Internalization of Aspergillus fumigatus into pulmonary epithelial cells: joint action of host and pathogen

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Chapter 1

General introduction

It is estimated that more than 200,000 cases of invasive aspergillosis (IA) induced by *Aspergillus* species occur each year, including more than 10% of patients with acute leukemia, bone marrow and other transplant patients (>105,000 cases), and 1.3% of COPD patients admitted to the hospital (60,000 confirmed cases). However, because of underdiagnosis, these estimates likely represent only 50 to 65% of actual existing cases. Invasive aspergillosis carries an overall 50% mortality rate even if diagnosed and treated, but if the diagnosis is missed or delayed, then it is nearly 100% fatal (1). *Aspergillus fumigatus* (*A. fumigatus*) is the most commonly isolated species (92%) and the lung represents the most frequent site of infection of invasive pulmonary aspergillosis (IPA) (2). IA is increasing because of the high incidence of cancer, the widespread use of immunosuppressive agents and the aggravation of the aging society (3,4). Besides IA, *A. fumigatus* can induce allergic asthma and allergic bronchopulmonary aspergillosis (ABPA) in immunocompetent individuals.

A. fumigatus

A. fumigatus is responsible for approximately 90% of human *Aspergillus* infections. It is a saprotrophic filamentous fungus widely distributed in nature and also the most prevalent airborne fungal human pathogen. It can produce a large number of tiny conidia (2-3μm). It is estimated that an individual inhales several hundred conidia per day (5). Conidial size, cell wall composition, secretion of secondary metabolites and special invasion mechanisms of *A. fumigatus* play an important role in its infection pattern (5-7). But the molecular mechanisms underlying the pathogenesis of aspergillosis remain poorly understood.

The composition of the *A. fumigatus* cell wall includes a variety of proteins, lipids, melanin, and polysaccharides, which are the most abundant molecules within this structure (8). As seen in Figure 1, the outer surface of the resting conidium is covered by a layer of tightly organized proteins known as hydrophobins, of which RodA is the most important and well-characterized (9). The other important outer component is dihydroxynaphtalene (DHN)-melanin, which is responsible for the grayish-green color of *A. fumigatus*. Conidial swelling causes dissolution of the rodlet layer and melanin and subsequently exposure of main components of the *A. fumigatus* cell wall including various polysaccharides (10). Among them, β-1,3-glucan is the most important one. In addition, *A. fumigatus* also secretes various toxins, phosphatases and proteases. These factors enable *A. fumigatus* to infect host by interfering with defense mechanisms of the host, mediating its adhesion and internalization, and inhibition of immunocyte phagocytosis (11-13). Gliotoxin is one of the well-known virulence factors produced from *A. fumigatus* (14). Also some phospholipases (15) are known to damage host cells and thereby to facilitate tissue infection.

Currently, it is generally accepted that there is no unique essential virulence factor for *A. fumigatus*, and its virulence appears to be under polygenetic control (3,16). Until now, key virulence factors are still unclear.

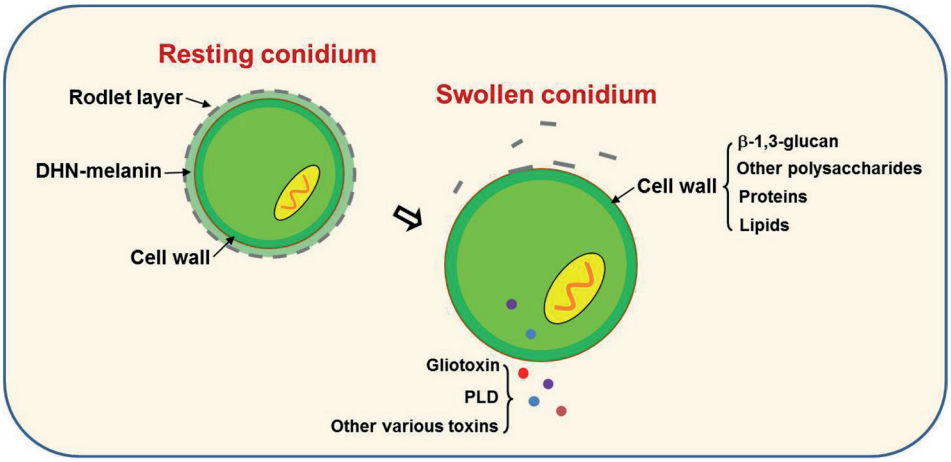

Figure 1. Molecular features of *A. fumigatus* conidia. Schematic representation of resting conidia (left) and swollen conidia (right). The organization of conidia is depicted, with specific cell wall and secreted components.

Pulmonary Epithelial Cell

The respiratory tract is a complex organ system that is divided into the upper and lower respiratory tract. The primary function of the mammalian lung is gas exchange. The lower respiratory tract comprises the conducing airway (the trachea, bronchi and bronchioles) and the alveoli, in which gas exchange takes place. The mature alveolar epithelium consists of type I and type II alveolar epithelial cells (ACE I and ACE II), also called type I and type II pneumocytes (17). Next to gas exchange, one of the major roles of the pulmonary epithelium is its function as a physical barrier to defend pathogens, allergens and other noxious substances. In addition to their function as mechanical barrier, pulmonary epithelial cells have been demonstrated to act as an important member of the first defense line of the host innate immune system like alveolar macrophages, neutrophils and others (18). They play an essential role in releasing inflammatory factors, presenting signals to lymphocytes and even directly killing microbes (19). Because of lack of a better system, the involvement of pulmonary epithelial cells in innate immunity responses against infections has been studied in the lung carcinoma A549 cell line which represents type II-like lung epithelial cells covering only less than 5% of the alveolar surface (20).

A. fumigatus Internalization into Pulmonary Epithelial Cells

A. fumigatus airborne conidia are inhaled from the environment and then colonize within the lung alveoli. Like many intracellular bacterial pathogens (21), *A. fumigatus* conidia are able to bind to and internalize into pulmonary epithelial cells. Consequently, *A. fumigatus* conidia survive and disseminate within these normally non-phagocytic host cells (22-24). Thereby immune evasion as well as dissemination may occur (18,25,26). To date, it has been shown that the internalization of *A. fumigatus* conidia into type II A549 pulmonary epithelial cells is closely related to host cell cytoskeletal dynamics, which induce the invagination of the host cell membrane and the engulfing of the conidia by pseudopods (26,27). The process of *A. fumigatus* internalization into pulmonary epithelial cells is represented in Figure 2. To date, the mechanism associated with *A. fumigatus* internalization into pulmonary epithelial cells including possible host cellular receptors and intracelluar signaling pathways remains largely unclear.

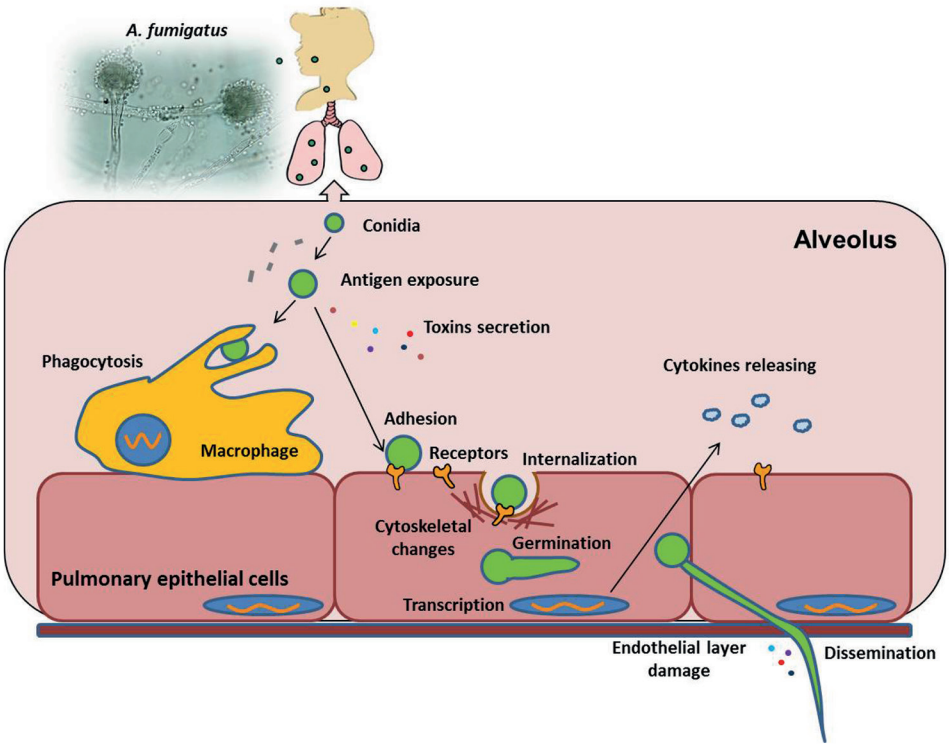

Figure 2. Schematic diagram of *A. fumigatus* internalization into pulmonary epithelial cells. For further explanation, please see text.

Virulence Factors and Components of *A. fumigatus*

β-*1,3-glucan*

The core component of the *A. fumigatus* is β-1,3-glucan. It is generally accepted that the recognition and induction of inflammatory responses to *A. fumigatus* by host alveolar macrophages rely on the obligate stage-specific exposure of β-1,3-glucan during conidial germination (28-30), which is characterized by conidial swelling, dissolution of the rodlet layer, and appearance of polysaccharide moieties on the cell wall (10). It is well-known that a major mammalian receptor for β-1,3-glucan is dectin-1, which is expressed predominantly by myeloid cells (31-33). In addition, mammalian toll-like receptors (TLR) (34), mannose receptors (35) and complement receptor 3 (CR3) (36) have all been implicated in the recognition of the cell wall components of *A. fumigatus* conidia and hyphae.

Phospholipase D (PLD)

PLD hydrolyzes the phosphodiester bond in the phospholipid backbone through its highly-conserved HKD motifs to yield phosphatidic acid (PA) and choline or ethanolamine, depending on the specific phospholipid species involved, i.e. phosphatidylcholine or phosphatidylethanolamine (37-39). Currently, mammalian PLDs are recognized as key enzymes in intracellular signaling involved in processes such as inflammation, endocytosis and cell shape changes (40), while bacterial PLDs from *Corynebacterium pseudotuberculosis* and *Acinetobacter baumannii* have been shown to acts as critical virulence determinants of these organisms (41-43). In fungi, PLD appears to be closely related to fungal cell shape changes, such as sporulation in Saccharomyces cerevisiae (44) and the dimorphic transition of *C. albicans* (45). Moreover, *C. albicans* PLD1-deficient mutants exhibit a substantially reduced ability to internalize into epithelial cells and a low virulence in immunodeficient mice, indicating that PLD may also be an important virulence factor in fungal pathogenesis. To date, three PLD isoforms, PLD, PLD1 and PLDA, have been reported in *A. fumigatus*, but their extracellular existence remains undetermined, and their role in pathogenesis has not yet been studied. Compared to PLD1 and PLDA, PLD of *A. fumigatus*, encoded by the *pld* gene, is rather specific and more distinct from the PLDs in other medically important fungi by phylogenetic analysis (46,47). Therefore, in this thesis we explore the function of the *pld* gene in development and virulence of *A. fumigatus*.

Gliotoxin

Gliotoxin is one of the well-known members of the epipolythiodiopiperazine class of metabolites produced by *A. fumigatus*. It is characterized by a disulfide briage across a piperazine ring which is essential for its toxicity. It should be noted that gliotoxin possesses multiple immuno-suppressive activities (48). In murine models of invasive aspergillosis (IA), gliotoxin has been shown to inhibit specifically the nuclear transcription factor NF-κB (49,50), which consequently induces host cell apoptosis (51,52) and suppresses the cytotoxic T-cell response

(53,54). Moreover, gliotoxin is also able to inhibit macrophage and polymorphonuclear cell function, including phagocytosis (55) and respiratory bursts (56,57). It has been demonstrated that gliotoxin from *A. fumigatus* affects the process of phagocytosis and actin cytoskeleton rearrangement of human neutrophils through distinct signaling pathways, which involve cyclic adenosine monophosphate (cAMP) and arachidonic acid signals, respectively (58). Recently, an immune-active role of gliotoxin has been demonstrated and linked to its antifungal properties and ability to activate platelets (59). In epithelial cells, it has been shown that gliotoxin reduces ciliary movement and alters membrane permeability thereby leading to epithelial cell damage (55,60,61). At low concentrations (<50 ng/ml), gliotoxin seems also to reduce the amount of transforming growth factor β1 (TGFβ1), interleukin-6 (IL-6) and interleukin-8 (IL-8) levels in A549 lung epithelial cells (61). However, much less is known about the effect of gliotoxin on *A. fumigatus* internalization into lung epithelial cells and warrants more research on this aspect.

DHN-Melanin

The DHN-melanin is responsible for the grayish-green color of *A. fumigatus* and is a protective factor for conidia by limiting the activation of the complement cascade and neutrophils, providing resistance against reactive oxygen species and masking the antigens on the surface of conidium (62). Most studies proposed that that *A. fumigatus* melanin acts as an immunological inert material and works by covering components with immune activity (63). For example, a *A. fumigatus* mutant lacking DHN-melanin is able to expose polysaccharides on its surface, such as β-glucan and mannose, to induce the release of inflammatory factors from peripheral blood mononuclear cells (64). However, DHN-melanin seems not only to act as an inert component but also to directly regulate innate immune response. Recent data showed that DHN-melanin seem to act as an important pathogenicity factor able to significantly activate platelets and thus to influence immune response and inflammation in infected patients (65). Thus, the functions of DHN-melanin need to be elucidated during *A. fumigatus* internalization into pulmonary epithelial cells.

Host Cellular Receptors and Intracellular Signaling Molecules

Dectin-1

Dectin-1 is a transmembrane protein present in leukocytes, with the highest levels of cell surface expression in neutrophils, macrophages, and dendritic cells (66). Expression has also been reported on other cell types including humans B cells and eosinophils (67). Dectin-1 contains a single c-type like domain in the extracellular region and an immunoreceptor tyrosine-based activation-like motif (ITAM) within its intracellular tail. By interacting with a particulate glucan, ITAM is phosphorylated, triggering several biological effects, including the production of superoxide, increase of phagocytosis, and induction of cytokines or anti-fungal effectors (32).

Dectin-1 appears to play various roles in different fungal species, such as *Candida albicans* (68), *A. fumigatus* (69) and *Cryptococcus neoformans* (70). However, the functions of dectin-1 during *A. fumigatus* internalization into pulmonary epithelial cells are presently unknown.

PLD in host cell

PLD is an enzyme that catalyzes the hydrolysis of the most abundant membrane phospholipids, phosphatidylcholine (PC) to produce PA and choline (71). In above paragraph, *A. fumigatus* PLD has been introduced. Here, we will introduce PLD in the host. Two mammalian PLD isoforms, PLD1 and PLD2, have been identified. Stimulation of PLD has been described in many cellular systems in response to a large variety of agonist-activated tyrosine kinase receptors and receptors coupled to heterotrimeric G proteins (40,72). In mammalian cells, PLD activity has been found to be closely related to actin dynamics (73-75). PLD is recognized as an effector of small GTPases and cofilin, both signaling families are central regulators of cellular actin dynamics (76,77). In addition, it has been demonstrated that macrophage phagocytosis might be coordinately regulated by PLD1 and PLD2 (78,79). Therefore, we hypothesized that host cellular PLD might play an important role during *A. fumigatus* internalization into pulmonary epithelial cells.

Cofilin

The dynamic processes of the actin cytoskeleton have been proposed to be highly regulated by various factors, among which the ADF (actin depolymerizing factor)/ cofilin family plays an essential and conserved role (80). In mammalian cells, the ADF/cofilin family consists of three similar members: cofilin-1, cofilin-2 (distributed specifically in muscle cells) and ADF (destrin) (81,82). Cofilin-1 is the most ubiquitous form and has been the most widely studied. Cofilin binds the minus end of actin and inhibits the formation of actin filaments (F-actin), whereas the Arp2/3 protein binds to the plus end of actin and activates the formation of F-actin (83,84). When the third amino acid of the conserved N-terminus (Ser) is phosphorylated, cofilin loses its actin depolymerizing activity, leading to the inhibition of F-actin severing and the production of filopodia/lamellipodia. The threonine kinase family LIM kinases (LIMK) phosphorylate and deactivate cofilin. Accordingly, dephosphorylation by the slingshot phosphatases (SSH) results in reactivation of the actin binding activity of cofilin (85). Recent studies have shown that cofilin activity is required for entry into host cells by many pathogens, including HIV (human immunodeficiency virus), *Cryptococcus neoformans*, and *Listeria monocytogenes* (74,86,87). Due to the vital role of cofilin in the invasion process of host cells by pathogens, studies on the involvement and function of cofilin in host cells during *A. fumigatus* infections is of considerable importance.

cAMP

cAMP is an important intracellular second messenger being widely studied before (88,89). A plenty of studies showed that host cell cAMP plays important roles in many lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), pulmonary fibrosis and other lung inflammatory diseases (90-92). The most important downstream signaling molecules of cAMP are exchange protein directly activated by cAMP (Epac) (93,94) and protein kinase A (PKA) (95). Host cell cAMP also plays important roles during the interactions between pathogens and hosts. Some studies reported that increased intracellular cAMP levels inhibited *Escherichia coli* internalizing into renal tubular epithelial cells (96) and bladder epithelial cells (97). Gliotoxin, a toxin of *A. fumigatus,* interferes with the intracellular cAMP homeostasis and inhibits phagocytosis of neutrophils to *A. fumigatus* (58). But still unclear are the underlying mechanisms leading to the alterations in regulation and function of host cell cAMP, the components of *A. fumigatus* being involved in this process, host cellular receptors and intracellular signalling molecules during *A. fumigatus* internalization into pulmonary epithelial cells.

Diagnosis and Treatments for Aspergillosis

Aspergillus spp. can cause a wide range of diseases ranging from allergies to invasive infections in human. Invasive aspergillosis is the most serious infection caused by it and the lung is the most common site of *Aspergillus* infections. Early diagnosis and correct antifungal treatment have a direct impact on patient recovery and survival (98). Currently, the diagnosis of *Aspergillus* infections mainly depends on conventional methods (99), biomarker tests (100), PCR-based DNA detection assays (101) and other new biomarker detection technologies (102). The conventional methods, cultures along with histopathologic detection of the fungus on biopsy specimens are still the gold standard methods for the diagnosis of infections. However, biopsies are not always obtained and cultures of respiratory specimens have relatively low positive predictive value (103). Treatment options are limited to three antifungal drug classes: polyenes (amphotericin B), azole drugs and echinocandins. Among them, azoles are the first-line drugs in treating disease caused by *Aspergillus* spp (104). However, reports in *A. fumigatus* azole resistance is markedly increasing (105,106) and antifungal resistance became an emerging problem in recent years (107). In addition, *A. fumigatus* might be able to adapt to the human host during infection and such adaptation may contribute to treatment failure and persistence of the fungus (108). Nowadays *Aspergillus* infections become more and more serious because of the high incidence of cancer, organ (lung) transplantation and other diseases known to suppress or damage the host immune system (108,109). Therefore, it is very important to identify some novel possible molecular targets to develop better early diagnostic methodologies or new antifungal drugs. In this thesis, we aim to provide some new insights.

Scope of the Thesis

The objective of this thesis is to investigate possible novel mechanisms during *A. fumigatus* internalization into pulmonary epithelial cells**.** We studied molecular mechanisms of two aspects, the pathogenic fungus and the host. **In chapter 2,** we mainly focused on PLD as the host intracellular signaling molecule. We found that β-1,3-glucan stimulated PLD activity of type II pneumocytes A549 cells to improve the internalization of conidia. PLD activation and conidia internalization were inhibited by an anti-dectin-1 antibody. We proved that dectin-1 was indeed expressed in A549 cells. Followed, **in chapter 3,** we demonstrated using a *pld* gene deletion strategy that PLD of *A. fumigatus* act as an important virulence factor on its own. Our results suggested that PLD of *A. fumigatus* regulated its internalization into lung epithelial cells and may represent an important novel virulence factor during *A. fumigatus* infections. **In chapter 4,** we studied gliotoxin. Our data indicated that gliotoxin modulates *A. fumigatus* internalization into epithelial cells through activation of host intracellular PLD1 and actin cytoskeleton rearrangement. Furthermore, **in chapter 5,** we studied the role of host intracellular cofilin. The results indicated that cofilin enable *A. fumigatus* internalization through the RhoA-ROCK-LIM kinase pathway**. In chapter 6,** we investigated the effects of DHN-melanin on the surface of *A. fumigatus* resting conidia on host intracellular cAMP and its downstream signaling molecules in pulmonary epithelial cells. Current data indicated that DHN-melanin significantly decreased the cAMP level whereas the expression level of both Epac1 and Epac2 were increased. To collect overall informations, **in chaper 7**, we used RNA-seq technology to assess the transcriptome profiles of A549 cells following direct interactions with conidia of *A. fumigatus*. We found several differentially expressed genes. In agreement with elevated immunological responses upon *A. fumigatus* infections, our results provided important insights into dynamic changes of gene expression in lung epithelial cells. The findings presented in this thesis will support the identification of yet unknown pathogenic mechanisms of *A. fumigatus* and host cell responses, and provide some important scientific insight to unravel new possible antifungal drug targets. Finally, **in chaper 8**, we summarize and discuss the novel findings presented in this thesis and provide future perspectives.

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