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

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Chapter 3

Disruption of the phospholipase D gene attenuates virulence of Aspergillus fumigatus

Xianping Li1,2‡, Meihua Gao1‡, Xuelin Han2‡, Sha Tao2, Dongyu Zheng2, Ying Cheng3, Rentao Yu2, Gaige Han2, Martina Schmidt4, Li Han2

1 Department of Immunology, Medical College of Qingdao University, Qingdao, China
2 Department for Hospital Infection Control & Research, Institute of Disease Control & Prevention of PLA, Academy of Military Medical Sciences, Beijing, China
3 Department of Hospital Acquired Infection Control and Prevention, Institute for Communicable Disease Control & Prevention, Chinese Center for Disease Control & Prevention, Beijing, China
4 Department of Molecular Pharmacology, University of Groningen, Groningen, The Netherlands

‡: The authors contributed equally to this article.

Abstract

Aspergillus fumigatus (A. fumigatus) is the most prevalent airborne fungal pathogen inducing serious infections in immunocompromised patients. Phospholipases are key enzymes in pathogenic fungi that cleave host phospholipids, resulting in membrane destabilization and host cell penetration. However, knowledge of the impact of phospholipases on A. fumigatus virulence is rather limited. In this study, disruption of the pld gene encoding phospholipase D (PLD), an important member of the phospholipase protein family in A. fumigatus, was confirmed to significantly decrease both intracellular and extracellular PLD activity of A. fumigatus. The pld gene disruption did not alter conidial morphological characteristics, germination, growth and biofilm formation, but significantly suppressed the internalization of A. fumigatus into A549 epithelial cells without affecting conidial adhesion to epithelial cells. Importantly, the suppressed internalization was fully rescued in the presence of 100 µM phosphatidic acid, the PLD product. Indeed, complementation of pld restored the PLD activity and internalization capacity of A. fumigatus. Phagocytosis of A. fumigatus conidia by J774 macrophages was not affected by the absence of the pld gene. Pre-treatment of conidia with 1-butanol and a specific PLD inhibitor decreased the internalization of A. fumigatus into A549 epithelial cells, but had no effect on phagocytosis by J774 macrophages. Finally, loss of the pld gene attenuated the virulence of A. fumigatus in mice immunosuppressed with hydrocortisone acetate, but not with cyclophosphamide. These data suggest that the PLD of A. fumigatus regulates its internalization into lung epithelial cells and may represent an important virulence factor for A. fumigatus infection.
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Introduction

Aspergillus fumigatus (A. fumigatus), an airborne fungal pathogen, causes a wide range of diseases, including allergic bronchopulmonary aspergillosis, aspergilloma and invasive aspergillosis (54,58). Inhalation of conidia and their colonization in the alveoli may result in their germination and growth in the lung, and in immunocompromised individuals, the pathogen may spread and result in the development of invasive aspergillosis associated with high mortality. However, the molecular mechanisms underlying the pathogenesis of invasive aspergillosis remain poorly understood. 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 (38,58). At least four groups of molecules and genes of A. fumigatus associated with its virulence have been studied intensively (2,54), including cell wall components required for massive growth during infection (46), such as β (1-3)-glucan, galactomannan (Afmp1 and Afmp2) (70); stress-response genes and molecules which have been implicated in the evasion from the host immune response, such as cAMP-dependent protein kinases (pkaR, pkaC) (54) and mitogen-activated (MAP) kinases (mpkA) (67); a number of genes and molecules to allow A. fumigatus to compete in its environmental niche, such as genes involved in iron (9) and phosphorous acquisition (10,50); toxins and allergens, as well as enzymatic proteins secreted by A. fumigatus, such as alkaline serine proteases (Alp and Alp2) (53) and phospholipases (59) that damage host cells and facilitate tissue infection. Phospholipases hydrolyze ester linkages in glycerophospholipids, which are one of the major chemical constituents of the host cell envelope, and, hence, may destabilize the host cell membrane to mediate microbe entry into host cells and tissues. Phospholipases constitute of a heterogeneous group of enzymes, including phospholipase A (PLA), B (PLB), C (PLC) and D (PLD) (21). It has been shown that phospholipases, especially PLB, are essential virulence factors in the pathogenesis of several important pathogenic fungi, including Candida albicans (41) and Cryptococcus neoformans (12). Although activity of A. fumigatus phospholipases and three A. fumigatus plb genes have been characterized (5,59), our current knowledge of the impact of A. fumigatus phospholipases, in particular of PLD, on the virulence of this pathogen is rather limited (1).

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 (3,17,21). Currently, mammalian PLDs are recognized as key enzymes in intracellular signaling involved in processes such as inflammation, endocytosis and cell shape changes (27), while bacterial PLDs from Corynebacterium pseudotuberculosis and Acinetobacter baumannii have been shown to be the critical virulence determinants of these organisms (25,29,44). In fungi, PLD appears to be closely related to fungal cell shape changes, such as sporulation in Saccharomyces cerevisiae (55) and the dimorphic transition of C. albicans (14). Moreover, C. albicans PLD1-deficient mutants exhibit a substantially reduced ability to be
internalized by epithelial cells and 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 yet to be 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 (26,31). Therefore, in this study we chose to explore the function of the *pld* gene in development and virulence of *A. fumigatus*.

### Materials and Methods

#### Fungal and Bacterial Strains, Media and Chemical Reagents

The strains used in this study are listed in Table S1. *A. fumigatus* B5233 was used as the wild-type strain for all *in vitro* and animal model experiments. All *A. fumigatus* cultures were grown in *Aspergillus* minimal medium (AMM) at 37°C unless otherwise specified. *Escherichia coli* DH-5α was used for routine cloning and was grown in Luria-Bertani broth at 37°C. *Agrobacterium tumefaciens* strains were grown either in Luria-Bertani broth supplemented with 50 mg/l kanamycin or induction medium supplemented with 200 μM acetosyringone (IMAS). Transformants were selected using AMM supplemented with 200 mg/l hygromycin (Roche, Mannheim, Germany) and 200 mg/l cefotaxime (62). The mammalian PLD1-specific inhibitor, VU0359595 (Avanti no. 857371) (42), and the PLD2-specific inhibitor, VU0285655-1 (Avanti no. 857372) (39) and PA (Avanti no. 840101P) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Hydrocortisone acetate (Bio Basic Inc, Markham, Canada) and cyclophosphamide (Sigma Aldrich, Saint Louis, MO, USA) were used for immunosuppression.

#### Construction of Δ*pld* and Δ*pldc* Strains of *A. fumigatus*

The Δ*pld* mutant of *A. fumigatus* B5233 was constructed by *A. tumefaciens*-mediated transformation as described previously (62,66). Briefly, the deletion vector was constructed by cloning a 5.8-kb sequence, including a 1,220-bp fragment upstream and a 1,032-bp fragment downstream of the coding region of the *A. fumigatus* *pld* gene, into pDHt/SK.2 to produce plasmid A using primers P1 and P2 (Table S2). Subsequently, a 2.9-kb PCR product of the hygromycin resistance gene (*hph*) was amplified from pDHt-hph-hindIII-sacI (62) using primers P3 and P4 (Table S2) to add the *Bgl* II and *Asu* II restriction sites. The *hph* fragment, which was digested with *Bgl* II and *Asu* II, was ligated into plasmid A to create plasmid B (43,62). Plasmid B was transformed into competent *A. tumefaciens* EHA105 using the freeze-thaw method (52,62). The resulting strain of *A. tumefaciens* was designated as the EHA105h strain. To obtain the Δ*pld* strain, conidia of B-5233 and EHA105h were cultivated together at a ratio of 1:10 (conidia to bacteria). A total of 100 μl of the *Agrobacterium* culture was mixed with 100 μl of B-5233
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conidia and spread onto a nylon filter placed on an AMM agar plate supplemented with 0.2 mM acetosyringone. Plates containing the filters were incubated at 24°C in the dark for 48 h. To select transformants, filters containing the transformants were transferred to AMM agar plates supplemented with 200 μg/ml hygromycin and 200 μg/ml cefotaxime and incubated at 37°C for 72 h (62). Transformants were scraped from the nylon filter and transferred onto Sabouraud medium plates. The Δpld strain was initially screened by PCR with primers designed to amplify the regions of pld (primers P7 and P8 [Table S2]) that should have been deleted in the Δpld strain. An additional PCR screen was performed to amplify the junctions of homologous sequences and hph to indicate replacement and homologous recombination (primers P9 and P10) (Table S2).

To ensure that the mutant phenotype obtained could be attributed to the specific desired deletion, the Δpld strain was reconstituted by integration of the B5233 pld allele to create a complementation strain, pldc. Briefly, the 5.8-kb Spe I - Kpn I DNA fragment containing the partial pld promoter, pld ORF and pld terminator was cloned into pDHt/SK to produce plasmid C. The 2.7-kb PCR product of a phleomycin resistance gene (ble cassette) was then amplified from pAN8.1 using primers P5 and P6 (Table S2) and subcloned into plasmid C (19). The resulting plasmid was linearized at the unique Kpn I site. Protoplasts of the pld mutant were transformed with 10 μg of this linear construct. Transformants were selected on 375 μg/ml phleomycin plates. To confirm the constructed strains, Southern blot analysis was performed with Xba I digested genomic DNA. A 454-bp fragment of the pld gene was used as a probe for Southern hybridization (primers P11 and P12) (Table S2).

**PLD Activity Assay**

The conidia (2.5 × 10⁸/ml) from each strain were inoculated in 50 ml of liquid Sabouraud medium and incubated at 37°C with shaking at 150 rpm for 4 h and 8 h to obtain the swollen conidia and hyphae, respectively. The swollen conidia or hyphae and supernatants were separated by centrifugation at 10,000 × g for 2 min and prepared for PLD activity assays. After washing three times with phosphate buffered saline (PBS), the swollen conidia or hyphae were resuspended in a volume of 300 μl of lysis buffer consisting of PBS with 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor and 100 μM phenylmethylsulfonyl fluoride. Cell extracts were prepared by adding an equal volume of 0.5-mm glass beads (Sigma Aldrich) to the cells, followed by shaking at 1,800 rpm in a IKA-Vibrax shaker at 4°C for 15 min. The cell lysates were collected by centrifugation at 10,000 × g for 2 min (11). PLD activity was determined with the Amplex Red phospholipase D assay kit (Invitrogen, Eugene, Oregon, USA) (29). Each PLD reaction contained 50 μM of Amplex Red reagent, 1 U/ml HRP, 0.1 U/ml choline oxidase, 0.25 mM lecithin and 100 μl of sample. The reaction lasted for 30 min, and fluorescence was excited at 530 nm and measured at 590 nm using a fluorescence microplate reader (SpectraMax M5). In parallel, the extracellular PLD activities in the Sabouraud medium culture supernatants were also determined with the same methods.
Morphological Characterization and Measurement of Mycelial Growth Rate

A total of $5 \times 10^2$ conidia (5 μl) were inoculated centrally in AMM, Sabouraud medium and egg yolk lecithin medium agar plates and cultured at 37°C for 72 h. Every 12 h, the colonies of each strain were observed and photographed under an inverted microscope (Olympus Leica DMI3000 B). Additionally, the colony diameter was measured every 12 h, and the mycelial growth rate was determined as the increase in colony diameter per hour (mm/h).

Biofilm Formation Assay

Quantification of the biofilm formation for *A. fumigatus* was performed as described in a previous report (22). Briefly, 100 μl of liquid AMM containing $2 \times 10^4$ conidia per well were added to a 96-well plate and incubated for 24 h at 37°C. The medium was removed, and the wells were washed three times with PBS. Subsequently, 150 μl of 0.5% crystal violet solution were added to stain the residual material in the well for 5 min. Excess stain was gently removed under running water, and the biofilm was then destained by adding 200 μl of 95% ethanol. The biofilm density was measured by determining the absorbance of the destaining solution at 570 nm using a microplate reader (SpectraMax M5).

In vitro Internalization and Phagocytosis Assay

Internalization of *A. fumigatus* into lung epithelial cells and phagocytosis of the pathogens by macrophages were analyzed as described in a previous report (68). Briefly, human A549 lung epithelial cells or J774 murine macrophages was grown to confluence in 96-well plates (approximately $2 \times 10^4$ cells per well). Subsequently, $2 \times 10^5$ conidia were added and incubated at 37°C under 5% CO$_2$ to induce internalization or phagocytosis. After 4 h for internalization or 1 h for phagocytosis, the cell monolayers were washed three times with PBS, and 100 μl DMEM supplemented with 10% fetal calf serum and 20 μg/ml nystatin were added to each well and incubated for 3.5 h to kill non-internalized conidia. The cell monolayers were then washed and treated with 100 μl of PBS containing 0.1% Triton X-100 for 15 min at 37°C to induce cell lysis and the release of internalized conidia. The released conidia were diluted onto Sabouraud plates and incubated at 37°C for 20 h. Colonies were counted to determine the total bound and intracellular conidia (29). The internalization rate was determined as the percentage of intracellular conidia colonies compared to the initial inoculum of conidia. To ensure that the nystatin had eliminated extracellular conidia, the supernatants of the cell cultures were plated onto Sabouraud plates as control.

Adherence Assay

The adherence capacity of *A. fumigatus* to epithelial cells was determined as described previously (22,60). The 6-well plates were pre-coated with 1 ml of 0.01 mg/ml fibronectin in PBS (without Ca$^{2+}$/Mg$^{2+}$) and incubated at 37°C for 4 h. A549 cells were seeded in this fibronectin-coated well and grown for 24 h. The conidia ($1.5 \times 10^3$) or germlings (germinated for 8 h) in
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Hank's balanced salt solution (HBSS)/Tween-20 0.01% were added into the wells and incubated at 37°C for 30 min, followed by three washes with HBSS to remove non-adherent organisms and overlaid with AMM agar at 45°C. The number of adherent organisms was quantified by colony counting. The adhesion rate was determined as the percentage of adherent colonies compared to the total amount of conidia.

**In vivo Virulence Assay**

Institutional Animal Care and Use Committee of the Academy of Military Medical Sciences approved the animal experiments. The wild-type, Δ*pld* and Δ*pldc* strains were used for experimental infections in white male BALB/c mice weighing 18–22 g. Mice were immunosuppressed by one of two methods. One was subcutaneous injection of 5 mg hydrocortisone acetate (Sigma Aldrich) in 300 μl PBS-0.1% Tween 20 on days -4, -2, 0, 2, and 4 of infection (64); the other was intraperitoneal injection of 3 mg cyclophosphamide (Sigma Aldrich) on days -4, -1, and 3 of infection and a single intraperitoneal injection of 4 mg hydrocortisone on day 0 (15). Mice were housed under sterile conditions and provided with sterile drinking water containing 500 μg/ml tetracycline hydrochloride. On day zero, mice were anesthetized by inhalation of diethyl ether and infected intranasally with 5 × 10⁶ conidia in 30 μl of PBS-0.01% Tween 20. Control mice were also immunosuppressed with hydrocortisone or cyclophosphamide and inoculated intranasally with 30 μl PBS-0.01% Tween 20. Eight mice per group were used. Morbidity and mortality were monitored for up to 16 days, and the Kaplan-Meier survival with log rank test was used for comparison among the groups. To determine the lung fungal burden, immunosuppressed mice were infected intranasally with 5 × 10⁵ conidia in 30 μl. Twelve mice of each group were used. Mice were sacrificed at 2, 72 and 120 h post-infection. One ml of lung lavage fluid was harvested and centrifuged at 10,000 × g for 3 min. The precipitate was resuspended, serially diluted and plated on Sabouraud agar plates for incubation at 37°C. The number of colonies in lung lavage fluid (CFU/ml) was counted and calculated after 20 h. In parallel, the lung tissues were weighed, homogenized and then serially diluted onto Sabouraud agar plates for incubation at 37°C. After 20 h the number of colonies in lung tissues (CFU/g) was also counted and calculated. For histopathological examination, the lung tissue sections from mice of each group at 72 h post-infection were dissected, fixed in 10% (v/v) formaldehyde and stained with hematoxylin and eosin (H&E) and Periodic Acid-Schiff (PAS), respectively. Images were observed and captured under light microscopy (OLYMPUS BX51). The area of the sites that contained fungal hyphae with inflammatory cell infiltration was determined by use of Image-Pro Express (Version 6.0, Media Cybernetics). Briefly, the software was utilized to outline lesions and the outlined area was determined for 8 sites of infection for each strain tested (61). In addition, we determined the number of lesions per lung for 4 mice that had been infected with B5233 wild-type (WT), Δ*pld*, or Δ*pldc* strains, respectively (61).
Pre-treatment of *A. fumigatus* Conidia with Chemical Inhibitors

Samples of $2 \times 10^5$ conidia of different *A. fumigatus* strains were pre-treated in 100 μl of DMEM in the presence of 1 μl DMSO, 1% 1-butanol, 4 nM VU0359595 (mammalian PLD1-specific inhibitor), 400 nM VU0285655-1 (PLD2-specific inhibitor), or both 4 nM PLD1 inhibitor and 400 nM PLD2 inhibitor. After 4 h at 37°C, the conidia were washed three times with PBS for a subsequent internalization and germination assay.

Statistical Analysis

Data shown in the Figures are either from a representative experiment or presented as mean ± standard error (s. e.) of 3–4 independent experiments performed in triplicate. For experiments comparing groups, single-factor analysis of variance (ANOVA) was performed and Student’s paired t-test was performed between two groups. Survival curves were analyzed using the log rank test. The values of $P < 0.05$ were considered statistically significant.

Results

1. Construction of Δ*pld* and *pldc* Strains of *A. fumigatus*

   To disrupt the *pld* gene using homologous recombination, a binary plasmid pDHt/Δ*pld::hph* with a hygromycin resistance gene (*hph*) and 5’ and 3’ flanking sequences of *pld* was first constructed for homologous recombination (Table S1, Figure S3). As *Agrobacterium tumefaciens* is able to transfer a fragment of DNA between the left and right borders of its tumor-inducing plasmid to the *A. fumigatus* genome at a high homologous recombination rate (62), it was used in this study to transfer pDHt/Δ*pld::hph* to *A. fumigatus* B5233. Overall, approximately 75% of the transformants included the *hph* locus but no detectable *pld* loci, as shown in Figure S3B. To complement the *pld* defect in the Δ*pld* strain, a PCR product containing the entire B5233 *pld* locus with an additional 1.2 kb of 5’ and 3’ flanking sequences and a 2.7-kb bleomycin resistance gene was transformed into the recipient Δ*pld* strain (Figure S3C). The gene deletion and complementation were confirmed by Southern blot. As shown in Figure S3D, *A. fumigatus* B5233 wild-type strain presented a 4.0-kb band; meanwhile, the *pld*-deleted strain exhibited a band at this position of 2.3 kb, and the complemented strain had two blot bands, one at 2.3 kb and the other at 7.6 kb (43). A randomly-selected *pld*-deleted strain and a complemented strain were designated as Δ*pld* and *pldc*, respectively, and used throughout this study (51).

2. Disruption of the *pld* Gene Reduces both Intracellular and Extracellular PLD Activities of *A. fumigatus*

   As it remains not clearly determined whether *A. fumigatus* secretes PLD outside the mycelium (14), we tested both the intracellular and extracellular PLD activities of the *A. fumigatus* B5233 wild-type, the Δ*pld* and the *pldc* strains. As shown in Figure 1A, the intracellular PLD activity...
of Δpld swollen conidia that had germinated for 4 h was significantly lower (~56% decrease) than that of wild-type swollen conidia. Importantly, upon complementation of the pld gene, intracellular PLD activity was fully restored in the pldc strain (Figure 1A). The intracellular PLD activities in the hyphae of conidia that had germinated for 8 h were obviously higher than that of the swollen conidia that had germinated for 4 h, and they were lowered by disruption of pld gene as well (Figure 1A). On the other hand, the extracellular PLD activity of Δpld in the supernatants of the A. fumigatus cultures of conidia germinated for 4 h or 8 h, respectively, exhibited a striking decrease (~60%) compared to the activities of the wild-type and pldc strains (Figure 1B). The extracellular PLD activities in different strains increased along with the germination as well (Figure 1B). These results indicate that A. fumigatus secretes the PLD enzyme, and disruption of the pld gene in A. fumigatus reduces both intracellular and extracellular PLD activities.

Figure 1. Pld gene disruption inhibits both intra- and extracellular PLD activities of A. fumigatus.
Resting conidia from A. fumigatus B5233 wild-type (WT), Δpld and pldc strains were inoculated and cultured in liquid Sabouraud medium. After 4 h and 8 h, the swollen conidia and hyphae were harvested by centrifugation, respectively. Intracellular PLD activity in swollen conidia or hyphae (A) and extracellular PLD activity in culture medium (B) were measured by an Amplex Red reagent-based assay. Data shown here are the mean ± s.e. (n= 3-4). Depicted are the differences in fluorescence intensity between the wild-type and pld gene mutant strains. *P < 0.05.

3. Disruption of the pld Gene Does Not Affect Conidial Morphological Characteristics, Hyphal Growth and Biofilm Formation of A. fumigatus
As the PLD signal is associated with cell shape changes in mammalian cells and other fungi (14,27,55), we studied potential morphological alteration in A. fumigatus induced upon the pld gene disruption. Compared to the wild-type and the pldc strains cultured on AMM plates (Figure 2A), Δpld did not exhibit any morphological changes, including the pigmentation
of colonies. Similar results were also observed on the Lecithine medium plates that contain lecithin as the sole carbon source (data not shown). The hyphal growth rate of the Δpld mutant were indistinguishable from those of the wild-type and the pldc strains on AMM, Sabouraud and Lecithin medium plates at 37°C (Figure 2B) and 28°C (data not shown). No differences were found in the biofilm density under static culture among the Δpld, the wild-type and the pldc strains (Figure 2C). Taken together, the results suggest that the pld gene of A. fumigatus plays a minor role in its morphology and growth.

Figure 2. Disruption of the pld gene does not alter the conidia morphological characteristics, hyphae growth and biofilm formation.

Resting conidia from the wild-type (WT) strain, the Δpld strain and the pldc strains of A. fumigatus B5233 were cultured on AMM, Sabouraud and Lecithine plates at 37°C, respectively. (A) At 24, 36 and 72 h, the colonies of different strains were compared by microscopy. Pictures shown are characteristic colonies on AMM plates for the independent experiments. (B) Every 12 h, the diameter of each colony of different strains were measured. The hyphal growth rates were calculated on the three media types. (C) Resting conidia from the wild-type (WT) strain, the Δpld strain, the pldc strains of A. fumigatus B5233 were inoculated into 96-well plates and cultured for 24 h at 37°C, and biofilm density was determined by measuring absorbance using a microplate reader. Data shown here are the mean ± s. e. (n = 3~4). Depicted are the differences in the hyphal growth rate or biofilm density absorbance between the wild-type and pld gene mutant strain. * P < 0.05.
4. Disruption of the pld Gene Reduces *A. fumigatus* Internalization but has No Effect on Adhesion on A549 Cells and Phagocytosis by Macrophages

Internalization into non-phagocytic epithelial cells is an important mechanism for *A. fumigatus* to avoid host immune attack and spread its infection, while the phagocytosis of *A. fumigatus* conidia by host macrophages represents a critical innate immune reaction against fungal infection. Therefore, we studied the impact of the *pld* gene disruption in *A. fumigatus* on the internalization into lung epithelial cells and phagocytosis by macrophages *in vitro*. As shown in Figure 3A, the internalization of ∆*pld* into A549 cells decreased significantly compared to that of the wild-type strain (0.60 ± 0.07% versus 1.24 ± 0.05%; *P* < 0.05), whereas the *pldc* strain reconstituted the internalization level to that of the wild-type (1.22 ± 0.07%). By contrast, the ∆*pld* mutant was phagocytosed by J774 macrophages as efficiently as the wild-type and *pldc* strains (Figure 3B). Next we studied whether the reduced internalization of ∆*pld* into epithelial cells may be due to a defect of its adhesion on A549 epithelial cells. As shown in Figure 3C, there was no significant difference in the adhesion of conidia and hyphae to A549 cells between the ∆*pld* and wild-type strains. These results indicate that *A. fumigatus* PLD may improve internalization into lung epithelial cells without affecting the conidial adherence to epithelial cells.

![Figure 3](image-url)

**Figure 3.** *Pld* gene disruption suppresses the internalization of *A. fumigatus* into A549 cells, but does not alter the phagocytosis of *A. fumigatus* by J774 macrophages and the adhesion of *A. fumigatus* on A549 cells.

A549 cells (A) and J774 macrophages (B) were infected with the wild-type (WT) strain, the ∆*pld* strain and the *pldc* strain of *A. fumigatus* B5233 at an MOI of 10, respectively. The internalization of *A. fumigatus* into A549 cells and phagocytosis by J774 macrophages were analyzed by the nystatin protection assay. (C) A549 cells were incubated with conidia or hyphae (germination for 8 h) from the wild-type (WT) strain, the ∆*pld* strain and the *pldc* strain of *A. fumigatus* B5233 at 37°C for 30 min and the adhesion on A549 cells was measured. Data shown here are the means ± s.e. (*n* = 3~4). Depicted are the differences in the percentage of total inoculated conidia between the wild-type and the *pld* gene mutants. “*”, *P* < 0.05.
5. Pre-treatment of *A. fumigatus* Conidia with PLD Inhibitors Suppresses their Internalization into A549 Cells

The significant decreases in PLD activity and internalization of ∆*pld* mutant prompted us to speculate that pharmacological inhibition of PLD activity of *A. fumigatus* may also suppress its internalization ability into lung epithelial cells. To test this hypothesis, we pre-treated the conidia of wild-type *A. fumigatus* with the non-specific PLD inhibitor 1-butanol and isoforms-specific mammalian PLD inhibitors. As shown in Figure 4A, the conidia pre-treated with

![Figure 4. Pharmacological PLD inhibitors reduce the internalization of *A. fumigatus* into A549 cells.](image)

Conidia of the wild-type strain *A. fumigatus* B5233 were pretreated for 4 h with 1×PBS (Control), 1% 1-butanol, 4 nM of the mammalian PLD1-specific inhibitor VU0359595, 400 nM of the mammalian PLD2-specific inhibitor VU0285655-1 or their indicated combination, respectively. Thereafter, the internalization of *A. fumigatus* into A549 cells (A) and the phagocytosis of *A. fumigatus* into J774 macrophages (B) were analyzed by the nystatin protection assay. The conidial survival rate (C) and the germination rate (D) of *A. fumigatus* after pretreatment with the PLD inhibitors were also analyzed. Data shown here are the means ± s.e. (n = 3~4). Depicted are the differences in the percentage of total inoculated conidia between the wild-type and the PLD inhibitor-pretreated group. ***,* P < 0.05.
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either 1% 1-butanol or 4 nM mammalian PLD1-specific inhibitor VU0359595 internalized less efficiently into A549 cells compared to the control group pre-treated with PBS. This result was consistent with the effects of pld gene disruption on A. fumigatus internalization. Pre-treatment with the mammalian PLD2-specific inhibitor VU0285655-1 had no effect on A. fumigatus internalization into A549 cells. These data gave a hint that the structure and function of A. fumigatus PLD might be more similar to mammalian PLD1 but not PLD2. Moreover, the phagocytosis of conidia by J774 macrophage cells was not affected by pre-treatment with any of the inhibitors (Figure 4B), indicating again that the pld gene function of A. fumigatus does not interfere with macrophage phagocytosis of A. fumigatus. The inhibitor concentrations used were shown not to compromise the fungal cell viability and conidial germination (Figure 4C and 4D). These findings indicate that pharmacological inhibition of PLD function in A. fumigatus may impair its internalization into lung epithelial cells.

6. Impact of Phosphatidic Acid on the Internalization of Δpld into A549 Cells

Next, we asked whether the PLD product, PA, regulates the internalization of A. fumigatus into A549 cells (71). As shown in Figure 5, the presence of PA significantly promoted the internalization of wild-type and Δpld strains into A549 cells in a dose-dependent fashion, peaking at a concentration of 100 µM. Intriguingly, PA did not cause any significant difference on internalization rates between wild-type and Δpld strains with the exception that at a concentration of 100 µM PA was able to restore the internalization of the Δpld mutant to a level of the wild-type strain. Although an effect of exogenous PA on host cellular signal regulation could not be excluded, our data indicate that extracellular addition of PA can improve the internalization of A. fumigatus into A549 cells.

Figure 5. Impact of phosphatidic acid on the internalization of Δpld into A549 cells.

A549 cells were infected with the resting conidia from the wild-type (WT) strain and the Δpld strain of A. fumigatus B5233 at an MOI of 10 at 37°C for 4 h in the presence of the indicated phosphatidic acid concentrations. The internalization of A. fumigatus into A549 cells was analyzed by the nystatin protection assay. Data shown are the means ± s.e. (n = 3–4). Depicted are the differences in the percentage of total inoculated conidia between the wild-type strain and Δpld strain. ***, P < 0.05.
7. PLD is Required for the Virulence of *A. fumigatus* in Mice Immunosuppressed with Hydrocortisone Acetate but not with Cyclophosphamide.

To determine the contribution of PLD to the virulence of *A. fumigatus*, freshly harvested conidia from the wild-type, the Δ*pld* and the *pldc* *A. fumigatus* B5233 strains were inoculated into two groups of BALB/c mice that were immunosuppressed either by hydrocortisone acetate or cyclophosphamide. Mice mortality was monitored for 16 days post-inoculation. Mice immunosuppressed with hydrocortisone acetate possessed a clear difference in the survival rates between the groups infected with the wild-type and Δ*pld* strains (*P* = 0.009) (Figure 6A). Nearly all wild-type or *pldc*-infected mice (15/16) died within a week, whereas only 60% of the Δ*pld*-infected mice died in the same period. At day 16 post-infection, approximately 25% of the Δ*pld*-infected mice were still alive. At 72 h post-infection, the number of *A. fumigatus* in lung lavage fluid from the Δ*pld*-infected mice was significantly lower than that of wild-type or *pldc*-infected mice (Figure 6B). Similarly, a significant difference of fungal burden in lung tissue homogenates was also found between the wild-type or Δ*pld*-infected groups (Figure 6C). By contrast, mice immunosuppressed with cyclophosphamide possessed no significant difference between the mortality of the wild-type and Δ*pld* strains (*P* = 0.272) (Figure 6D). In line with this result, no alterations in fungal burden in either lavage fluid or lung homogenates was found between the wild-type and Δ*pld* groups (Figure 6E and 6F). These results suggest that the virulence of the Δ*pld* strain compared to the wild-type strain was reduced in hydrocortisone acetate-treated, but not in cyclophosphamide-treated mice. Histological analysis of the lung sections confirmed the reduced virulence of the Δ*pld* strain in hydrocortisone acetate-immunosuppressed mice. As revealed by H&E stainings at 72 h post-infection, hyphae were seen primarily within the bronchioles of mice infected with the wild-type or *pldc* strains (Figure 7A and S5A). The lung sections from mice infected with the wild-type and *pldc* strains had multifocal necrosis and inflammatory cell infiltration, the typical characteristics of invasive pulmonary aspergillosis (63) (Figure 7A). Though hyphae were also observed in the lung tissues from mice infected with the Δ*pld* conidia, the fungal growth and the size and the number of lesions per lung were reduced significantly in Δ*pld*-infected mice relative to wild-type infected mice (Figure 7A, 7C, 7E and S5A). Importantly, the reduced Δ*pld*-induced pulmonary lesions in comparison to that of the wild-type strain was corroborated by the survival data (Figure 7A, and 6A). In mice immunosuppressed with with cyclophosphamide, hyphae and necrosis with inflammatory cell infiltration were also easily observed in the lung sections prepared 72 h post-infection with the wild-type, Δ*pld* and *pldc* strains (Figure 7B and S5B). In comparison to the infections with the wild-type and *pldc* strains, the fungal growth and the size and the number of lesions per lung caused by the Δ*pld* strain was not significantly altered (Figure 7D, 7F), which was also in line with the survival data (Figure 6D). Taken together, these results indicate that disruption of the *pld* gene significantly attenuates the virulence of *A. fumigatus* in mice immunosuppressed with hydrocortisone acetate, but not with cyclophosphamide.
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**Figure 6.** Disruption of the *pld* gene attenuates the virulence of *A. fumigatus* in hydrocortisone acetate-treated mice, but not in cyclophosphamide-treated mice.

BALB/c mice were immunosuppressed with either hydrocortisone acetate (A, B, C) or cyclophosphamide (D, E, F) and inoculated intranasally with 30 μl PBS-0.01% Tween 20 (control) or 30 μl PBS-0.01% Tween 20 containing 5×10⁵ conidia of *A. fumigatus* B5233 wild-type (WT) strain, the Δ*pld* strain, and the *pldc* strain, respectively. The Kaplan and Meier survival with log rank test was used to compare the survival rates between the groups (A and D). Data shown are from two independent experiments. Mice immunosuppressed with either hydrocortisone acetate (B and C) or cyclophosphamide (E and F) were inoculated intranasally with 30 μl PBS-0.01% Tween 20 (control) or 30 μl PBS-0.01% Tween 20 containing 5×10⁵ conidia of *A. fumigatus* B5233 wild-type (WT), Δ*pld*, or *pldc* strains. Mice were sacrificed at 72 h postinfection to measure the fungal burden in lung lavage fluid (B and E) and lung tissues (C and F).

Data shown here are the means ± s. e. (n = 3–4). Depicted are the differences in the percentage of total inoculated conidia between wild-type and pld gene mutant strain. **"**, P < 0.05.
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Discussion

Targeted gene disruption mediated by homologous recombination is a powerful tool that has been used to investigate the function of many genes and molecules in A. fumigatus virulence. However, none of the genes encoding extracellular phospholipases from A. fumigatus had previously been disrupted and evaluated for its role in virulence (1). In the present study, we successfully constructed a pld gene-deleted mutant of A. fumigatus B5233 using A. tumefaciens-mediated transformation (ATMT). In our experiments, approximately 75% of the obtained transformants exhibited pld gene disruption with mitotic stability. Although this percentage was not as high as that found in a previous study (62), the efficiency was enough to allow production and isolation of the pld deficient and complemented strains of A. fumigatus. Furthermore, the mechanism of PLD secretion is still poorly understood; however, the detection of PLD activity in the A. fumigatus culture medium and the obvious decrease of extracellular PLD activity of the Δpld strain compared to the wild-type strain are consistent with previous indications that A. fumigatus is able to secrete PLD (6).

In the present study, disruption of the pld gene in A. fumigatus had no influence on conidial morphology, hyphal growth rate and biofilm formation but it obviously attenuated virulence in hydrocortisone-treated mice. These findings are similar with the functional analysis of other genes of A. fumigatus, such as laeA (63), Sho1 (43), MpkA (67) and HdaA (40). In mammalian cells PLD interacts closely with the actin cytoskeleton to regulate cell shape changes. In S. cerevisiae, Spo14, a phosphatidylcholine-specific PLD, was found to be essential for sporulation (55), while C. albicans PLD1 was found to be required for dimorphic transition (45). However, further phylogenetic analysis showed that A. fumigatus PLD has low homology with Spo14 and C. albicans PLD1 (31). A. fumigatus PLD lacks two critical regulatory domains, the PX (Phox homology) and PH (pleckstrin homology) domains, at its NH₂-terminus, which
is significantly different from *A. fumigatus* PLD1, *C. albicans* PLD1, Spo14 and mammalian PLDs. Therefore, it is tempting to speculate that *A. fumigatus* PLD may not be able to interact with phosphoinositides, especially phosphatidylinositol-4,5-biphosphate (PIP$_2$) and phosphatidylinositol-3,4,5-trisphosphate (PIP$_3$), which are known cytoskeleton-modulator phosphor–inositides (13,56). From our findings, it could be deduced that *A. fumigatus* PLD, as one of three PLD isoforms in *A. fumigatus*, may be not the isoforms that is predominantly involved in morphological changes and mycelial growth. Disruption of other isoforms of PLD in *A. fumigatus*, such as *A. fumigatus* PLD1, is being pursued in future studies to test this hypothesis.

The internalization of many infectious particles (e.g. *Listeria monocytogenes*, *Candida albicans*, *Cryptococcus neoformans*) and nanoparticles into non-phagocytic cells is a deliberate interaction process between particles and its host (20). It is known that *A. fumigatus* conidia must cross the anatomical barriers of respiratory epithelial cells after inhalation to traverse tissues and cause invasive disease (47). However, little is known about the role of *A. fumigatus* PLD in this process. Compared to the wild-type strain, the Δpld strain exhibited a decrease in uptake by A549 cells, whereas in the pldc strain, this ability was fully restored, indicating that the activity of *A. fumigatus* PLD may be required to facilitate internalization of the conidia. However, the adherence of *A. fumigatus* to A549 cells was not affected by pld gene disruption, suggesting that *A. fumigatus* PLD probably plays only a minor role in this first interaction step. This observation was consistent with previous findings showing that several laminin- or galactofuranose-associated proteins may mediate the adhesion between *A. fumigatus* and epithelial cells (37). In contrast to internalization into epithelia, the phagocytosis of *A. fumigatus* by macrophages was not altered by either pld gene disruption or pre-treatment with PLD inhibitors in *A. fumigatus*. In the phagocytosis assay, we choose to quantify the germinated conidia in our experiments at 1 h post-infection, because it takes approximately 2 h for macrophages to kill *A. fumigatus* conidia via phagolysosome acidification (28). Although PLD activity has been closely associated with the intracellular survival of pathogens (44), the survival of *A. fumigatus* conidia in macrophages at 4 h post-infection was also not altered by pld gene disruption (data not shown). These results indicate that *A. fumigatus* PLD does not contribute to virulence by promoting phagocytosis by host macrophages and subsequent survival in the macrophages. As fungal phospholipases may regulate various host immune signals, such as inducing host cell-associated phospholipase A2 activation in *C. albicans* (65), the involvement of *A. fumigatus* PLD in other host immune responses of leukocytes, such as interleukin release or ROS responses, can currently not be excluded.

Interestingly, *A. fumigatus* PLD was involved in the virulence of *A. fumigatus* in hydrocortisone- but not cyclophosphamide-immunosuppressed mice. This *in vivo* effect of pld gene disruption on virulence was quite similar as that of other two important factors of *A. fumigatus*, gliotoxin (36) and DvrA (16), which had also shown significant interference on survival of hydrocortisone-immunosuppressed mice but not of cyclophosphamide-treated
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mice. These findings suggest that, like gliotoxin, the effect of PLD on *A. fumigatus* virulence may be also related to neutrophils, which would render PLD to be less important for *A. fumigatus* virulence in neutropenic mice that lack this interaction. Further investigations are needed to clarify the exact role of PLD on the interaction of *A. fumigatus* with lung epithelia or neutrophils *in vivo*.

In the present study chemical inhibitors of mammalian PLD decreased the internalization of wild-type conidia, while the internalization of Δ*pld* conidia was rescued by exogenous PA at the concentration of 100 µM. These results hinted that PLD may be rather evolutionarily conserved in many eukaryotic cells, at least in its HKD motif (18); meanwhile, the PLD enzymatic product PA may be a critical modulator of *A. fumigatus* internalization into lung epithelia. As an important second messenger in the cell, PA is a fusogenic lipid and can induce negative curvatures to promote membrane fission (33,57,69). Although the distribution of PA in the reaction system is still poorly understood, it is usually added in the concentrations of 50, 100 or and 200 µM to restore the cellular downregulation of PLD activity *for in vitro* studies (8,34,35). It is rather difficult to decipher here the rationale that the maximum of *A. fumigatus* internalization and rescue of the Δ*pld* strain internalization both occurred with PA at the concentration of 100 µM; however, this phenomenon was similar as our recent findings that too high PLD activity (more localized PA) may inhibit the internalization of a typical intracellular pathogen, *Listeria monocytogenes* into epithelial cells (23). These results might be explained as followed. The higher concentration of PA could induce host stress fiber formation so as to hinder the internalization (4,30,32,49). Likewise, localized PA concentration, which rises initially and then descend, correlates deliberately with phagocytic process during phagocytosis in macrophage. Thus, increased phosphatidic acid concentration may disturb this relationship and ultimately suppress *A. fumigatus* internalization (7,48). Interestingly, our recent data indicated that beta-1,3-glucan on the surface of germinated conidia is able to stimulate host cell PLD activity and this activation is important for the efficient internalization of *A. fumigatus* into A549 lung epithelial cells (24). It could be deduced that local PA production driven by PLD from *A. fumigatus* itself may also contribute to the internalization. Nevertheless, the exact role of PLD and PA in *A. fumigatus* internalization need to be further explored.

In summary, we demonstrated for the first time that PLD of *A. fumigatus* modulates the internalization of *A. fumigatus* into epithelial cells and may be a virulence factor of *A. fumigatus* in invasive aspergillosis under immunosuppression by corticosteroids.
Acknowledgements

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Supplemental materials

Table S1. Strains and plasmids used in the study.

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Table S2. Primers used in the study.

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Figure S3. Schematic map of pld gene deletion and complementation for the pld gene in A. fumigatus B5233. (A) pld locus in wild-type (WT) A. fumigatus B5233. White boxes indicate the 5’ and 3’ flanking sequences of the pld gene used for homologous recombination. (B) Δpld locus. The pld gene was deleted and replaced with the hygromycin resistance gene (hph). (C) pldc locus. The pld gene from wild-type A. fumigatus B5233 flanked by the bleomycin resistance gene (ble) was integrated into the flanking DNA of the Δpld strain to complement the deletion. (D) The genome of wild-type, Δpld and pldc strains of A. fumigatus B5233 were digested with XbaI and detected with a 454-bp probe by Southern blot. A representative southern blot from 3-4 experiments is shown.
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Figure S4. Deletion of the *pld* gene reduces lung fungal burden in hydrocortisone acetate-treated mice, but not in cyclophosphamide-treated mice.

BALB/c mice were immunosuppressed with either hydrocortisone acetate (A, C, E, G) or cyclophosphamide (B, D, F, H) and inoculated intranasally with 30 μl PBS-0.01% Tween 20 (control) or 30 μl PBS-0.01% Tween 20 containing $5 \times 10^5$ conidia of *A. fumigatus* B5233 wild-type (WT) strain, the Δ*pld* strain, and the *pldc* strain, respectively. Mice were sacrificed at 2 h and 120 h postinfection to measure the fungal burden in lung lavage fluid (A, B, E, F) and lung tissues (C, D, G, H). Data shown here are the means ± s.e. (n = 3~4). Depicted are the differences in the percentage of total inoculated conidia between wild-type and *pld* gene mutant strain. ***, P < 0.05.**
Figure S5. Deletion of the *pld* gene reduces the hyphae growth of *A. fumigatus* in Periodic Acid-Schiff (PAS) stain section in hydrocortisone acetate-treated mice, but not in cyclophosphamide-treated mice.

Mice immunosuppressed with either hydrocortisone acetate (A) or cyclophosphamide (B) were inoculated intranasally with 30 μl PBS-0.01% Tween 20 (control) or 30 μl PBS-0.01% Tween 20 containing $5 \times 10^5$ conidia of *A. fumigatus* B5233 wild-type (WT), Δ*pld*, or *pldc* strains. At 72 h postinfection the lung tissue sections from mice were dissected, fixed in 10% (v/v) formaldehyde, stained with PAS and observed under microscopy (OLYMPUS BX51). The red filaments indicated by black arrows are the hyphae of *A. fumigatus*. 
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References


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