488 Streptavidin was used for detection. C. C57Bl/6 bone marrow macrophages were incubated 6h with ΔrodA or HF treated conidia at a ratio of 1:50 (MOI=50), and CXCL1, CXCL2 and TNF-α secretion was measured by ELISA. Bar represents 2µm. Experiments were performed at least twice with similar results. Reprint permission obtained from the publisher. [4]
**Figure S2.1** Removal of RodA leads to exposure of surface β-glucan and α-mannose. Dormant conidia from *A. fumigatus* G10 and ΔrodA strains were incubated with a dectin-1 Fc fusion protein to detect β-glucan and with ConA to detect α-mannose. Experiment was repeated twice. Reprint permission obtained from the publisher. [4]
As the RodA protein confers resistance to phagocytosis [133], we examined cytokine production in macrophages incubated with \( \Delta \text{rodA} \) and HF treated conidia. Figure 2.1C shows significantly higher CXCL1, CXCL2, and TNF-\( \alpha \) production by macrophages incubated with Aspergillus \( \Delta \text{rodA} \) and Fusarium HF treated conidia compared with the G10 strain or untreated Fusarium conidia.

These data indicate that the hydrophobic RodA protein masks surface \( \beta 1,3 \)-glucan and \( \alpha \)-mannose in Aspergillus conidia, and that HF treatment removes molecules that share a similar function as RodA on Fusarium conidia.

**Dectin-1 and Dectin-2 are required for conidia-induced macrophage NFkB translocation and cytokine production in the absence of RodA**

To determine if the increased cytokine production in the absence of RodA protein is related to macrophage binding or uptake of conidia, we incubated C57BL/6, Dectin-1\(^{-/-}\) and Dectin-2\(^{-/-}\) bone marrow macrophages with \( \Delta \text{rodA} \) conidia at a multiplicity of infection (MOI) of 100, and the number of conidia bound or internalized by macrophages was imaged by DIC microscopy and quantified by direct counting. As shown in Figures 2.2A-B, the average number of associated \( \Delta \text{rodA} \) conidia per macrophage was significantly higher than G10 conidia.

To assess the role of c-type lectins in conidia-induced macrophage activation in the absence of RodA, Dectin-1\(^{-/-}\) and Dectin-2\(^{-/-}\) bone marrow macrophages were incubated 1h with \( \Delta \text{rodA} \) conidia, and nuclear translocation of
the p65 subunit of NFκB was detected by immunofluorescence and DAPI nuclear stain. As shown in Figures 2.2C-D, following LPS incubation, p65 was detected in the nucleus of C57Bl/6, Dectin-1\(^{-/-}\) and Dectin-2\(^{-/-}\) macrophages. In contrast, nuclear localization of p65 was detected in C57Bl/6, but not Dectin-1\(^{-/-}\) or Dectin-2\(^{-/-}\) macrophages incubated with \(\Delta\)rodA conidia, whereas the G10 strain failed to induce p65 translocation in C57Bl/6, Dectin-1\(^{-/-}\) or Dectin-2\(^{-/-}\) macrophages.

To determine if Dectin-1 and Dectin-2-dependent p65 translocation also affects cytokine production, macrophages were incubated for 6h with \(\Delta\)rodA and HF treated *Fusarium* conidia, and cytokine production was measured by ELISA. Figure 2.2E shows significantly less CXCL1, CXCL2, and TNF-α production by Dectin-1\(^{-/-}\) and Dectin-2\(^{-/-}\) compared with C57Bl/6 macrophages stimulated with \(\Delta\)rodA mutant strain. Consistent with the absence of p65 translocation, cytokines were not detected following incubation with the G10 parental strain. These results indicate that the *Aspergillus* RodA protein and HF sensitive *Fusarium* molecules mask Dectin-1 and Dectin-2 recognition of conidia by macrophages, resulting in impaired cytokine production.
Cytokine production induced by ΔrodA and HF conidia is dependent on spleen tyrosine kinase

Spleen tyrosine kinase (Syk) mediates Dectin-1 and Dectin-2 signaling in macrophages and dendritic cells [56]. Syk contains two SH-2 domains that allow it to bind phosphorylated tyrosines in the ITAM-like motif of Dectin-1 and in the ITAM motif of the Fc receptor that associates with Dectin-2. Syk kinase activity then mediates downstream signaling and activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) to activate transcription of target genes [11,55,57].

To examine the role of Syk in conidia-induced cytokine production, C57Bl/6 bone marrow macrophages were incubated with LPS (which signals through TLR4), curdlan, ΔrodA, and Fusarium HF treated conidia in the presence of the Syk inhibitor Piceatannol. As shown in Figure 2.3, LPS-induced cytokine production was not inhibited, consistent with the TLR4 pathway being mostly syk-
Figure 2.3 Role of spleen tyrosine kinase (Syk) pathway in macrophage cytokine production. C57Bl/6 macrophages were incubated 6h with ΔrodA or HF treated conidia at a ratio of 1:50 (MOI=50) in the presence of a Syk inhibitor (Piceatannol). CXCL1, CXCL2 and TNF-α were measured by ELISA. P values are *<0.05, **<0.001, ***<0.0001. Experiments were performed twice with similar results. Reprint permission obtained from the publisher [4].
independent (although internalized TLR4 activates Syk). In contrast curdlan-induced production of CXCL1, CXCL2, and TNF-α was significantly lower in the presence of Piceatannol. Cytokine production induced by ΔrodA and Fusarium HF treated conidia was also significantly lower in the presence of Piceatannol, indicating that the Syk-dependent pathway is essential for the increase in cytokine production. As before, cytokines were not detected following stimulation with G10 or HF untreated conidia.

**RodA protein enhances fungal survival in vivo**

While ΔrodA mutants were shown to induce more lung inflammation than G10 [35], the role of RodA in fungal survival and clinical disease has yet to be determined. To examine the role of RodA, we used a murine model of *A. fumigatus* corneal infection in which conidia are injected directly into the corneal stroma of immunocompetent, C57BL/6 mice. Conidia germinate and spread throughout the cornea, stimulating a pronounced neutrophil infiltrate and corneal opacification [121]. C57BL/6 mice were then infected with G10 or ΔrodA conidia, and disease progression, neutrophil infiltration, and fungal viability were assessed.

As shown in Figure 2.4A-C, corneas infected with the G10 parent strain developed progressively increasing corneal opacification over 72h (quantification of corneal opacity is described in Supplemental Figure 2.2), which is consistent with our earlier findings using other *A. fumigatus* strains [113], and in our model of *Fusarium* keratitis [134]. In contrast, corneas infected with the ΔrodA mutant
exhibited significantly higher percent and total corneal opacity at 24h, but had significantly less disease at 48h and 72h. Consistent with the 24h opacification data, we found that neutrophil and macrophage infiltration into the corneal stroma was significantly elevated in mice infected with the ΔrodA mutant at 24h post-infection compared with the G10 parent strain (Figure 2.4D, E). Further, there was significantly less colony forming units (CFUs) in the ΔrodA compared with G10 infected corneas at each time point. Four of the five mice given the ΔrodA strain completely cleared the infection by 96h (Figure 2.4F).

We conclude from these data that the ΔrodA strain induced more rapid cellular infiltration and fungal killing than the parent strain, with the cellular infiltration causing more corneal opacity at 24h. Conversely, the host response to the parent G10 strain is delayed due to expression of RodA, causing a more protracted infiltrate and prolonged corneal opacity.

These findings clearly demonstrate that the RodA hydrophobin enhances fungal survival in vivo resulting in prolonged and exacerbated corneal disease.
Figure 2.4 Virulence of *A. fumigatus* ΔrodA and G10 strains in infected corneas. 5x10^4 live G10 and ΔrodA conidia were injected into the corneal stroma of C57Bl/6 mice, and corneal opacity, neutrophil infiltration and CFUs were examined. **A.** Representative corneas at 24h, 48h and 72h post infection with strains G10 or ΔrodA, **B, C.** Percent and integrated corneal opacification scores quantified by image analysis (Figure S2). **D, E.** Total number of neutrophils and macrophages in the cornea at 24h post infection. Quantification was done by flow cytometry. **F.** Colony forming units (CFU) from infected eyes with G10 and ΔrodA strains at indicated time points. P value is *<0.05, **<0.001, ***<0.0001. **B, C, F:** data points represent individual corneas or eyes; **D, E:** Mean +/-SD of five corneas per group. Experiments were repeated twice with similar results. Reprint permission obtained from the publisher [4].
Figure S2. Quantification of corneal opacification. A circular region of the same constant area was centered on the image of each cornea to standardize the area (upper panels), and photos of each cornea were converted into pseudocolor images of corneal opacification using Metamorph software (lower panels). A color visualization of corneal opacity ranging from purple (no opacity) to red (maximum opacity) that corresponds to increasing pixel intensity level. Areas of glare were also demarcated and then set to zero, thereby eliminating glare from the subsequent analysis. To set the threshold for no opacity (transparency), we used images of at least four naïve corneas (left panels). All values above this threshold were included in analyses of Percent Corneal Opacity (area over the threshold value / area of circle – glare x100). Total Corneal Opacity (integrated pixel intensity above the threshold) was calculated using Metamorph software. Reprint permission obtained from the publisher [4].
**ΔrodA induces Dectin-1 and Dectin-2 dependent increased cytokine production and neutrophil recruitment to the cornea**

Results of our previous studies on *Aspergillus* and *Fusarium* keratitis suggested a sequence of events in which Dectin-1 dependent cytokine production by resident macrophages induces neutrophil recruitment to the cornea stroma and mediates hyphal killing [1]. To examine if the enhanced clearance of ΔrodA conidia from infected corneas is due to a more rapid or robust cytokine response and neutrophil infiltration, and to determine the role of Dectin-1 and Dectin-2, we infected Dectin-1−/− and Dectin-2−/− mice with G10 or ΔrodA conidia. After 6h, cytokine production and neutrophil infiltration were quantified.

**Figure 2.5A** shows that at 6h post infection, there were significantly higher CXCL1, CXCL2, and IL-6 levels in C57BL/6 corneas infected with ΔrodA compared to the G10 strain. Further, whereas there were no significant differences in cytokine production among Dectin-1−/−, Dectin-2−/− and C57BL/6 corneas infected with the G10 strain, production of each cytokine was significantly lower in ΔrodA infected corneas of Dectin-1−/− and Dectin-2−/− compared with C57Bl/6 mice.

As normal corneas are avascular and have resident macrophages, but not neutrophils, we examined neutrophil infiltration using the Ly6G antibody NIMP-R14 to identify these cells in 5µm corneal sections. Consistent with the chemokine data, the amount of neutrophils in G10 infected Dectin-1−/− and Dectin-2−/− corneas at 6h post-infection was not significantly different from C57BL/6
Figure 2.5 The role of Dectin-1 and Dectin-2 in the early host response following corneal infection with ΔrodA and G10 conidia. A. Cytokine production in the cornea at 6h post-infection. C57Bl/6, Dectin-1^−/−, and Dectin-2^−/− mice were injected intrastromally with 5x10^4 G10 or ΔrodA conidia as before, and after 6h, corneas were dissected and homogenized, and CXCL1/KC, MIP-2, and IL-6 were measured by ELISA. B, C. Histological sections (5 µm) were stained with PASH, or immunostained with NIMP-R14 to detect neutrophils. B. Representative sections near the peripheral cornea at the site of neutrophil infiltration. Images were taken at 40X magnification C. Quantification of NIMP-R14 staining by image analysis (Metamorph) shows average pixel intensity of the entire corneal section (mean+/−SD of five mice per group). P value is ^*<0.05, ^**<0.001, ^***<0.0001. Bar represents 20µm. Experiments were repeated twice with similar results. Reprint permission obtained from the publisher [4].
corneas (Figure 2.5B, C). In contrast, ΔrodA infected C57BL/6 corneas had a significantly higher neutrophil infiltrate compared with the G10 strain, which was significantly lower in Dectin-1\(^{-/-}\) and Dectin-2\(^{-/-}\) corneas. These data indicate that the early cytokine production and neutrophil recruitment induced by ΔrodA conidia are dependent on Dectin-1 and Dectin-2.

_Dectin-1 and Dectin-2 regulate corneal opacification and fungal killing following infection with ΔrodA conidia_

To determine if there is a role for Dectin-1 and Dectin-2 in fungal clearance of ΔrodA and G10 from infected corneas, Dectin-1\(^{-/-}\) and Dectin-2\(^{-/-}\) mice were infected intrastromally with live conidia as before, and examined after 24h when there was a clear difference in CFU, cellular infiltration and corneal opacity (Figure 2.5).

_Figure 2.6A_ shows that as in Figure 5, there were significantly less CFUs in ΔrodA compared with G10 infected corneas of C57Bl/6 mice at 24h post-infection, indicating more efficient killing of the ΔrodA strain. In contrast, CFU of Dectin-1\(^{-/-}\) and Dectin-2\(^{-/-}\) corneas infected with ΔrodA were significantly higher than C57Bl/6 mice, indicating an essential role for Dectin-1 and Dectin-2 in fungal killing. There was no significant difference in CFU between C57BL/6 Dectin-1\(^{-/-}\) and Dectin-2\(^{-/-}\) infected with G10 at this time point.
Figure 2.6 The role of Dectin-1 and Dectin-2 in corneal infection with *A. fumigatus ΔrodA*. C57Bl/6, Dectin-1^+/−, and Dectin-2^+/− mice were infected intrastromally with ΔrodA or G10 conidia, and CFU, corneal opacity and neutrophil infiltration were assessed after 24h. A. CFU in eye homogenate (data points represent individual eyes). B,C. Percent and total corneal opacification was quantified by image analysis (data points represent individual eyes). D. Representative corneas at 24h post infection. E. Total neutrophils in infected corneas. Corneas were dissected and digested with collagenase. Cells were incubated with NIMP-R14 and total neutrophils were quantified by flow cytometry. Data are mean±SD of five mice per group. P values are *<0.05, **<0.001, ***<0.0001, and these experiments were repeated twice with similar results. Reprint permission obtained from the publisher [4].
Conversely, Figures 2.6B-D show that corneal opacification in $\Delta$rodA infected C57Bl/6 mice was significantly lower in Dectin-1$^{-/-}$ and Dectin-2$^{-/-}$ mice, indicating that these receptors also mediate corneal disease in the absence of RodA. Interestingly, the parent G10 strain, which based on our prior studies using related A. fumigatus strains would have germinated and partially exposed surface $\beta$-glucan 24h post infection [113], has significantly less corneal opacity in Dectin-1$^{-/-}$ compared with C57BL/6 corneas. Consistent with these findings and with the role for neutrophils in corneal opacification, there were also significantly less neutrophils in G10 and $\Delta$rodA infected Dectin-1$^{-/-}$ and Dectin-2$^{-/-}$ corneas compared with C57BL/6 mice (Figure 2.6E). Furthermore, there was significantly more neutrophil infiltration in $\Delta$rodA compared with G10 infected C57BL/6 corneas, consistent with increased corneal opacity.

Taken together, these data indicate that the RodA hydrophobin masks Dectin-1 and Dectin-2 recognition of exposed fungal cell wall $\beta$-glucan and $\alpha$-mannose, impairing neutrophil recruitment to the cornea and facilitating fungal survival.
Discussion

The innate immune system recognizes microorganisms through a limited number of germline-encoded pathogen recognition receptors, which regulate the outcome of microbial infections [135]. In fungal infections, the C-type lectins are the largest and most diverse lectin family and comprise receptors that can bind to fungal glycan ligands in a calcium-dependent manner [75,136]. In the current study, we show that the *A. fumigatus* RodA hydrophobin masks surface β1,3-glucan and α-mannose in dormant *A. fumigatus* and *A. flavus* conidia in strains isolated from patients with corneal ulcers. This hydrophobic protein blocks Dectin-1 and Dectin-2 recognition of conidia, thereby evading Syk-dependent cytokine production by macrophages in addition to phagocytosis. *In vivo*, we demonstrate that during corneal infection with *A. fumigatus*, blockade of Dectin-1 and Dectin-2 signaling leads to decreased neutrophil infiltration and enhanced survival in the cornea. Consistent with our findings using the *A. fumigatus* ΔrodA mutant, we show that HF treated *Fusarium* conidia results in exposure of surface β1,3-glucan and α-mannose, which are then recognized by Dectin-1 and Dectin-2 on macrophages and result in cytokine production. This indicates a novel role for the RodA protein in promoting fungal survival during *A. fumigatus* infection, and implies that other pathogenic filamentous fungi that express RodA or other molecules have a similar role in evading host immune responses.

Our findings are consistent with the elevated macrophage phagocytosis of *A. fumigatus* LaeA mutants (*ΔlaeA*), which have lower RodA expression than the parent strain [137]. Our data also extend our understanding of the role of the
rodA protein described by Latge et al [35]. These investigators showed that the \( \Delta \text{rodA} \) mutant induced increased IL-6 and TNF-\( \alpha \) production by macrophages, and more severe pulmonary inflammation in mice infected with \( A. \ fumigatus \ \Delta \text{rodA} \) compared with the G10 parent strain. We confirmed these observations using a model of corneal infection, and increased our understanding of the mechanism by which hydrophobins prevent potentially damaging host cell responses to common airborne spores. Our \textit{in vitro} and \textit{in vivo} findings indicate that the RodA hydrophobin impairs macrophage recognition of spores by Dectin-1 and Dectin-2, which would otherwise induce elevated cytokine production by recognition of \( \beta 1,3 \)-glucan and \( \alpha \)-mannose, in addition to increased phagocytosis. By blocking macrophage production of pro-inflammatory and chemotactic cytokines, recruitment of neutrophils to the site of infection is impaired, resulting in increased fungal growth. Masking of immunogenic cell wall components appears to be a broad strategy of fungal survival during infection as dormant yeasts such as \textit{Candida albicans} and \textit{Histoplasma capsulatum} do not expose \( \beta 1,3 \)-glucan on the surface until they form bud scars during the process of germination [138,139].

Although there is a clear role for Dectin-1 in recognizing \( \beta 1,3 \)-glucan and mediating the host response to germinating \textit{A. fumigatus} [140,141], less is known about the role of Dectin-2 during fungal infection. Our results showing a role for Dectin-1 and Dectin-2 in protection against infection with \( A.\ fumigatus \ \Delta \text{rodA} \) are in agreement with the protective role of Dectin-2 in controlling \textit{C. albicans} infection by inducing IL-23 production by dendritic cells generating a protective
Th17 response [81]. Consistent with this report, Dectin-2 activates the c-Rel subunit of NF-κB in human dendritic cells, which selectively induces IL-23 and IL-1α gene expression, and which is distinct from Dectin-1-mediated activation of all NF-κB subunits [79]. In contrast, we found that Dectin-1 and Dectin-2 are the main receptors required for translocation of the p65 subunit of NF-κB to the nucleus, although we have yet to examine other subunits or the direct effect of p65 on cytokine gene expression. Further, blockade of Syk by Piceatannol did not exhibit an additive effect in cytokine production, suggesting that Syk-independent pathways downstream of Dectin-1 and Dectin-2 could also be contributing to the inflammatory response.

In the current study, we found that RodA protein enhances fungal viability and prevents Dectin-1 and Dectin-2 mediated recognition of conidia in vivo. These results suggest that opsonization of RodA protein with antibodies could potentiate phagocytosis and improve the outcome of infection. Also, since RodA protein is degraded at the site of germination, antibodies to RodA could potentially activate Fc receptors on macrophages and neutrophils to induce production of reactive oxygen species, which we found are essential for killing fungal hyphae [121].

In conclusion, the more rapid fungal killing of the ∆rodA strain in infected corneas indicates that this protein functions as a virulence factor that mediates fungal survival in vivo by masking Dectin-1 and Dectin-2 recognition by resident tissue macrophages. Impaired macrophage cytokine production and neutrophil recruitment to the site of infection allows time for spores to germinate and invade
the tissue before an effective host response can develop. Therefore, future studies of the molecular pathways underlying hydrophobin formation and degradation, in addition to an increased understanding of the role of the host response may identify potential therapeutic targets that could abrogate the consequences of infection with these pathogenic fungi.
Chapter 3

Neutrophil Acidic Mammalian Chitinase (AMCase) and *Aspergillus fumigatus* chitin synthases regulate fungal growth during infection
Abstract

Chitin is a polysaccharide found in fungal cell walls that has been shown to modulate the immune system; however, the role of chitin in fungal virulence is not well understood. We therefore examined the effect of chitin degradation or inhibition of chitin synthesis in *A. fumigatus* pathogenesis. Using a murine model of corneal infection, we found expression of acidic mammalian chitinase (AMCase), which was produced by infiltrating neutrophils. *In vitro*, mouse and human neutrophils expressed AMCase in response to *Aspergillus* hyphal extract (AspHE), which was dependent on the Dectin-1/Syk pathway. IL-6 and IL-23 also induced high levels of AMCase expression in neutrophils. Moreover, inhibition of AMCase using Bisdionin F or C blocked hyphal killing *in vitro*. Conversely, chitin synthase mutant strains were more susceptible to neutrophil killing and were less viable during infection. We also found that a chitin deacetylase mutant which has elevated cell wall chitin, was more resistant to neutrophil killing, and exhibited augmented virulence *in vivo*. Consistent with these findings, inhibition of chitin synthesis with nikkomycin Z reduced *Aspergillus* virulence during infection and increased neutrophil-mediated killing *in vitro*. Further, inhibition of chitin and β-glucan synthesis using nikkomycin Z and caspofungin resulted in increased susceptibility to neutrophil killing. Together, these data indicate that neutrophil AMCase is an essential mediator of anti-fungal responses, and identifies chitin as a virulence factor that protects *A. fumigatus* from neutrophil killing.
Introduction

Chitin is a polymer of β-(1-4)-N-acetyl-D-glucosamine and is the most abundant polysaccharide in nature after cellulose. It is present in the cyst walls of amoebae, the exoskeleton of crustaceans and insects, the gut lining of parasitic nematodes, and in the cell walls of bacteria and fungi [142-144]. In fungal cell walls, chitin is an essential molecule that provides strength and rigidity to protect the organism under cell wall stress conditions. After its synthesis and polymerization, chitin fibrils covalently attach to β (1, 3)-glucans to reinforce and strengthen the cell wall and protect the fungus against harsh environments [145]. Chitin can also regulate immune responses by blocking innate immune recognition and potentially serve as a mechanism of immune evasion by fungal pathogens [93]. The function of chitin in the fungal cell wall and its ability to modulate innate immune responses suggests that chitin could serve as a potential therapeutic target during disease.

Cell wall chitin can be degraded by enzymes known as chitinases. There are six chitinases identified in humans and seven in mice. In humans, Acidic mammalian chitinase (AMCase) and Chitotriosidase (CHIT-1) are the only two enzymatically active chitinases that hydrolyze chitin [83,97]. The other four chitinases contain amino acid substitutions on their catalytic sites, making them non-catalytic. Chitotriosidase is thought to be part of immune responses against fungi as it is up-regulated following systemic infection with A. fumigatus in guinea pigs and in neonates [146-148]. In contrast, AMCase is highly up-regulated in the lungs of asthma patients and in keratoconjunctivis patients, where its expression
in macrophages is dependent on the secretion of the Th2 cytokine IL-13 [10,99,149-151]. Furthermore, AMCase neutralization ameliorated Th2 inflammation and airway hyper-responsiveness [99]. However, there are no reports that implicate AMCase as part of an anti-fungal immune response.

In the current study, we utilized two approaches to determine the role of chitin in *A. fumigatus* pathogenesis. First, we examined the effect of chitin hydrolysis in the fungal cell wall by neutrophil AMCase and show increased susceptibility to neutrophil killing. Second, we inhibited chitin synthesis using chitin synthase mutants or nikkomycin Z, and demonstrate that reduced cell wall chitin content impairs *A. fumigatus* virulence in a murine model of corneal infection. Here, we demonstrate for the first time a role for neutrophil AMCase in mediating anti-fungal responses and identify chitin as a virulence factor that promotes resistance to neutrophil-mediated killing during infection.
Results

**AMCase is expressed by neutrophils during corneal infection with A. fumigatus**

To determine if AMCase is expressed during fungal infection, corneas of C57BL/6 mice were abraded, and injected with $4 \times 10^4$ live *A. fumigatus* conidia, and after 24h AMCase was detected by western blot. **Figure 3.1A** shows no AMCase in uninfected corneas, indicating no constitutive expression; however, after 24h AMCase is abundant in infected corneas. As neutrophils are the predominant cell type infiltrating the cornea during fungal infections, we examined if these cells are the source of AMCase by immunostaining histological sections. We found that AMCase in the corneal stroma of infected mice has the same distribution as neutrophils (**Figure 3.1B**), indicating that AMCase is produced by neutrophils during infection.

**Neutrophil AMCase expression is induced by Aspergillus hyphae, and mediates fungal killing**

We reported recently that IL-6 and IL-23 (IL-6/23) induce expression of IL-17A and IL-17RC in a sub-population of neutrophils, and that this population produces more reactive oxygen species and is more efficient at killing *A. fumigatus* hyphae than unstimulated neutrophils [82]. To determine the role of IL-6/23 on neutrophil AMCase expression, bone marrow neutrophils from C57BL/6 mice were incubated with rIL-6 and IL-23, and AMCase expression was examined by quantitative PCR. As shown in **Figure 3.2A**, although there was
Figure 3.1 Neutrophil AMCase expression during A. fumigatus infection. A) Western blot analysis of C57BL/6 mice uninfected or infected corneas with 4x10⁴ A. fumigatus live spores for 24h. B) Immunohistochemistry staining for Neutrophils and AMCase in sections from 24h infected corneas with A. fumigatus. Images were taken at magnification of 10x and 40x. Experiments were performed at least twice with similar results. Reprint submission obtained from the publisher.
constitutive AMCase expression that was slightly up-regulated in the presence of Aspergillus hyphal extract (AspHE), incubation with IL-6/23 with or without AspHE induced a profound increase in AMCase mRNA expression. Similarly, AMCase expression in human peripheral blood neutrophils was elevated after incubation with IL-6/23 alone or in combination with AspHE, whereas AspHE alone had only a slight effect (Figure 3.2B). These findings were also detected at the protein level, as AMCase expression was increased in bone marrow derived neutrophils incubated with either AspHE (Figure 3.2C), or IL-6/23 (Figure 3.2D).

To determine if AMCase has a direct role in the ability of neutrophils to inhibit *A. fumigatus* hyphal growth, peripheral blood neutrophils were incubated with *A. fumigatus* hyphae in the presence of the chitinase inhibitors Bisdionin C, which has broad activity for chitinases, or Bisdionin F, which is specific for AMCase [152]. Neutrophils were incubated with live *A. fumigatus* hyphae for 16h, and fungal mass was measured by adding the metabolic XTT dye and measuring absorbance as described [153]. We found significantly less hyphal mass when neutrophils were added to growing hyphae, demonstrating neutrophil mediated fungal killing (Figure 3.2E). However, in the presence of either inhibitor, fungal mass was significantly higher, indicating impaired killing, and that neutrophil AMCase has an important role in killing *A. fumigatus* hyphae. Similar results were found when IL-6/23-stimulated neutrophils were incubated with *A. fumigatus* hyphae in the presence or absence of chitinase inhibitors. Figure 3.2F shows that although these neutrophils are more effective at killing hyphae, Bisdionin C and Bisdionin F treated neutrophils had significantly higher fungal
Figure 3.2 Neutrophil AMCase up-regulation in response to AspHE and IL-6/23. A) Expression of AMCase mRNA in C57BL/6 bone marrow neutrophils and B) human neutrophils after 1h stimulation with AspHE alone, IL-6/23 alone or in combination with AspHE. C) Western blot analysis for AMCase in lysates from bone marrow neutrophils stimulated with AspHE or D) increasing concentrations of IL-6/23. E) Human neutrophils incubated with A. fumigatus hyphae with or without Bisdionin F or Bisdionin C for 16h. Fungal viability was measured by incubating hyphae with the XTT dye. F) Human neutrophils incubated with A. fumigatus hyphae with or without Bisdionin F or C and IL-6/23 for 16h. Fungal viability was measured by incubating hyphae with the XTT dye. P values are: *<0.05, **<0.001, ***<0.0001. Experiments were performed at least twice with similar results. Reprint submission obtained from the publisher.
mass, indicating that AMCase has an important role in anti-fungal activity in this neutrophil population. These results also indicate that AMCase expression is increased by AspHE, which signals through c-type lectins, and by IL-6/23, which signals through the STAT3 pathway.

*Aspergillus induces neutrophil AMCase expression via the Dectin-1/Syk pathway*

A recent report showed that curdlan stimulates production of chitotriosidase in human neutrophils, suggesting that Dectin-1 regulates AMCase expression [154]. We therefore examined if Dectin-1 mediates AMCase expression upon recognition of surface β (1, 3)-glucan. We then incubated WT and Dectin-1−/− bone marrow neutrophils with AspHE and observed decreased AMCase protein expression in Dectin-1 deficient neutrophils at 30 and 60min of stimulation compared to the WT neutrophils (Figure 3.3A). Furthermore, pharmacological inhibition of Syk which gets activated downstream of Dectin-1 lead to a decrease in AMCase protein expression in WT neutrophils incubated with AspHE (Figure 3.3B). To further confirm these results, we incubated the neutrophils with Curdlan, which only activates Dectin-1, in the presence or absence of the Syk inhibitor and also observed decreased AMCase protein expression (Figure 3.3C). These results indicate for the first time that Dectin-1 regulates AMCase expression in neutrophils and that chitin degradation increases the ability of neutrophils to eliminate *A. fumigatus.*
Figure 3.3 Regulation of AMCase expression in neutrophils by Dectin-1 and Syk. A) C57BL/6 and Dectin-1/- bone marrow neutrophils were incubated with AspHE for 30 and 60min and AMCase protein expression was detected by western blot. B) Stimulation of C57BL/6 bone marrow neutrophils stimulated with AspHE or C) curdlan for 1h in the presence or absence of R406. AMCase protein expression was detected by western blot. Experiments were repeated at least twice. Reprint submission obtained from the publisher.
Chitin synthase mutants are more susceptible to neutrophil killing and are less virulent in vivo

Chitin production by *Aspergillus* requires activation of a series of chitin synthase enzymes [155]. To examine the role of chitin on *A. fumigatus* resistance to neutrophil killing, we incubated human neutrophils with chitin synthase mutants ChsA/B/C/G and ChsE/EB/D/F, and measured metabolic activity of hyphae using XTT. As shown in Figure 3.4A, chitin synthase mutants have no impaired growth in RPMI; however, following incubation with neutrophils, the chitin synthase mutants were less viable than the WT Ku80 strain.

To examine the role of *A. fumigatus* chitin during infection, ChsA/B/C/G and ChsE/EB/D/F live conidia were injected into C57BL/6 mouse corneas, and fungal survival was quantified by CFU. We found that the CFU of the chitin synthase mutants were significantly lower than the WT strain after infection (Figure 3.4B), indicating that chitin synthesis is required for *A. fumigatus* growth and survival during infection. Consistent with lower CFU, we observed significantly less corneal opacity at 24 and 48h after infection with the chsE/EB/D/F strain compared to the WT strain, but no difference with the ChsA/B/C/G strain (Figure 3.4C-E).

Chitin deacetylase regulates susceptibility to neutrophil killing

Chitin can be deacetylated to form chitosan, which is not degraded by mammalian chitinases [156]. As shown in Figure 3.5A, chitin is a polymer of N-acetylglucosamine and chitosan is its N-deacetylated derivative. Chitin
Figure 3.4 Role of chitin synthases in *A. fumigatus* survival during infection. A) Human neutrophils incubated with WT, ChsA/B/C/G, and ChsE/EB/D/F strains for 16h. Fungal viability was measured with the XTT dye. B) 48h infected eyes were dissected and plated onto SDA agar plates overnight for CFU measurement. C) Representative images of 24h and 48h infected corneas from C57BL/6 mice with WT, ChsA/B/C/G, and ChsE/EB/D/F strains. D, E) Quantification of percentage and total corneal opacity in 24h and 48h infected corneas. ChsA (ChsA/B/C/G) and ChsE (ChsE/EB/D/F). P values are: *<0.05, **<0.001, ***<0.0001. Experiments were performed at least twice with similar results. Reprint submission obtained from the publisher.
deacetylase is the enzyme that catalyzes this reaction. To determine the role of chitin deacetylation in *A. fumigatus* resistance to neutrophil killing, we utilized a chitin deacetylase mutant (Δcda1-6) that does not convert chitin into chitosan. Hence, this strain has more cell wall chitin than the parent strain. Peripheral blood human neutrophils were incubated with growing Δcda1-6 hyphae, and fungal viability was measured using XTT. The Δcda1-6 strain had significantly higher XTT than the parent strain, thereby exhibiting more resistance to neutrophil-mediated killing (Figure 3.5B). To assess the role of chitosan during infection, corneas of C57BL/6 mice were infected with live Δcda1-6 conidia, and CFU and corneal opacity were quantified as before. Consistent with the *in vitro* findings, there was significantly higher CFU from Δcda1-6 compared to WT infected corneas (Figure 3.5C). Similarly, we found significantly increased corneal opacity scores in Δcda1-6 infected corneas compared to those infected with the WT strain (Figure 3.5D, E). Taken together, these data indicate that chitin contributes to virulence and is essential for *A. fumigatus* pathogenesis.

*Inhibition of chitin synthase activity facilitates A. fumigatus killing in vitro and in vivo*

As a second approach to examine the role of chitin synthases in *A. fumigatus* virulence, fungi were incubated with Nikkomycin Z, which inhibits chitin synthase activity [157]. Human neutrophils were incubated with *A. fumigatus* hyphae in the presence of nikkomycin Z, and fungal viability was quantified using the XTT method. We found no effect of nikkomycin Z on growth in media alone.
Figure 3.5 Effect of increased cell wall chitin in *A. fumigatus* pathogenesis. 
A) Chemical structure of chitin (top) and chitosan (bottom). B) Human neutrophils incubated with WT and ΔcdA1-6 strains for 16h. Fungal viability was measured with the XTT dye. C) 48h infected eyes were dissected and plated onto SDA agar plates overnight for CFU measurement. D) Quantification of total corneal opacity in 24h and 48h infected corneas. E) Representative images of 24h and 48h infected corneas from C57BL/6 mice with WT and ΔcdA1-6 strains. P values are: *<0.05, **<0.001, ***<0.0001. Experiments were performed at least twice with similar results. Reprint submission obtained from the publisher.
However, in the presence of human neutrophils, nikkomycin Z-treated hyphae exhibited significantly less metabolized XTT (Figure 3.6A), indicating increased hyphal killing.

To examine the effect of nikkomycin Z in vivo, corneas of C57BL/6 mice were infected with an RFP-expressing strain of A. fumigatus and after 6h mice were injected intrastromally with nikkomycin Z or vehicle. As shown in Figure 3.6B-D, there was significantly less RFP hyphae and CFU in infected corneas given nikkomycin Z compared with vehicle alone. Taken together with in vitro susceptibility, these data demonstrate that inhibition of chitin synthases using nikkomycin Z makes A. fumigatus more susceptible to neutrophil-mediated killing.

*Inhibition of chitin and β-glucan synthesis leads to enhanced neutrophil killing of A. fumigatus hyphae in vitro*

To determine the effect of combined inhibition of chitin synthesis and β-glucan synthesis on neutrophil killing, we incubated the chitin synthase mutants ChsA/B/C/G and ChsE/EB/D/F with neutrophils in the presence of caspofungin, which inhibits β-glucan synthesis, and examined hyphal growth using XTT. As shown above, the chitin synthase mutants were more susceptible to neutrophil killing than the WT strain (Figure 3.7A); however, in the presence of caspofungin, both the WT and the mutants were more susceptible to neutrophil killing than in
Figure 3.6 Inhibition of *A. fumigatus* chitin synthases *in vivo*. A) Human neutrophils incubated with *A. fumigatus* for 16h in the presence or absence of nikkomycin Z. Fungal viability was measured with the XTT dye. B) Representative dsRed images of 24h *A. fumigatus* infected corneas injected with vehicle or nikkomycin Z from C57BL/6 mice. C) Quantification of *A. fumigatus* RFP fluorescence with Metamorph software. D) 48h infected eyes were dissected and plated onto SDA agar plates overnight for CFU measurement. P values are: *<0.05, **<0.001, ***<0.0001. Experiments were performed at least twice with similar results. Reprint submission obtained from the publisher.
the absence of caspofungin, and the mutants were more susceptible than the WT strain (Figure 3.7A).

As caspofungin inhibits WT *A. fumigatus* hyphal growth in the presence of neutrophils, we examined the effect of combining nikkomycin Z and caspofungin. As shown in Figure 3.7B, hyphal mass was significantly lower in the presence of nikkomycin Z together with caspofungin, indicating that inhibition of chitin and β-glucan synthesis confers increased susceptibility of *A. fumigatus* hyphae to killing by neutrophils.
Figure 3.7 Effect of inhibition of chitin and β-glucan synthesis in the susceptibility of *A. fumigatus* to neutrophil responses. A) Human neutrophils incubated with WT, ChsA/B/C/G, and ChsE/EB/D/F strains for 16h in the presence or absence of caspofungin (1µM). Fungal viability was measured with the XTT dye. B) Human neutrophils incubated with *A. fumigatus* for 16h in the presence or absence of nikkomycin Z in combination with caspofungin. Fungal viability was measured with the XTT dye. P values are: *<0.05, **<0.001, ***<0.0001. Experiments were performed at least twice with similar results. Reprint submission obtained from the publisher.
Discussion

Expression of pathogen recognition receptors (PRRs) confers the ability of host cells to recognize and respond to live fungi and fungal cell wall products [135,158,159]. Fungal cell wall chitin is not recognized by PRRs; however, small fragments of chitin (<40µm) can suppress the immune response by inducing IL-10 production by macrophages [160]. In contrast, intermediate fragments (40-70 µm) are able to induce both pro-inflammatory responses in macrophages [95,96,142]. Chitin particles are recognized by alveolar NK cells in SCID mice to induce IFN-γ, which activates alveolar macrophages to increase phagocytosis [94]. In this study, we show that A. fumigatus chitin is important in the neutrophil anti-fungal responses. We also show that neutrophils express AMCase, which is required for effective anti-fungal responses against A. fumigatus. Lastly, we targeted the biosynthetic pathway of chitin by inhibiting chitin synthases using the antibiotic nikkomycin Z, and demonstrating increased susceptibility to neutrophil killing to identify this drug as a potential therapeutic treatment during fungal infection, and show that a combination of drugs that inhibit chitin and β-glucan synthesis increases susceptibility to neutrophil killing which can lead to the elimination of fungal growth during disease.

Our findings also showed that inhibition of AMCase using Bisdionin F or C results in impaired fungal killing by neutrophils in vitro, thereby demonstrating a non-redundant role for AMCase. However, the role of AMCase on fungi has not been well characterized. Purified AMCase has activity in vitro against C. albicans and A. fumigatus by hydrolyzing chitin [98]. We now demonstrate that
neutrophils express AMCase during fungal infection and that this expression is required for neutrophils to kill A. fumigatus. Our group and others have reported that reactive oxygen species (ROS) generation by neutrophils is essential to eliminate fungal hyphae during infection [161]. However, the inhibition of AMCase activity resulted in less neutrophil-mediated fungal killing without impairing their ability to make ROS, indicating that AMCase is a major player of the innate anti-fungal response in addition to ROS.

We found that AMCase expression by mouse and human neutrophils is dependent on Dectin-1 recognition of β-glucan and Syk activation. These results are also consistent with our studies of fungal keratitis showing that Dectin-1 activation occurs early in infection [162], and indicates that Dectin-1 mediated AMCase expression is part of the initial anti-microbial responses in neutrophils. In addition to AMCase expression in neutrophils being regulated by Dectin-1, we showed that AMCase expression is also induced following incubation with IL-6 and IL-23. Neutrophils produce IL-17 after incubation with IL-6 and IL-23, and these cells are more efficient at killing A. fumigatus by generating more ROS [82]. Our findings show that AMCase production by these cells is also essential to kill A. fumigatus, as Bisdionin C and F impair fungal killing.

AMCase has an important role in the pathology of asthma by inducing neutrophil and eosinophil infiltration and airway hyper-responsiveness [10,163]; however, this role has yet to be established during fungal infection. AMCase is overexpressed in ovalbumin sensitized mice that develop airway hyper-responsiveness and in lung tissue from asthmatic patients [99]. Moreover,
chemical inhibition of AMCase ameliorated allergic inflammation by decreasing eosinophil and neutrophil recruitment in the lungs of ovalbumin-sensitized mice [10]. Although AMCase contributes to the pathogenesis of asthma by inducing airway inflammation and hyper-responsiveness [10], we have shown in this study a protective role for AMCase during fungal infection by degrading chitin.

As a second approach to understanding the role of chitin in fungal infections, we showed that chitin synthase mutants were more effectively killed by neutrophils, and were less virulent during corneal infection. These findings are in contrast to a recent study showing that in an immunocompromised model of lung infection, these mutants were as virulent as the WT strain [164], and thereby supporting that the role for chitin during infection is to help survive the host immune response. This conclusion is also supported by the chitin deacetylase mutant, which has more cell wall chitin and was more resistant to neutrophil killing in vitro and in vivo. Our studies using the chitin synthase inhibitor nikkomycin Z further support this conclusion. Hyphae grown in the presence of nikkomycin Z were more susceptible to neutrophil killing, and in vivo injection of nikkomycin Z at the time of infection resulted in impaired fungal growth. Nikkomycin Z is a potent inhibitor of chitin synthases [145], where it impairs Candida albicans growth by inhibiting the chitin synthases Chs1 and Chs3 [157,165]. Hence, nikkomycin Z has fungistatic rather than fungicidal activity against C. albicans in vitro [166]. We show here that inhibition of chitin synthesis augments susceptibility to the neutrophil anti-fungal response.
During cell wall stress, chitin can be synthesized in excess to provide integrity to the cell wall and prevent cell lysis [167]. Further, the β-glucan synthesis inhibitor caspofungin leads to increased chitin synthesis in *C. albicans* [168,169]. We showed that combined treatment with caspofungin and nikkomycin Z increased susceptibility of *A. fumigatus* to neutrophil mediated killing, which is consistent with earlier studies. [167,170,171]. This study revealed that besides providing cell wall integrity for fungal growth, synthesis of β-glucan and chitin provides resistance to neutrophil anti-fungal responses. Further studies will determine the mechanism by which these two carbohydrates promote resistance against neutrophils and their effect as therapeutic agents during infection.

In summary, the current study identified a novel anti-fungal role for neutrophil AMCase against *A. fumigatus* by hydrolyzing cell wall chitin and demonstrated that its expression can be regulated by the Dectin-1/Syk pathway and stimulation of IL-6 and IL-23 in combination. These findings suggest that AMCase levels in the blood could be utilized as a potential diagnostic for fungal infection. Since mammalian cells don’t make chitin, administration of purified AMCase would be an excellent approach to treat fungal infections. We also showed that chitin synthases can be targeted by nikkomycin Z *in vivo* to eliminate *A. fumigatus* during infection and that a combination of nikkomycin Z and caspofungin significantly increases susceptibility to neutrophil killing *in vitro*. Together, these findings increase our understanding of the role of neutrophil AMCase and chitin in *A. fumigatus* infections, and identified targets for therapeutic intervention.
Materials and Methods

Mice and reagents

All animals were treated in accordance with the guidelines provided in the Association for Research in Vision and Ophthalmology ARVO statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by Case Western Reserve University IACUC. C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were used between the ages of 5-12 week old. Dectin-1<sup>−/−</sup> mice were provided by Yoichiro Iwakura (University of Tokyo, Japan). All animals were bred under specific pathogen-free conditions and maintained according to institutional guidelines.

Curdlan (Invivogen) and AspHE were used at 100µg/ml for all experiments. Antibodies used were AMCase (Proteintech), β-actin (cell signaling), and NIMP (Abcam). The inhibitors bisdionin C, nikkomycin Z, and caspofungin were purchased from Sigma. Bisdionin F was purchased from EMD Millipore. Mouse recombinant IL-6 and IL-23 were purchased from eBioscience and human recombinant IL-6 and IL-23 from R&D Systems.

Fungal strains, media, and growth conditions

Aspergillus strains used in this study were cultured in Vogel’s minimal media (VMM) with 4% agar at 37C/5% CO<sub>2</sub> unless stated otherwise. The chsA/B/C/G, chsE/EB/F/D, and Δcda1-6 mutant strains were provided by Jean Paul-Latge (Institut Pasteur, Paris, France).
**Murine model of A. fumigatus keratitis**

Mice were infected as previously described [4,172]. _A. fumigatus_ strains were cultured in VMM agar in 25cm² tissue culture flasks. Dormant conidia were disrupted with a bacterial L-loop and harvested in 5ml PBS. Pure conidial suspensions were obtained by passing the culture suspension through PBS-soaked sterile gauges placed at the tip of a 10ml syringe. Conidia were quantified using a hemacytometer and a stock was made at a final concentration of 2.5x10⁴ conidia/µl in PBS. Mice were anaesthetized by intraperitoneal (IP) injection of 0.6% Tribromoethanol, 1.2% tert-butyl alcohol, and PBS. Corneal epithelium was abraded with a 30 gauge needle. Through the abrasion was inserted a 33-gauge Hamilton needle and a 2 µl injection containing 4x10⁴ conidia (Optimal inoculum to study the disease was determined by preliminary studies; data not shown) was released into the corneal stroma.

For the drug experiments, nikkomycin Z was suspended in cyclodextran and 2µl were injected into the corneal stroma at 6h post-infection. For this experiment, nikkomycin Z was used at 10mg/ml.

**Imaging corneal opacity**

Mice were euthanized by CO2 asphyxiation and positioned in a three-point stereotactic mouse restrainer. Corneal opacity (Brightfield) was visualized in the intact cornea using a high resolution stereo fluorescence MZFLIII microscope (Leica Microsystems) and Spot RT Slider KE camera (Diagnostics Instruments).
All images were captured using SpotCam software (RT Slider KE; Diagnostics Instruments).

**Quantification of colony forming units (CFUs)**

For assessment of fungal viability, whole eyes were homogenized under sterile conditions in 1 ml PBS, using the Mixer Mill MM300 (Retsch, Qiagen, Valencia, CA) at 33 Hz for 4 min. Subsequently, serial log dilutions were performed and plated onto Sabouraud dextrose agar plates (Becton Dickenson). Following incubation for 24h at 37° C, the number of CFUs was determined by direct counting.

**Human and bone marrow derived neutrophil isolation**

For bone marrow derived neutrophil isolation, mice were euthanized by CO2 asphyxiation, and femurs and tibias were dissected. Cells were flushed out by placing the tip of a needle containing sterile PBS in one end of the bones and releasing the PBS. Cells were centrifuged at 1,300 rpm for 5min at 4C. Red blood cells (RBCs) were lysed in 5ml of RBC lysis buffer (eBioscience), and neutrophils were isolated from the white blood cell population using a mouse neutrophil enrichment kit (STEM CELL Technologies). Cells were then counted and harvested.

For human neutrophil isolation, 20mL of blood were withdrawn from healthy individuals and incubated with 3% Dextran for 20min at room temperature. White blood cells were then transferred into a fresh tube containing 10mL of Ficoll Plaque™ PLUS (GE Healthcare) and centrifuged at 1,700 rpm for
20min at 4C. Pellet was dissolved in RPMI and cells were counted and harvested.

**Growth Inhibition Assay**

Isolated conidia from *A. fumigatus* WT or mutant strains was prepared as a stock of 10,000 conidia/200ul of SDA and cultured in a black 96-well plate with an optical clear bottom (CoStar 3720) for 6h at 37C to obtain germ tubes. Supernatant was removed and cells were washed 3X with H2O. Cells were then incubated with RPMI alone or RPMI + human neutrophils (200,000 neutrophils/well) and incubated at 37C for 16h. For drug studies, neutrophils were incubated with the drug for 30min at 37C prior to the stimulation and then added to the plates. Next day, cells were washed 3X with ddH2O and fungal viability was measured by incubating hyphae with the XTT dye as previously described [153].

**Statistical Analysis**

Statistical analysis was performed using an unpaired t test (Prism’ GraphPad). A p value of <0.05 was considered significant.
Chapter 4

Discussion
**Research Summary**

We have demonstrated that the RodA protein masks the surface β-glucan and α-mannose in the outer conidial cell wall of *A. fumigatus* and *F. oxysporum*. Blocking the exposure of these polymers prevented macrophage activation *in vitro*, leading to decreased CXCL1, CXCL2, and TNF-α production. In contrast, we found that NFκB was increased in macrophages stimulated with a RodA deficient *A. fumigatus* (ΔrodA) strain and this was dectin-1 and dectin-2 dependent. This indicates that exposure of β-glucan and α-mannose in the conidial cell wall of the ΔrodA strain leads to activation of their receptors, dectin-1 and dectin-2. Phagocytosis was also significantly decreased in Dectin-1⁻/⁻ and Dectin-2⁻/⁻ compared to Wt macrophages stimulated with the ΔrodA mutant. Utilizing our murine model of corneal infection, we found that C57BL/6 mice infected with the ΔrodA strain were more efficient at recruiting neutrophils and fungal killing than mice infected with the Wt strain. During early infection (6h), Dectin-1⁻/⁻ and Dectin-2⁻/⁻ mouse corneas infected with the ΔrodA strain exhibited decreased CXCL1, CXCL2, and IL-6 production than the Wt corneas. This was accompanied by decreased neutrophil infiltration. There was no difference in cytokine production and neutrophil infiltration in the Wt, Dectin-1⁻/⁻, and Dectin-2⁻/⁻ infected corneas with the Wt strain because RodA masks the immune response. At 24h post-infection, Dectin-1⁻/⁻ and Dectin-2⁻/⁻ mouse corneas infected with the ΔrodA strain had impaired fungal killing and neutrophil infiltration compared to Wt mice, indicating that the RodA protein masks Dectin-1 and Dectin-2 activation in resident corneal macrophages to enhance fungal survival during infection.
We also studied the anti-fungal role of AMCase against *A. fumigatus* and found that it is highly expressed during corneal infection. Further, AMCase production co-localized with infiltrating neutrophils in the corneal stroma. AMCase expression in mouse and human neutrophils was induced by *Aspergillus* hyphal extract (AspHE) and IL-6 and IL-23 (IL-6/23), although IL-6/23 were much more potent inducers of AMCase expression. Chemical inhibition of AMCase in human neutrophils resulted in decreased killing of *A. fumigatus* hyphae *in vitro* in the presence or absence of IL-6/23. Expression of AMCase was regulated by Dectin-1 activation and this was dependent on spleen tyrosine kinase (Syk). This indicates that Dectin-1 mediates fungal recognition and expression of AMCase to promote fungal killing by neutrophils.

We identified chitin as a virulence factor in *A. fumigatus* by showing that two chitin synthase mutants were more susceptible to neutrophil killing *in vitro*, and were also less virulent than the parent strain *in vivo*. A chitin deacetylase strain which has increased cell wall chitin than the parent strain exhibited increased resistance to neutrophil anti-fungal responses. *In vivo*, this mutant strain caused more corneal opacity and it was significantly more viable than the parent strain. This suggests that a loss of chitin synthesis decreases fungal virulence *in vivo*. Pharmacological inhibition of chitin synthases with nikkomycin Z rendered *A. fumigatus* more susceptible to neutrophil killing than in the absence of drug. Intrastromal injection of nikkomycin Z at six hours post-infection led to increased fungal killing but not corneal opacity, indicating that inhibition of chitin synthesis *in vivo* decreases fungal virulence and does not affect leukocyte
recruitment. This also identifies nikkomycin Z as a potential anti-fungal drug to treat fungal infections in the cornea. Finally, we found that dual inhibition of chitin and β-glucan synthesis further increases *A. fumigatus* susceptibility to neutrophil killing by a yet unknown mechanism.

### 4.1. Immune evasion during fungal disease

Recognition of fungal pathogens by tissue resident cells represents an important line of defense during infection. However, fungi have developed mechanisms of immune evasion to mask recognition by the host. We have shown here that *A. fumigatus* conidia can avoid host immune recognition by expressing the RodA protein, which masks the exposure of β-glucans and α-mannose (Figure 4.1).

![Figure 4.1 Role of the RodA protein in promoting fungal survival in vivo.](image)

In the absence of RodA, conidia that gains access to the corneal stroma have increased exposure of β-glucan and α-mannose, which then gets recognized by Dectin-1 and Dectin-2 in the surface of macrophages. This leads to accelerated neutrophil recruitment in a CXCL1 and IL-1β dependent manner and increased hyphal killing. Reprint permission obtained from the publisher [1].
A recent report showed that *A. fumigatus* hyphae but not conidia express galactosaminogalactan (GAG), which can mask β-glucan in the cell wall [37]. We hypothesized that during infection the RodA protein promotes survival of conidia and its germination by masking β-glucan, while GAG becomes expressed by hyphae to continue suppressing Dectin-1 activation through the masking of β-glucan.

We also showed that chitin can protect hyphae from neutrophil anti-fungal activity by a yet unknown mechanism since inhibition of chitin degradation or synthesis leads to increased fungal growth (Figure 4.2). In contrast to RodA, which masks underlying conidial cell wall carbohydrates and proteins, chitin appears to be inert to the immune system [96]. Our findings show that inhibition of chitin synthesis renders *A. fumigatus* hyphae susceptible to neutrophil killing, indicating that chitin has a protective role in host immune recognition. The inert properties of chitin have also been shown in *C. albicans* [93], indicating that this phenomena happens not just in *Aspergillus* species, but also in other fungal pathogens.

Other human pathogenic fungi also possess mechanisms to circumvent host immune recognition and promote fungal survival during disease. For example, in the human pathogen *Histoplasma capsulatum*, α-glucans in the cell wall shield β-glucan polymers to prevent host immune recognition through Dectin-1 activation [173]. In *Blastomyces dermitatidis*, the concentration of cell
wall β-glucan increases from yeast to mycelium, thus dectin-1-mediated immune responses are weaker against yeast [174]. Further, Candida albicans can also prevent Dectin-1 activation because the mannoprotein layer covers the β-glucan layer of the cell wall, which blocks β-glucan-dectin-1 interactions [175]. Similar to

Figure 4.2 Role of chitin in protection against neutrophil anti-fungal responses. AMCase expression is induced by Dectin-1 or IL-6 and IL-23 signaling in neutrophils. Blockade of AMCase activity with Bisdionin F or C leads to decreased chitin degradation in the cell wall and impairment of fungal killing by neutrophils. Similarly, pharmacological inhibition of chitin synthesis with nikkomycin Z increases susceptibility to neutrophil killing.
A. fumigatus, these fungal pathogens also mask β-glucan exposure and thereby block dectin-1 activation during infection.

Other mechanisms of immune evasion utilized by human fungal pathogens involve the degradation of molecules with anti-fungal activity released by innate immune cells. Cryptococcus neoformans undergoes phenotypic switching in vivo, which results in an altered polysaccharide capsule, a dysfunctional host immune response and decreased phagocytosis by alveolar macrophages, and enhanced virulence [176]. C. albicans and A. fumigatus can evade complement attack by degrading the host complement components C3b, C4b, and C5b, and thus inhibiting the membrane attack complex (MAC) formation [177,178]. Another important mechanism of immune evasion is the production of enzymes with anti-oxidative activity. A. fumigatus, C. albicans, and C. neoformans express the superoxide dismutase (SOD) enzyme, which converts superoxide (O$_2^-$) radical into molecular oxygen (O$_2$) or hydrogen peroxide (H$_2$O$_2$) [121,179,180]. Further, A. fumigatus express the enzyme catalase to convert hydrogen peroxide into water and oxygen for protection against ROS [121].

4.2. Dectin-1 signaling during fungal infection

Dectin-1 is the most widely studied c-type lectin receptor during fungal infection. The findings performed in Chapters 2 and 3 show that Dectin-1 is essential for the induction of neutrophil recruitment in resident corneal macrophages and AMCase expression in neutrophils to enhance hyphal killing.
An earlier study done by our lab shows that Dectin-1 is part of the primary innate immune response against *A. fumigatus* [113]. These results are supported by reports that individuals with genetic polymorphisms in Dectin-1 have increased susceptibility to mucosal fungal infections [114, 181, 182]. Moreover, Dectin-1 can regulate intestinal inflammation by recognizing β-glucan in the cell walls of commensal fungal species from the mycobiome [183].

As discussed in Chapter 1, Dectin-1 activation leads to the expression of pro-inflammatory cytokines that recruit neutrophils and other inflammatory cells. Dectin-1 also induces phagocytosis and ROS formation, and can lead to NLRP3 inflammasome activation [184-186]. We show that Dectin-1 also mediates up-regulation of AMCase expression in bone marrow derived neutrophils. Neutrophils have four types of granules that contain anti-microbial peptides which are released during infection [187], and AMCase could potentially be a part of this arsenal. The mechanism by which the chitin polymer is initially degraded has yet to be determined, but it is thought that degraded chitin particles have the ability to induce chitinase expression [142]. Our findings suggest that initial fungal recognition by Dectin-1 triggers AMCase expression, which degrades the biologically inert chitin to initiate an immune response against it. AMCase expression in neutrophils therefore has an important role in degrading fungal cell wall chitin and thereby increasing susceptibility to neutrophil anti-fungal responses.

4.3. Role of Dectin-2 during fungal infection
In contrast to Dectin-1, the role of Dectin-2 during infection is not well understood. Our findings from Chapter 2 show that Dectin-2 expression is essential to mediate anti-fungal innate immune responses in the cornea during infection with \textit{A. fumigatus}. This is consistent with a recent report from our lab that shows increased neutrophil killing of \textit{A. fumigatus} after up-regulation of Dectin-2 expression, which regulates IL-17RC expression and production of ROS [82]. Dectin-2 has mostly been studied in the context of Th17 cell differentiation, thus these are the first two reports that show a role for Dectin-2 in innate anti-fungal immunity. Unlike Dectin-1, there are no reported human polymorphisms for Dectin-2 that are associated with increased susceptibility to fungal infections. However, several reports using mouse models show a role for Dectin-2 in the induction of Th17 cell differentiation during \textit{C. albicans} infection [80,81]. Other reports show that Dectin-1 induces Th17 cell differentiation while Dectin-2 only enhances the response, but is not essential [79]. Since Th17 cells have been shown to have a protective role in fungal infections [188], and Dectin-1 and Dectin-2 have similar signaling pathways described to date (REF), the specific signaling pathways leading to Th17 cell differentiation have yet to be identified. Thus, the relative contribution of each receptor is still not completely understood.

4.4. \textbf{AMCase during fungal infection}

As discussed earlier, the role of AMCase during fungal infection remains undetermined. There is only one report that shows that purified AMCase has anti-fungal activity against \textit{A. fumigatus} and \textit{C. albicans in vitro} [98]. Our findings further expand on this by showing that blockade of AMCase with
pharmacological inhibitors significantly decreases neutrophil-mediated killing of
*A. fumigatus* hyphae. Further, AMCase expression has been shown in
macrophages and epithelial cells, and we now demonstrate expression in
neutrophils. Chitinase expression is a common adaptation that not just mammals
but also plants have developed for protection against chitin-containing pathogens
[189,190], indicating that AMCase is highly conserved as an important anti-fungal
response. The essential role for neutrophils in killing hyphae supports the
concept that neutrophil AMCase has an important in protection against
pathogenic fungi. AMCase can be induced through TLR-2 receptor activation or
by IL-17 signaling in macrophages [95]. We show that surface β-glucan can also
induce expression of AMCase in neutrophils as well as the cytokines IL-6 and IL-
23. Although not tested, it is more likely that the IL-17 generated by neutrophils
after stimulation with IL-6 and IL-23 will further enhance the expression of
AMCase.

4.5. Chitin as a potential therapeutic target for fungal infection

Since chitin is an essential structural component of the fungal cell wall
which is not produced by mammalian cells, chitin represents a potential
therapeutic target for fungal infections. There are multiple chitin inhibitors
commercially available, the most potent of which is the peptidyl nucleoside
nikkomycin Z [145]. The fungistatic activity of nikkomycin Z against *A. fumigatus*
or *C. albicans* has been reported [157,165]. However, these studies did not
address the *in vitro* effect of this drug in fungal susceptibility to host immune
cells. We show here that inhibition of chitin synthesis in *A. fumigatus* with
nikkomycin Z increases neutrophil-mediated killing *in vitro* and *in vivo*. The mechanism by which the neutrophils kill *Aspergillus* hyphae after inhibition of chitin synthesis remains unknown, but we hypothesize that it makes hyphae susceptible to neutrophil ROS.

Although we observed increased fungal killing after administration of nikkomycin Z, the clinical potential as a therapy for infected patients should be evaluated in clinical trials. The molecular weight of nikkomycin Z is considerably high (495.44g/mole) and this could severely diminish the amount of drug that could penetrate the tight junctions in the corneal epithelium to enter the stroma. In a mouse model of coccidiomycosis, nikkomycin Z sterilized the lungs of 7 out of 8 mice [191], suggesting that this drug might be more effective than currently used anti-fungal drugs. The safety of nikkomycin Z has been tested in healthy individuals; where there were no dose-related adverse events noted with doses up to 2,000mg [192]. Taken together, our findings further support the idea that nikkomycin Z could act as a very efficient anti-fungal drug and determine the negative effect of targeting chitin synthesis in *A. fumigatus* against neutrophils. Previously it has been shown that a combination of the β-glucan inhibitor micafungin and nikkomycin Z improved survival in mice with systemic aspergillosis [193]. Our results further enhance our understanding by showing that inhibition of β-glucan and chitin synthesis not just reduce fungal growth, but also increases susceptibility to neutrophil killing. Since these drugs do not possess fungicidal activity and neutrophils are still required to kill the fungus, their efficiency should be tested in neutropenic mice.
4.6. Potential caveats of experimental approaches

Bisdionins are xanthine derivatives that have the ability to inhibit the enzymatic activity of mammalian chitinases [152]. We utilized Bisdionin F and C to block AMCase and chitotriosidase activity in neutrophils and observed less fungal killing \textit{in vitro} in the presence of either drug. However, fungi also express chitinases to rearrange chitin in the cell wall [194,195], and at high doses both chitinase inhibitors could also block the activity of fungal chitinases. An alternate experiment to overcome this caveat is to isolate bone marrow derived neutrophils from AMCase\textsuperscript{−/−} mice and measure fungal mass after incubation with hyphae.

We also used the R401 chemical inhibitor to block the kinase activity of Syk \textit{in vitro}. R401 binds to the ATP-binding pocket of Syk, which leads to inhibition of Syk kinase activity. Besides having this effect on Syk, R401 also has non-specific effects as it can inhibit JNK kinases. To circumvent this caveat we could perform siRNA knockdown for Syk to confirm the results obtained with the R401 inhibitor.

We showed that nikkomycin Z and caspofungin do not act as fungicidal agents against \textit{A. fumigatus} hyphae \textit{in vitro} as we did not observe inhibition of fungal growth unless neutrophils were added. To determine if these drugs have any anti-fungal activity against \textit{A. fumigatus}, we will incubate hyphae with increasing concentrations of each drug without the addition of neutrophils. To test this \textit{in vivo}, we will administer the drug by sub-conjunctival injection into infected
mice immunosuppressed with cyclophosphamide. This will allow us to determine if both drugs are fungicidal against *A. fumigatus* in the absence of cell infiltration.
Chapter 5

Future Directions
5.1. Chitin fragments, but not chitin, regulate expression of AMCase

Exp 5.1.1 Role of Dectin-1 in binding to large chitin polymers

Although chitin polymers in the cell wall are biologically inert [142], it is clear now that the inflammatory response leads to degradation of the chitin to generate smaller fragments with the ability to modulate the immune response. A recent report showed that intranasal administration of purified cross-linked chitin and β-glucan from the cell wall of *A. fumigatus* into C57BL/6 mice induces high amounts of AMCase expression [196]. In a guinea pig model of systemic infection with *A. fumigatus*, the amount of AMCase produced correlates with the amount of the inoculum [147], suggesting that the up-regulation of AMCase is induced by the fungus.

Our data show that binding of Dectin-1 to its ligand β-glucan induces up-regulation of AMCase expression in bone marrow derived neutrophils, but its ability to bind to chitin remains undetermined. Work performed by Underhill et al., showed that Dectin-1 can bind to both soluble and particulate β-glucan polymers, but signaling is only activated upon binding to particulate β-glucan [58]. The underlying mechanism is the presence of CD45 and CD148 phosphatases, which keep the tyrosine residues in the ITAM chains of Dectin-1 de-phosphorylated. These phosphatases are excluded after binding of Dectin-1 to β-glucan. In contrast, binding of soluble β-glucan does not lead to exclusion of these phosphatases. We hypothesized that similar to β-glucan, there is signal specificity with regards to chitin size where large chitin fragments do not lead to
exclusion of CD45 and CD148, but smaller fragments do. To test this, SBPc-tagged Dectin-1-expressing RAW264.7 cells will be incubated with purified chitin polymer or chitin fragments of different sizes and stained for CD45 or CD148. The exclusion of the phosphatases after stimulation will be detected by confocal microscopy. We anticipate observing exclusion of the phosphatases after stimulation with chitin fragments but not large chitin polymer, providing an explanation for why cell wall chitin does not activate Dectin-1.

**Exp 5.1.2 Induction of AMCase after binding of Dectin-1 to chitin fragments:**
Although direct binding of chitin to Dectin-1 has not been shown, small chitin fragments (<40µm) can induce IL-10 production in macrophages in a Dectin-1-dependent manner, and this is thought to mediate resolution of inflammation [96]. However, larger chitin fragments (40-70µm) can generate inflammatory responses and AMCase may further degrade the chitin polymers into smaller fragments. Hence, we hypothesize that Dectin-1 is able to bind larger fragments and up-regulate chitinase expression in neutrophils. To test this, we will incubate C57BL/6 and Dectin-1/- bone marrow neutrophils with large or small fragments and measure AMCase mRNA expression by qPCR. Western blots will also be performed to detect changes in AMCase protein expression. We predict that Dectin-1 deficient neutrophils will exhibit decreased AMCase mRNA and protein expression compared with C57BL/6 neutrophils.

**Exp 5.1.3 Role of IL-6 and IL-23, and chitin fragments in regulation of AMCase:** Our data shows that IL-6 and IL-23 (IL-6/23) together can induce up-regulation of AMCase in neutrophils and a previous study published by our lab
showed that stimulation with IL-6/23 leads to IL-17 production in neutrophils. Further, it was recently shown that IL-17 leads to up-regulation of the IL-17RA in macrophages and that IL-17 signaling induced AMCase expression [95]. Therefore, IL-17 might further enhance AMCase expression in neutrophils. We will stimulate C57BL/6 and IL-17−/− neutrophils with IL-6/23, IL-17, or both and AMCase expression will be detected by qPCR and western blot.

Chitin fragments have been shown to induce IL-17A expression in macrophages in vitro [95]. To determine if chitin fragments can induce expression of ROR-related orphan receptor gamma t (RORγt) to promote expression of IL-17, we will perform intracellular flow cytometry for RORγt in neutrophils stimulated with different sized chitin fragments to determine if chitin fragments induce RORγt expression. RORγt nuclear translocation will also be detected by western blot analysis of nuclear extracts. Flow cytometry will be conducted to detect the levels of intracellular IL-17 in the neutrophils after stimulation with chitin.

**Exp 5.1.4 Role of Dectin-2 in recognition of chitin fragments after IL-6/23 pre-treatment in neutrophils:** In addition to inducing IL-17 expression, IL-6/23 leads to up-regulation of Dectin-2 in neutrophils [82]. A recent report showed that the mannose receptor can bind small chitin fragments to produce IL-10 in macrophages [160]. Dectin-2 can also bind to high mannose structures and thus [77], we speculate that up-regulation of Dectin-2 after IL-6/23 pre-treatment could lead to AMCase expression in the neutrophils. Although they recognize different ligands, Dectin-1 and Dectin-2 mainly induce Syk activation as the
canonical pathway to activate gene transcription of target genes that play a role in the inflammatory response [48]. To test this, we will treat C57BL/6 and Dectin-2<sup>−/−</sup> neutrophils with small and large chitin fragments, and phosphorylation of Syk will be detected by western blot. This will be utilized as a marker of Dectin-2 pathway activation. The anticipated result is that Dectin-2<sup>−/−</sup> neutrophils will have less p-Syk. AMCase expression will also be studied by western blot and qPCR.

5.2. Galectin-3 and Dectin-1 interaction regulates the inflammatory response during A. fumigatus keratitis

5.2.1 Binding of Galectin-3 and Dectin-1 enhances the inflammatory response against A. fumigatus: Although Dectin-1 mediates a strong inflammatory response during fungal infection, Dectin-1 also collaborates with toll-like receptor 2 (TLR2) to enhance inflammation [197,198]. Recently, it was shown that Dectin-1 can associate with the lectin Galectin-3 to mediate fungal recognition in macrophages [199]. Galectin-3 is expressed in macrophages, immature dendritic cells, and neutrophils, and can directly interact with bacterial glycans to regulate leukocyte function and inflammatory responses [200]. However, Galectin-3 can directly induce death of C. albicans [201]. Since Galectin-3 can modulate leukocyte inflammatory responses, we will study the role of dectin-1/galectin-3 association in potentially enhancing the inflammatory response.

To determine if dectin-1 and galectin-3 regulate cytokine responses, we will incubate RAW264.7 cells transduced with galectin-3 shRNA with A.
fumigatus. CXCL1, CXCL2, and IL-1β production will be measured by ELISA compared with control cells not given shRNA. Cells will also be stimulated with curdlan as a control, since curdlan only binds to dectin-1. Cells will also be incubated with germinating conidia of A. fumigatus, and phagocytosis will be detected by fluorescent microscopy. Germinating conidia will be stained for β-glucan and then added to the cells. Calcofluor white will be utilized to stain extracellular germinating conidia. Germinating conidia stained with β-glucan will be counted as intracellular, whereas extracellular conidia will be excluded based on calcofluor staining. This will determine if association of dectin-1 with galectin-3 is required for phagocytosis.

5.2.2 Role of galectin-3 in neutrophil migration during A. fumigatus keratitis: During bacterial infection with Streptococcus pneumoniae, galectin-3 acts as an adhesion molecule that can mediate neutrophil adhesion to endothelial cells, suggesting a role for this protein in promoting neutrophil extravasation [202]. To test this, we will isolate bone marrow derived neutrophils from C57BL/6 and galectin-3−/− mice, and adoptively transfer them into CD18−/− mice as previously shown [121]. Recipient mice corneas with be infected with A. fumigatus and neutrophil infiltration will be measured by flow cytometry at 24h and 48h post-infection. We anticipate observing more neutrophil infiltration in mice that received C57BL/6 neutrophils compared to the recipients of galectin-3−/− neutrophils.

To determine the in vivo role of galectin-3 in anti-fungal defense, C57BL/6 and Galectin-3−/− mice will be infected with live A. fumigatus. Fungal viability will
be determined at 24h and 48h post-infection by measuring colony forming units (CFU). Histological sections of infected eyes will be stained with Grocott’s methenamine silver stain (GMS), which stains carbohydrates in the fungal cell wall. Histology sections will be imaged by bright field microscopy. To determine if galectin-3 in neutrophils is required to kill *Aspergillus* hyphae, C57BL/6 and galectin-3<sup>−/−</sup> neutrophils will be incubated with hyphae and fungal viability will be measured with the metabolic dye XTT. If the galectin-3<sup>−/−</sup> neutrophils exhibit less hyphal killing, recombinant galectin-3 (rGal-3) will be added exogenously to observe if it rescues the ability of galectin-3 deficient neutrophils to kill hyphae.

To determine if galectin-3/dectin-1 association in neutrophils is required to kill *Aspergillus* hyphae, Wt and galectin-3<sup>−/−</sup> neutrophils will be incubated with hyphae in the presence of a Dectin-1 blocking antibody and fungal viability will be measured with the metabolic dye XTT. We anticipate that there will be decreased fungal killing in galectin-3<sup>−/−</sup> neutrophils incubated with the dectin-1 blocking antibody compared to the WT or galectin-3<sup>−/−</sup> neutrophils. If the galectin-3<sup>−/−</sup> neutrophils exhibit less hyphal killing, recombinant galectin-3 (rGal-3) will be added exogenously to observe if it rescues the ability of galectin-3 deficient neutrophils to kill hyphae.
Concluding Remarks

In conclusion, the findings described here identify the RodA protein and chitin as two molecules essential to promote fungal survival during infection by preventing neutrophil and macrophage activation. Since these molecules are present in all pathogenic fungi, these findings have implications for other systemic fungal infections with high rates of mortality in humans. Data presented here can lead to the development of more efficient drugs that prevent fungal growth to improve the outcome of disease.
Selected Publications

Yan Sun, Steven de Jesus Carrion, Eric Pearlman. (2014) Dectin-1 directs neutrophil processing IL-1β by controlling Caspase-11/Caspase-1 activation through Raf-1, but not Syk. (Manuscript in preparation)

Steven de Jesus Carrion, Patricia Taylor, Sixto M. Leal Jr., Jean-Paul Latge, and Eric Pearlman. (2014) Neutrophil Acidic Mammalian Chitinase and Aspergillus Chitin Synthases Regulate Fungal Growth During Infection. (Submitted to Plos Pathogens)

Laetitia Muszkieta, Steven de Jesus Carrion, Pauline Robinet, Remi Beau, Carole Elbim, Eric Pearlman, Jean-Paul Latge. (2014) The protein phosphatase PhzA of Aspergillus fumigatus is involved in oxidative stress tolerance and fungal virulence. Fungal Genetics and Biology


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