

Surfactant protein D binding to *Aspergillus fumigatus* hyphae is calcineurin-sensitive

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Surfactant protein D (SP-D) plays a central role in pulmonary innate immune responses to microbes and allergens, often enhancing clearance of inhaled material. Although SP-D functions during bacterial and viral infections are well established, much less is known about its possible roles during invasive fungal infections. *Aspergillus fumigatus* is a prominent fungal pathogen in immunocompromised individuals, and can cause allergic or invasive aspergillosis. SP-D has been shown to be protective against both of these disease modalities. The moieties present on the fungal surface responsible for SP-D binding remain largely unclear, although cell wall 1,3- β -D-glucan is bound by SP-D in other fungal species. There is little information regarding the interaction of SP-D with *A. fumigatus* hyphae which are responsible for the invasive form of disease. Here, we show that SP-D binding to *A. fumigatus* hyphae is sensitive to the activity of the calcium-activated protein phosphatase calcineurin. Deletion of the catalytic subunit calcineurin A (Δ cnaA) or pharmacologic inhibition of calcineurin through FK506 abrogated SP-D binding. In contrast, SP-D binding to *Cryptococcus neoformans* was calcineurin-independent. Pharmacologic inhibition of *A. fumigatus* cell wall components by caspofungin (inhibits 1,3- β -D-glucan synthesis) and nikkomycin Z (inhibits chitin synthesis) increased SP-D binding to the wild-type strain. In contrast, SP-D binding increased in the Δ cnaA strain only after nikkomycin Z treatment. We conclude that SP-D binding to *A. fumigatus* hyphae is calcineurin-sensitive, presumably as a consequence of calcineurin's role in regulating production of key cell wall binding partners, such as 1,3- β -D-glucan. Elucidation of the interaction between lung innate immune factors and *A. fumigatus* could lead to the development of novel therapeutic interventions.

Keywords surfactant protein D, *Aspergillus fumigatus*, aspergillosis, hyphae, calcineurin

Introduction

Invasive aspergillosis (IA), caused largely by *Aspergillus fumigatus*, is a leading contributor to infectious mortality in immunocompromised patients [1,2]. Despite the use of newer antifungal agents, current IA therapy has a dismal 40–50% treatment success rate [3,4]. IA occurs when *A. fumigatus* conidia are inhaled into the lungs of immunocompromised patients and germinate into hyphae,

the critical growing and invading form of the fungus [5,6]. Paramount to the successful clearance of *A. fumigatus* hyphae in patients is host recognition of the invading fungus.

There is increasing evidence that lung surfactant protein D (SP-D) has a protective role in pulmonary defense against pathogens [7]. Mice deficient in SP-D have increased susceptibility to various microorganisms, including *Pseudomonas aeruginosa* and respiratory syncytial virus [7–10]. Furthermore, administration of exogenous SP-D was recently shown to be protective in a murine model of invasive pulmonary aspergillosis [11]. SP-D belongs to a group of C-type lectins called collectins, which have multiple and varied roles in host defense [12]. Collectins interact with carbohydrate

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structures present on the surfaces of a wide range of pathogens, including viruses, bacteria, and fungi, via carbohydrate recognition domains (CRDs) and enhance phagocytosis and killing by neutrophils and macrophages [13]. Therefore, appropriate pathogen recognition by SP-D represents an important first line of defense during host innate immune responses.

Calcineurin is a conserved serine-threonine-specific Ca^{2+} -calmodulin-activated protein phosphatase important in mediating cell stress responses [14]. It is a heterodimer composed of a catalytic A and a regulatory B subunit and, upon mobilization of calcium stores, the catalytic A subunit is bound by Ca^{2+} -calmodulin [15]. Calcineurin is the target of the immunosuppressants cyclosporine A (CsA) and tacrolimus (FK506) [16]. Although inhibiting human calcineurin has revolutionized modern transplantation through its powerful immunosuppressive role, calcineurin is also critical in fungal virulence [17].

We have previously shown that calcineurin is important in the formation of *A. fumigatus* hyphae and in the fungus' growth, invasion, and pathogenicity [18]. Our further work supports the link between the calcineurin pathway and the *A. fumigatus* cell wall, as we demonstrated significantly decreased cell wall 1,3- β -D-glucan content following calcineurin inhibition in the $\Delta cnaA$ mutant or the wild-type strain treated with FK506 [19]. Intriguingly, Lamaris *et al.* and Hohl *et al.* each recently described 1,3- β -D-glucan unmasking in hyphae preincubated with caspofungin, leading to more potent immune cell recognition [20,21]. Although hyphae are important for *in vivo* growth, it is unclear if calcineurin is required for host recognition of *A. fumigatus*. Here, we investigated SP-D binding to *A. fumigatus* as a measure of host immune recognition and sought to determine the role of the calcineurin pathway in host recognition through its ability to control products of putative cell wall binding structures.

Materials and methods

Isolation and fluorescent labeling of recombinant SP-D

Recombinant SP-D was isolated from Chinese hamster ovary cells expressing a clone of full-length rat SP-D, then purified using maltose affinity chromatography, and stored at 4°C in 5 mM Tris buffer pH 7.8 containing 2 mM EDTA as previously described [22]. Rat recombinant SP-D was fluorescently labeled using an Alexa Fluor 488 protein-labeling kit (Invitrogen, Carlsbad, CA). Alexa dyes were selected because their magnitude of fluorescence is constant from pH 4–10 and photobleaching occurs at a much lower level than comparable fluorescent

dyes. SP-D was dialyzed against PBS without Ca^{2+} and Mg^{2+} before and after labeling. In addition, the pH was raised for the labeling reaction by dialyzing SP-D against 0.1 M sodium bicarbonate pH 8.3 for 3 h prior to labeling. Protein concentrations were assessed using the BCA (bicinchoninic acid) reagents according to the manufacturer's instructions (Pierce, Rockford, IL). All proteins had a degree of labeling (DOL) efficiency of 5–15:1 (DOL, dye:protein ratio). Functionality of labeled SP-D was assessed by its ability to bind to *E. coli* HB101 [23,24].

A. fumigatus growth and preparation for flow cytometry

Freshly harvested conidia of *A. fumigatus* wild-type (AF293), $\Delta cnaA$, and $\Delta cnaA$ + *cnaA* strains previously described [18] were utilized in these studies. For conidial experiments, the fresh conidia were incubated with 1 $\mu\text{g}/\text{ml}$ AF448-labeled SP-D for 1 h at 37°C. For hyphal experiments, each strain was grown in 10 ml RPMI-1640 media with shaking at 200 rpm at 37°C for the indicated time periods (4, 8, 12 and 24 h). After growth, strains were prepared for labeling and flow cytometry by sonicating for 60 sec on ice to break up hyphae into fragments amenable to flow cytometry analysis. Sonicated strains were centrifuged at 1500 *g* for 5 min. The supernatant was removed and strains were washed twice in PBS + 0.9 mM CaCl_2 + 0.1% BSA and resuspended in the same buffer. Strains were counted using a hemocytometer and normalized to a concentration of 1×10^6 conidia or hyphal fragments per ml. Hyphal fragments were normalized based on number of fragments, as well as fragment size by assessing hyphal length using the hemocytometer, with hyphal length determined in relation to the hemocytometer quadrants for which the length was scored as equivalent to $\frac{1}{8}$, $\frac{1}{4}$, $\frac{1}{2}$, etc. of the quadrant. This technique enabled us to normalize inherent differences in fungal mass between strains and provided consistent results in three independent experiments, such that comparisons were appropriate.

Cryptococcus neoformans growth and preparation for flow cytometry

C. neoformans var. *grubii* strain H99 (wild-type, serotype A, mating type alpha) and the *cna1* Δ and *cnb1* Δ yeast strains, representing deletions of the calcineurin A and B subunits, respectively, were utilized [25]. The test strains were incubated in YPD broth with shaking for 18–20 h at 30°C, harvested, washed three times with PBS + 0.9 mM CaCl_2 + 0.1% BSA, and counted with a hemocytometer to determine cell concentrations.

Antifungal treatment of *A. fumigatus*

A. fumigatus strains were treated with caspofungin (1 µg/ml) to inhibit 1,3-β-D-glucan synthesis or FK506 (0.2 µg/ml) to inhibit calcineurin at clinical concentrations, as well as nikkomycin Z (16 µg/ml) to ensure chitin synthesis inhibition. Caspofungin was generously obtained from Merck, Inc., nikkomycin Z from Dr Richard Hector, and FK506 from the clinical pharmacy at Duke University Medical Center. Drugs were added to cultures at the time of inoculation and remained for the entire time period of the culture.

Assessment of SP-D binding

SP-D binding to *A. fumigatus* strains was assessed at a concentration of 1 µg/ml in PBS + 0.9 mM CaCl₂ + 0.1% BSA to block nonspecific binding. A 100 µl of each 1×10^6 stock (1×10^5 conidia or hyphal fragments) was incubated for 1 h with 1 µg/ml AF488-labeled SP-D in a final volume of 300 µl. Negative controls were maintained in buffer alone. Samples were incubated for 1 h at 37°C with shaking in the dark and then removed to 5 ml polystyrene round bottom FACs tubes (BD Biosciences Discovery Labware, Franklin Lakes, NJ) for analysis. Results were obtained using a LSR II Flow cytometry system (BD Biosciences, San Jose, CA). Analysis of results was performed using the FlowJo software (Treestar Inc. Ashland, OR). At least 5000 counts were assessed for hyphal fragments and at least 10,000 counts were assessed for conidia and 4 h cultures. Data represent experiments that were performed at least three times with similar results. *C. neoformans* yeast cells were assessed for SP-D binding as described above for *A. fumigatus*, except that 0.1 µg/ml of SP-D was used for all binding assays. We have previously established that 0.1 µg/ml of SP-D is sufficient to detect binding to *C. neoformans* cells [26].

Light and fluorescence microscopy

Microscopy was performed using a Zeiss Axioskop 2 Plus fluorescent microscope (Thornwood, NY) with an attached AxioCam MRM digital camera. All images were acquired at a fixed exposure.

Statistics

Data were analyzed using the two-way unpaired Student's *t*-test or Single Factor Analysis of Variance (ANOVA) as appropriate. All statistics were performed using the Microsoft Excel software package. Analyses with a resultant $P < 0.05$ were considered as statistically significant.

Results

SP-D binding to the *A. fumigatus* $\Delta cnaA$ strain is decreased compared to the wild-type

When the binding of SP-D to wild-type, $\Delta cnaA$, and complemented $\Delta cnaA + cnaA$ strains was assessed by flow cytometry, we discovered a role for calcineurin in SP-D binding. A time course was performed to examine SP-D binding to the three *A. fumigatus* strains at specific growth intervals (0, 4, 8, 12, and 24 h of incubation) to encompass important hyphal developmental landmarks such as unswollen conidia, swollen conidia, early germination, later germination, and full hyphal growth, respectively. Although SP-D binding to conidia (0 h growth) and hyphae grown for 4 h was similar for all strains assessed, significant differences became apparent after 8 h of growth (Fig. 1A). At this time point where hyphae were clearly present, the $\Delta cnaA$ strain demonstrated a significant reduction in SP-D binding compared to wild-type and $\Delta cnaA + cnaA$ strains, and these differences were maintained after 24 h of hyphal growth (Fig. 1A). All results were quantitatively assessed by flow cytometry and qualitatively confirmed by fluorescence microscopy (Fig. 1B). Interestingly, this binding pattern was not observed for *C. neoformans*, as the *C. neoformans* $cna1\Delta$ and $cnb1\Delta$ strains lacking the calcineurin A catalytic and calcineurin B regulatory subunits, respectively, demonstrated equivalent SP-D binding when compared to wild-type (H99) (Fig. 1C). SP-D binding to the *A. fumigatus* $\Delta cnaA$ hyphae grown for 24 h, which was also significantly decreased ($P < 0.05$) compared to the wild-type and $\Delta cnaA$ mutant + $cnaA$ strains (Fig. 1A), was examined to confirm that the effect observed at 8 and 12 h remained consistent for longer periods of hyphal growth. Sonication of hyphae grown for 24 h proved to be difficult, as previously indicated [27]. However, in numerous experiments conducted to exclude artifacts that could result from inconsistencies in the size of the hyphal fragments, none of the strains demonstrated any increase in agglutination, as assessed by the flow cytometry forward scatter profiles (data not shown) upon the addition of SP-D. Thus, we are confident there is a decreased SP-D binding to the $\Delta cnaA$ strain that is evident after hyphal growth begins and continues throughout growth.

Sonication marginally increases SP-D binding to *A. fumigatus* hyphae

Sonication was used to break hyphae into fragments to be able to utilize flow cytometry for our analysis. However, we wanted to verify that the sonication process itself did not alter our results by exposure of ligands that would typically be unavailable for SP-D binding. To examine

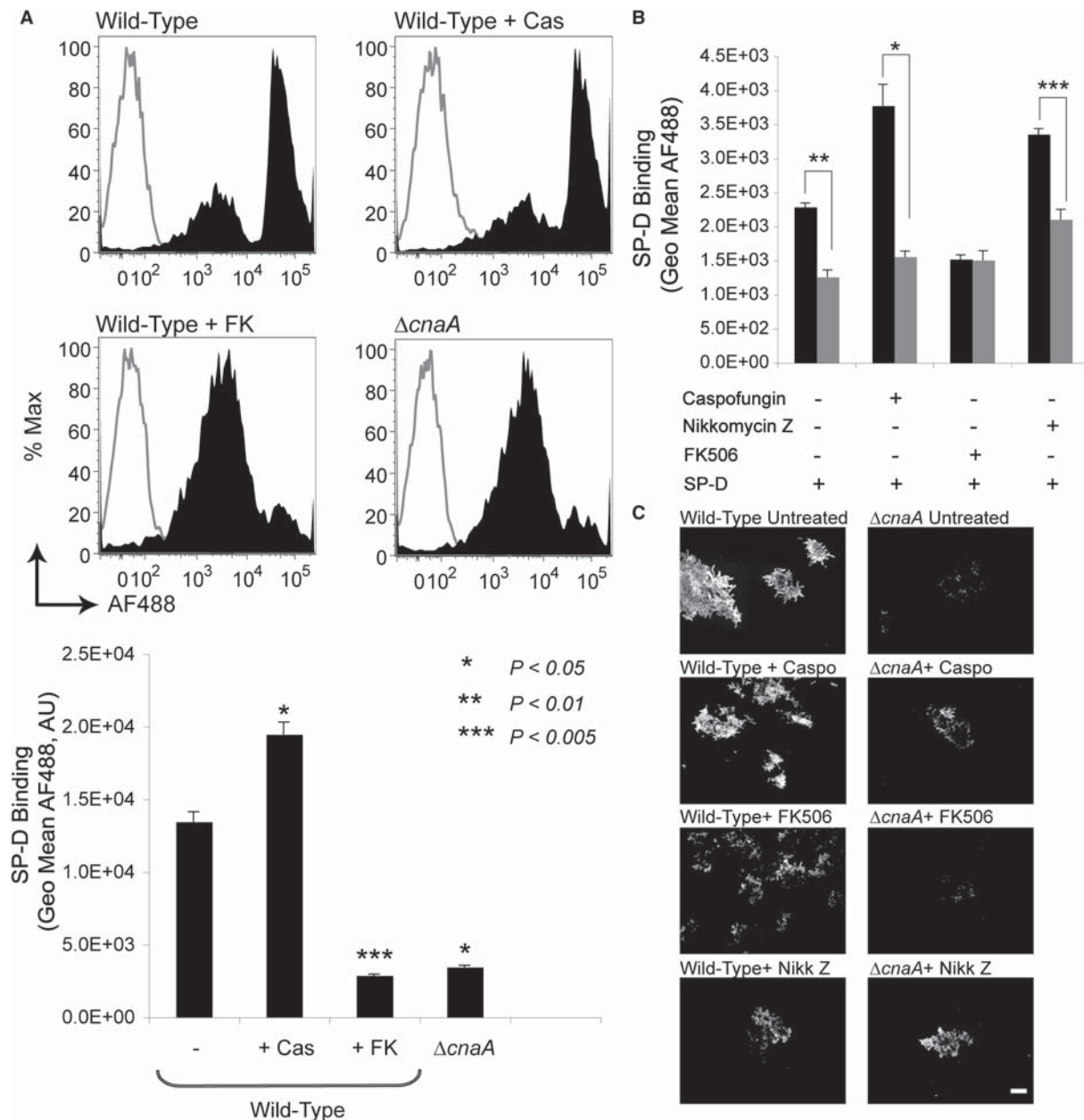


Fig. 1 Decreased SP-D binding to $\Delta cnaA$ becomes evident at 8 h post-inoculation. (A) Quantification of SP-D binding at the indicated time points post-inoculation by flow cytometry. Time point zero reflects SP-D binding to the conidia of the respective strains. Black filled bar = wild-type; grey filled bar = $\Delta cnaA$ mutant; grey filled bar with black cross-hatch = $\Delta cnaA$ mutant + *cnaA* A. AF488, Alexa Fluor 488 labeled SP-D binding detected by flow cytometry presented in arbitrary units (AU). (* $P < 0.05$, # $P < 0.001$ compared to wild-type). (B) Fluorescence microscopy showing SP-D binding at the indicated time points post-inoculation. Scale bar, 10 μ m. (C) SP-D binding to *C. neoformans* calcineurin mutants *cna1* Δ and *cnb1* Δ and the background wild-type control strain H99 was similar. %Max, Percent Maximum Fluorescence.

whether sonication of the hyphae affected the results, SP-D binding to both sonicated and unsonicated hyphae was assessed by fluorescence microscopy. Sonication did not change the pattern of SP-D binding (data not shown), although it may incrementally increase binding to the $\Delta cnaA$ strain. We predicted such an increase was likely, and postulate that sonication resulted in the exposure of

normally masked moieties that are recognized by SP-D. Yet, the differences in SP-D binding between sonicated wild-type and $\Delta cnaA$ were still statistically significant (data not shown), lending further support to our assertion that SP-D binding to *A. fumigatus* hyphae is calcineurin-sensitive. Therefore, we concluded that the $\Delta cnaA$ mutant exhibits impaired SP-D binding.

1,3-β-D-glucan and chitin synthesis inhibitor treatment of A. fumigatus increases SP-D binding in a calcineurin-sensitive manner

Strains were then incubated with caspofungin (1 µg/ml), FK506 (0.2 µg/ml), or nikkomycin Z (16 µg/ml) to selectively inhibit cell wall components, followed by examination of subsequent SP-D binding by flow cytometry. 1,3-β-D-glucan and chitin synthesis inhibitor treatment significantly increased SP-D binding ($P < 0.005$) to the wild-type strain (Fig. 2B), while calcineurin inhibitor (FK506) treatment decreased binding similar to the untreated $\Delta cnaA$ strain. However, caspofungin treatment did not increase SP-D binding to the $\Delta cnaA$ strain, whereas nikkomycin Z treatment did lead to a significant ($P < 0.005$) increase in binding (Fig. 2).

Discussion

Since the respiratory tract is the major portal of entry for the agents involved in IA, an understanding of the roles of lung innate immune mechanism mediators, such as SP-D, is important in elucidating host responses to this clinically important interaction. Previous studies have demonstrated that SP-D binds to *A. fumigatus* conidia in a calcium and carbohydrate-dependent manner [13,28,29]. It is thought that SP-D mediates host defense by binding carbohydrates on the surface of *A. fumigatus*, but the specific polysaccharides recognized remain unknown. Prior studies showing that binding of SP-D to *A. fumigatus* is inhibited by maltose are consistent with the possibility that the CRD regions of SP-D interact with carbohydrate structures on the *A. fumigatus* conidial cell wall [29]. A similar carbohydrate selectivity has been shown with SP-D and *C. neoformans* [30].

In our study, the hyphae from $\Delta cnaA$ or wild-type *A. fumigatus* strains treated with FK506 to inhibit calcineurin exhibited significantly decreased SP-D binding compared to the untreated wild-type strain. These results suggest that SP-D binding and immune recognition are at least partly calcineurin-dependent, as binding was decreased by both genetic and pharmacologic inhibition of calcineurin. However, there was no difference between SP-D binding to the conidia of $\Delta cnaA$ and wild-type *A. fumigatus* strains, suggesting that the differences may be solely hyphal-related given the substantial differences in hyphal morphology and length seen between the $\Delta cnaA$ mutant and wild-type *A. fumigatus* strain [18]. We postulate that the observed differences are a consequence of the downregulation of calcineurin-dependent cell wall components such as 1,3-β-D-glucan [31], as differences in fungal length and clumping between strains and conditions was accounted for using a hemocytometer and sonication, respectively, in our experiments.

Based on the observed calcineurin-sensitive binding differences and previous carbohydrate links to SP-D binding [32–35], we hypothesized that the SP-D binding ligand is a cell wall component whose product is calcineurin-regulated. We have previously shown that the $\Delta cnaA$ mutant or the wild-type strain treated with FK506 to inhibit calcineurin has significantly decreased 1,3-β-D-glucan content [19], suggesting calcineurin is involved in coordinating cell wall synthesis. We have also demonstrated that chitin inhibition through nikkomycin Z treatment leads to a compensatory increase in 1,3-β-D-glucan content [36]. To confirm this, we selectively pharmacologically inhibited two main cell wall components: 1,3-β-D-glucan and chitin. Here we found that the $\Delta cnaA$ mutant or the wild-type strain treated with FK506 to inhibit calcineurin has significantly decreased 1,3-β-D-glucan content [19]. Calcineurin inhibition led to decreased SP-D binding, suggesting that the binding ligand is absent or significantly diminished in the $\Delta cnaA$ mutant or the wild-type strain treated with the pharmacologic calcineurin inhibitor FK506. However, both 1,3-β-D-glucan inhibition through caspofungin and chitin inhibition through nikkomycin Z led to increased SP-D binding to the wild-type strain. The increased SP-D binding observed following caspofungin treatment is suggestive of an SP-D binding partner other than 1,3-β-D-glucan that is exposed and/or upregulated following this treatment.

We speculate that removal of these cell wall components exposed a ligand for additional binding. It is unclear if the 1,3-β-D-glucan or chitin inhibition exposed greater amounts of each individual component, or if the decreased synthesis of each component simply led to an unprotected cell wall shell where an unidentified ligand and/or 1,3-β-D-glucan was freely exposed for greater SP-D binding. Recent studies have shown increased 1,3-β-D-glucan exposure on hyphae after treatment with caspofungin, with a resultant increase in host recognition [20,21]. Consistent with these observations, we showed that SP-D binding to caspofungin-treated wild-type hyphae was significantly increased compared to untreated controls. Regardless, caspofungin-mediated 1,3-β-D-glucan synthase inhibition in the background of the $\Delta cnaA$ strain with decreased baseline levels of 1,3-β-D-glucan led to no statistical increase in SP-D binding, suggesting that the increase in SP-D binding following this cell wall stress is calcineurin-sensitive. It is important to point out that pleiotropic effects are likely incurred by calcineurin deficiency, making it difficult to provide explicit conclusions regarding the cause of the observed differences in SP-D binding. Because SP-D binds to the surface of the fungus, we feel that the data support the absence of key SP-D binding partners as a consequence of the lack of calcineurin activity. As ample evidence is available showing that SP-D binds to

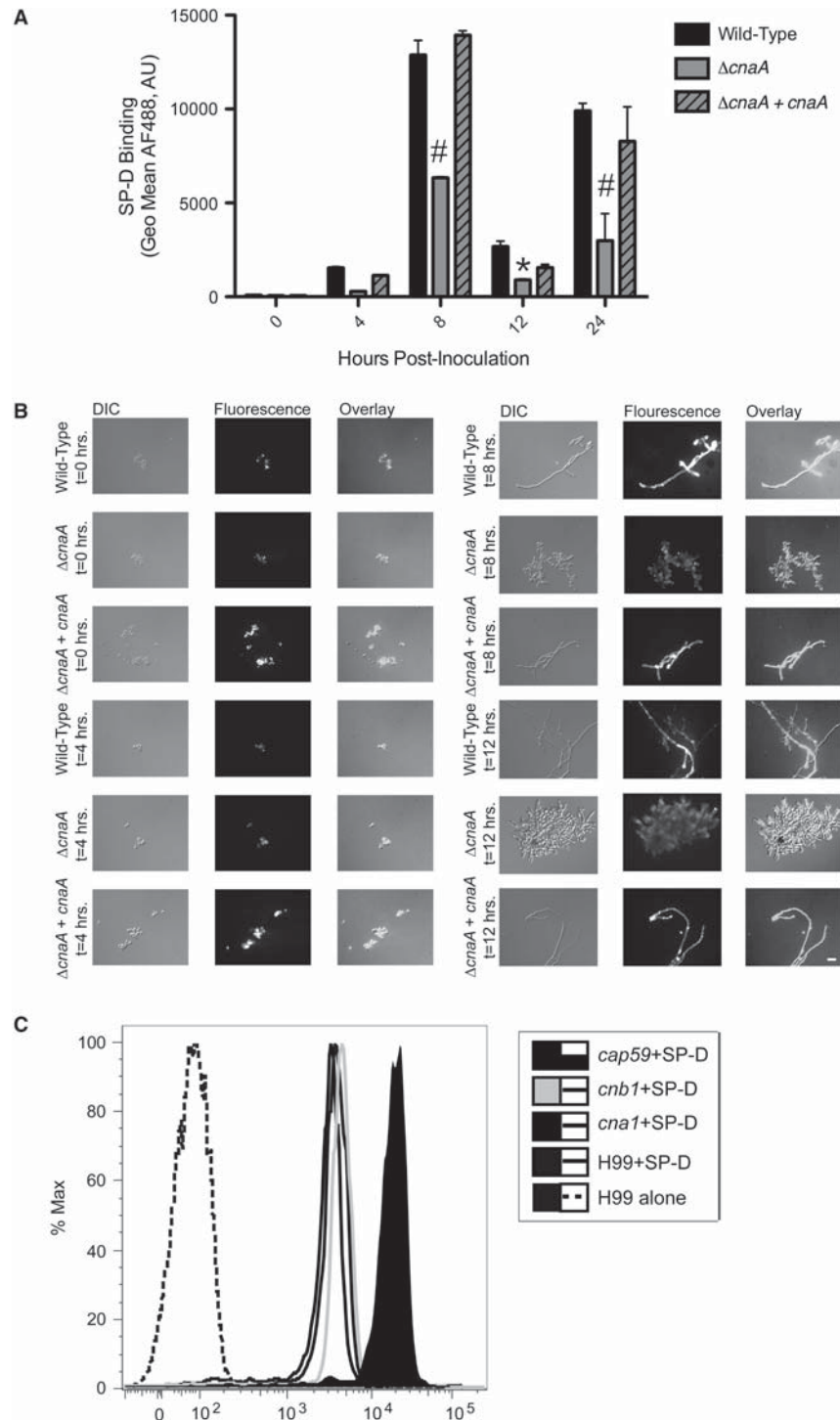


Fig. 2 Caspofungin and Nikkomycin Z treatment of wild-type *Aspergillus fumigatus* increases SP-D binding and FK506 treatment decreases binding, mimicking that observed for $\Delta cnaA$. Results of SP-D binding are shown for strains grown for 8 h. (A) Flow cytometry. Black line filled with white indicates negative (No SP-D) control; black line filled with black indicates the respective strain of *A. fumigatus* + SP-D. The results obtained by flow cytometry for SP-D binding to caspofungin and nikkomycin-treated wild-type hyphae were equivalent, and therefore only the data for the caspofungin treatment are presented. %Max, Percent Maximum Fluorescence. (B) Quantitation of SP-D binding by flow cytometry. Black bars indicate wild-type and grey bars indicate $\Delta cnaA$ mutant following treatment with either caspofungin, nikkomycin Z, or FK506. AF488, Alexa Fluor 488 labeled SP-D binding detected by flow cytometry presented in arbitrary units (AU). (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ compared to the untreated control). (C) Fluorescence microscopy showing SP-D binding to treated and untreated wild-type and $\Delta cnaA$ *A. fumigatus* strains. Scale bar, 20 μ m.

1,3- β -D-glucan [37] and we have previously demonstrated decreased 1,3- β -D-glucan content in the $\Delta cnaA$ strain [31], 1,3- β -D-glucan appears to be an important moiety recognized by SP-D on *A. fumigatus* hyphae. Any additional moiety(ies) on *A. fumigatus* recognized by SP-D remain(s) to be elucidated, but the present study demonstrates that this binding is affected by calcineurin expression.

Interestingly, SP-D binding to the *C. neoformans* *cna1* Δ and *cnb1* Δ mutants was equivalent to wild-type, suggesting potential differences in the carbohydrate moieties recognized by SP-D on the surface of *C. neoformans* compared to *A. fumigatus*. *A. fumigatus* lacks the polysaccharide-rich capsule that decorates the surface of *C. neoformans* yeast cells, and which serves as a major virulence factor that inhibits phagocytosis and SP-D binding [38,39]. *A. fumigatus* virulence is associated with hyphal growth, therefore recognition of hyphae by innate immune factors present in the lung, such as SP-D, may have important consequences for the host. For example, SP-D binding to 1,3- β -D-glucan on the surface of *Blastomyces dermatitidis* has been shown to inhibit the production of the pro-inflammatory cytokine tumor necrosis alpha, potentially alleviating an overzealous inflammatory response that could lead to irreversible damage to the delicate epithelial lining of the lung [40]. In light of these findings, differential binding patterns of SP-D to pathogens in the presence and absence of important virulence factors is of interest as it may provide insight into additional important innate immune recognition mechanisms. In support of this, we have demonstrated that SP-D binds to the acapsular *C. neoformans* strain, *cap59* Δ , to a significantly greater extent than to the wild-type strain (Fig. 1C), reaffirming the role of *C. neoformans* capsule in immune recognition [26,41].

SP-D binds to *A. fumigatus* conidia at concentrations comparable to physiological concentrations in the host [29], but there have been no previous studies to examine binding to growing hyphae. Infection with *A. fumigatus* begins with pulmonary inhalation of conidia, but the infection progresses when the conidia germinate into hyphae. Therefore, conidial adhesion is relevant for only the initial stage of disease. Hyphae are the clinically relevant form when determining antifungal therapy or possible adjunctive immune therapy. Consequently, in the present study, we sought to gain a better understanding of the interaction of SP-D with *A. fumigatus* hyphae. To achieve this goal, we utilized rat recombinant SP-D, which demonstrates high sequence homology and a similar structure and functional activity to human SP-D [42–46]. Furthermore, intranasal human and recombinant human SP-D has been shown to possess therapeutic efficacy in *Aspergillus*-infected mice [11], and conferred a survival benefit both alone and in conjunction with amphotericin B [13].

Elucidation of specific signaling pathways that modulate SP-D binding could have important consequences for clinical therapies targeting aspergillosis.

The *A. fumigatus* cell wall contains largely glucan, chitin, and galactomannan [47]. The fungal cell wall is at the crucial interface between the hyphae and the host and plays a critical role during fungal invasion. The cell wall also contains several receptor molecules for adhesion to host cells, and thus binding of SP-D to different carbohydrate structures could greatly influence *A. fumigatus* infection. SP-D binding has also been examined in *S. cerevisiae*, but the cell wall of *S. cerevisiae* differs considerably from that of *A. fumigatus*. Glucans are candidate SP-D binding moieties on the surface of *A. fumigatus* as the 1,6- β -glucan analog pustulan has been shown to effectively compete SP-D binding to *A. fumigatus* and *S. cerevisiae* [28], and both 1,3- β -D-glucan and 1,6- β -D-glucan have been shown to be important for SP-D binding to *B. dermatitidis* and *C. neoformans*, respectively [26,40].

This study is only the beginning of the explorations surrounding the mechanisms of host immune recognition and binding to *A. fumigatus* hyphae, and underscores the importance of future studies assessing the interactions of surfactant proteins with hyphae and the potential effect this has on disease progression and host defense. It seems clear that the calcineurin pathway of *A. fumigatus* plays a role in the context of SP-D-mediated host immune recognition, and interestingly this does not appear to be true for *C. neoformans*. The difference of a capsule in *C. neoformans* and the individual cell wall differences among the fungi offer several possible future directions for identifying the specific ligand(s) that SP-D binds to in these invasive fungal pathogens.

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