

## Antifungal activities of surfactant protein D in an environment closely mimicking the lung lining

Soledad R. Ordonez<sup>a</sup>, Martin van Eijk<sup>a</sup>, Natalia Escobar Salazar<sup>b</sup>, Hans de Cock<sup>b</sup>,  
Edwin J.A. Veldhuizen<sup>a</sup>, Henk P. Haagsman<sup>a,\*</sup>

<sup>a</sup> Department of Infectious Diseases and Immunology, Division Molecular Host Defence, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

<sup>b</sup> Microbiology & Institute of Biomembranes, Department of Biology, Utrecht University, Utrecht, the Netherlands

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### ABSTRACT

At the lung lining innate defenses protect our lungs against inhaled fungal cells that could pose a threat to our health. These defenses are comprised of mucociliary clearance, soluble effector molecules and roaming phagocytic cells, such as macrophages and neutrophils. How important each of these defenses is during fungal clearance depends on the specific fungal pathogen in question and on the stage of infection. In this study the localization and antifungal activity of the lung surfactant protein D (SP-D) was studied in an environment mimicking the lung lining. To this end Calu-3 cells were grown on an air-liquid interface allowing them to polarize and to produce mucus at their apical surface. Additionally, neutrophils were added to study their role in fungal clearance. Two fungal pathogens were used for these experiments: *Candida albicans* and *Aspergillus fumigatus*, both of clinical relevance. During fungal infection SP-D localized strongly to both fungal surfaces and stayed bound through the different stages of infection. Furthermore, SP-D decreased fungal adhesion to the epithelium and increased fungal clearance by neutrophils from the epithelial surface. These findings suggest that SP-D plays an important role at the different stages of pulmonary defense against fungal intruders.

### 1. Introduction

Fungal pathogens can cause life-threatening respiratory infections in immune-compromised individuals. Two well-studied organisms capable of causing such opportunistic infections are *Candida albicans* (*C. albicans*) and *Aspergillus fumigatus* (*A. fumigatus*). *C. albicans* yeasts are commonly found as commensals of mucosal surfaces but can cause opportunistic infections during immune suppression, as is the case for example for patients that receive treatment against AIDS (Stevens, 1990). *A. fumigatus*, another well-studied opportunistic pathogen, is the major cause of pneumonia in immune-compromised patients, often leading to disseminated infections (Walsh and Groll, 1999; Roilides and Simitopoulou, 2010). Furthermore, fungal infections are often persistent and not easy to treat mainly because they are eukaryotic, making it difficult to target them without affecting host cells. Currently there are only few antifungals available that can be used and there is an urgent need for alternatives.

The lung epithelium is coated with a stratified liquid layer (also known as the lung lining fluid or LLF). In the conductive airways, LLF together with ciliary movement and a plethora of secreted

antimicrobial molecules such as antimicrobial peptides, proteins and lipids, confer the first mechanisms of defense against inhaled microorganisms such as fungi (Ng et al., 2004; Berkebile and McCray, 2014). One of the proteins present throughout the lung lining and known to bind *C. albicans* and *A. fumigatus in vitro* is surfactant protein D (SP-D) (Madsen et al., 2000; Herias et al., 2007). SP-D belongs to the family of so-called ‘collectins’, multimeric glycoproteins that are characterized by the presence of a C-terminal Ca<sup>2+</sup>-dependent carbohydrate recognition domain (CRD), a neck region, and an N-terminal collagen-like domain in their polypeptide chain. Several publications have described the structure and processing of SP-D in detail (Crouch, 1998; Haagsman and Diemel, 2001). SP-D is mainly secreted as a large dodecameric or higher order oligomeric structure and to a lesser extent as a trimeric subunit structure. Binding of oligomeric SP-D to the surface of *C. albicans* and *A. fumigatus* has been described previously in simplified *in vitro* settings (Haagsman and Diemel, 2001; Brummer and Stevens, 2010). Binding of SP-D to fungal cells may also result in the modulation of the activity of immune cells and will vary depending on the type of fungus. Binding of SP-D to *C. albicans* yeast decreases phagocytosis by macrophages (van Rozendaal et al., 2000), in contrast with binding of SP-D to

\* Corresponding author at: Department of Infectious Diseases and Immunology, Division Molecular Host Defence, Utrecht University, Yalelaan 1, 3584 CL, Utrecht, the Netherlands.

E-mail address: [H.P.Haagsman@uu.nl](mailto:H.P.Haagsman@uu.nl) (H.P. Haagsman).

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*A. fumigatus* conidia, which results in increased phagocytosis and killing by macrophages and neutrophils (Madan et al., 1997). These contrasting outcomes are difficult to explain with our current knowledge. Moreover, the few studies that have been published do not consider the lung environment and the possible interactions between SP-D and other soluble innate defense molecules that can take place.

To our knowledge the antifungal activity of SP-D in an environment similar to that encountered at the lung lining, has not been studied. There are several components of the lung lining that can alter fungal recognition by this molecule (Jayaraman et al., 2001). Highly glycosylated proteins, such as mucins for example, could block fungal recognition by competing for binding with SP-D. In addition, it is possible that SP-D interacts with other soluble effector molecules such as antimicrobial peptides that, in this way, affect its antifungal activity.

In this study, we developed an *in vitro* system consisting of Calu-3 epithelial cells, multiple soluble molecules and immune cells, mimicking the environment of the lung lining of the conductive respiratory tract (Grainger et al., 2006). With the use of confocal microscopy and life imaging the localization of SP-D was visualized before and after fungal infection with two fungal pathogens: *C. albicans* and *A. fumigatus*. In addition, the effect of SP-D on the interactions of fungi with the epithelium and on the activity of neutrophils were also analyzed by measuring fungal adhesion, fungal clearance by neutrophils and cytokine production by Calu-3 cells.

## 2. Materials and methods

### 2.1. Fungal strains

Wild type *A. fumigatus* (AF 293) and a green fluorescent protein (GFP)-expressing strain A1258 (Wasylnka and Moore, 2002) (kindly provided by the Department of Medical Microbiology, Utrecht University), were grown on PDA agar (Becton, Dickinson, Le-Pont-De-Claix, France) plates for 3 days at 37 °C. Conidia were harvested with 0.85% (w/v) NaCl and filtered through 3 layers of miracloth (Merck Millipore Corporation, Darmstadt, Germany) to remove pieces of mycelium. Suspensions were adjusted to 10<sup>8</sup> conidia/ml after counting the conidia with a Bürker chamber. Cultures of *C. albicans* (CAI-4) were grown from a frozen glycerol stock in yeast malt broth (YM; SIGMA, St. Louis, MO, USA) agar plates. Yeasts were cultured at 30 °C in 10 ml YM broth until lag phase was reached. Growth was determined by measuring optical density (OD) at 620 nm and yeast suspensions were diluted to 2 × 10<sup>6</sup> CFU/ml in the same buffer. For minimal fungicidal concentration (MFC) experiments initial cell density was also checked by plating 10-fold dilutions in minimal YM broth (1:1.000 dilution of YM in HEPES buffer, pH 7.4; SIGMA St. Louis, MO, USA).

### 2.2. SP-D purification and labeling

Recombinant human surfactant protein D (SP-D) was produced in HEK293 cells as described previously (van Eijk et al., 2011). Fractionation of the produced SP-D into oligomeric and trimeric structures was performed by gel filtration using an ÄKTA purifier10 system, which was equipped with a Hiload 16/60 Superdex 200 PREP GRADE column. A trimeric CRD fragment of SP-D was expressed, purified and characterized as described previously (van Eijk et al., 2012) with small modifications. During the gel filtration step of SP-D and CRD, the elution buffer contained 5 mM HEPES (pH 7.4) and 150 mM NaCl. This buffer was also used as storage buffer (−20 °C). Amine labeling of SP-D fractions with Alexa 647 fluorescent label (A647) was done using a conjugation protein labeling kit from Molecular Probes, Rockford IL 61105, USA. Endotoxin levels were determined with the Toxinsensor LAL assay kit (Thermo Fisher, Rockford IL 61105, USA), ranging between 10 and 100 pg/μg of SP-D.

### 2.3. Flow cytometry

A suspension of yeast cells from *C. albicans* at a density of 1 × 10<sup>6</sup> CFU/ml or a suspension of *A. fumigatus* conidia at a density of 1 × 10<sup>6</sup> conidia/ml were used for these experiments. Cells were suspended in 5 mM HEPES buffer pH 7.4 supplemented with 150 mM NaCl and either 5 mM CaCl<sub>2</sub> or 5 mM EDTA. Cells were mixed with 10 μg/ml A647-hSP-D and incubated for 1 h at 37 °C. After incubation, cells were fixed with 2% PFA at room temperature for 30 min and analyzed by flow cytometry, using a BD FACS Calibur (BD Bioscience).

### 2.4. Calu-3 air liquid culture and neutrophil isolation

Calu-3 cells, a human airway epithelial cell line, were cultured as described by Grainger et al. (2006) with few modifications. Briefly, cells were purchased at ATCC (Rockville, MD, USA) and used between passages 5–20. Cells were cultured using Minimum Eagle medium (SIGMA, St. Louis, MO, USA) supplemented with 10% FCS in 75 cm<sup>2</sup> culture flasks (SIGMA, St. Louis, MO, USA), until 80% confluence was reached. Calu-3 cells were harvested and 250 μl of a 2 × 10<sup>5</sup> cell/ml suspension was added to a transwell chamber (Costar, Kennebunk, ME, USA) and grown until confluence. Before performing experiments, TEER measurements were performed and only wells with higher resistance values than 200 Ω were used.

Human neutrophils were isolated from whole blood of healthy donors following the Histopaque-Ficoll gradient protocol as described previously (Lehrer and Ganz, 1992). Written informed consent was obtained from all subjects and was provided according to the Declaration of Helsinki. Approval was obtained from the medical ethics committee of the University Medical Center Utrecht (Utrecht, The Netherlands).

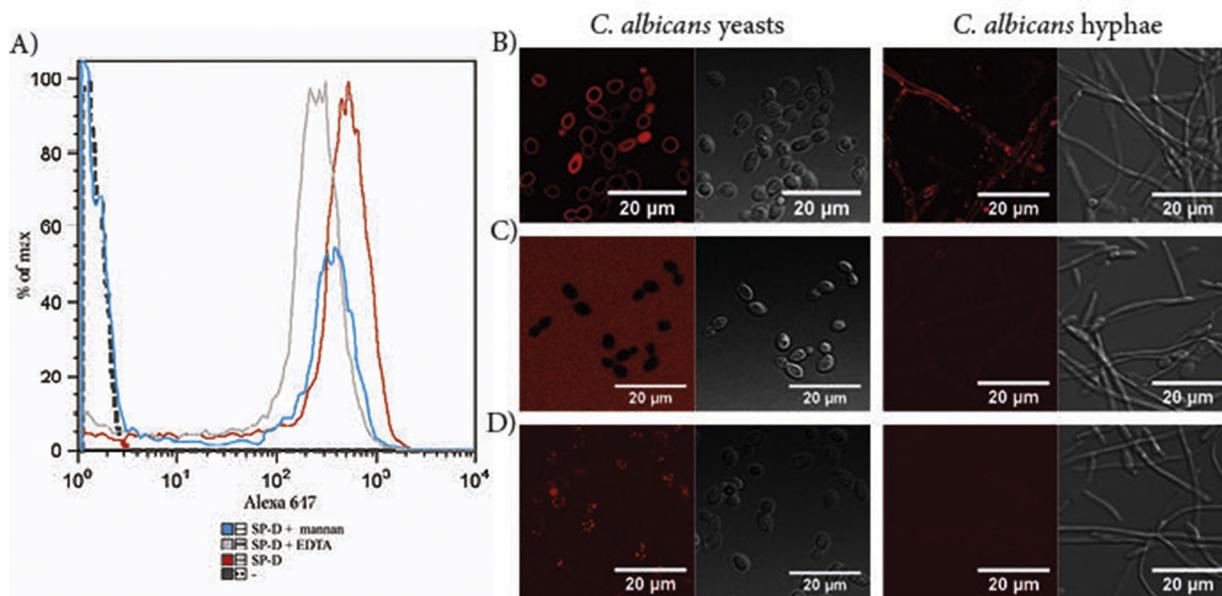
### 2.5. Fungal infection of Calu-3 cells

For the first experiments in which the localization and the effect of SP-D was measured, SP-D (final concentration: 20 μg/ml) was dissolved in 5 mM HEPES buffer pH 7.4 supplemented with 150 mM NaCl and 1 mM CaCl<sub>2</sub> (HEPES supp.), and added to the apical side of Calu-3 cells. Thirty minutes after treatment 100 μl of fungal suspension was added. For all experiments both *A. fumigatus* and *C. albicans* were re-suspended in 5 mM HEPES buffer, pH 7.4 supplemented with 150 mM NaCl and 1 mM CaCl<sub>2</sub> at a density of 1 × 10<sup>7</sup> and 2 × 10<sup>6</sup> CFU/ml, respectively. The infection was either visualized immediately by confocal microscopy (described in detail in the following section) or incubated for another 3 h period at 37 °C, 5% CO<sub>2</sub> and washed three times with HEPES supp. In order to assess fungal association to the epithelium, the apical side was washed and 200 μl of 1% Triton X (SERVA, Heidelberg, West Germany) in HEPES supp. was added. After 5 min at 37 °C the supernatant was collected and serially diluted for plating. Colonies of *C. albicans* were counted after 24 h incubation at 37 °C and colonies of *A. fumigatus* were counted after 48 h at 37 °C of incubation. For experiments using neutrophils, (2 × 10<sup>6</sup> cells/well) were added on the apical side of Calu-3 cells and incubated for 3 h followed by assessment of viable fungi (following the same protocol as for fungal association described earlier).

### 2.6. Confocal imaging

#### 2.6.1. Imaging with fluorescently labeled SP-D in buffer

Fungal cells were added to a glass bottom chamber (35-mm culture dishes (FluoroDish; WPI, Sarasota, FL)) and either grown until germination at 37 °C or imaged after 30 min at room temperature (*C. albicans* yeasts and *A. fumigatus* conidia). Images were taken 5 min after addition of 20 μg/ml fluorescently labeled A647-SP-D. Confocal images were acquired on a Leica SPE-II using the 63x ACS APO (NA = 1.3) or 40x PLAN APO (NA = 1.25-0.75) objectives. Imaging was performed using



**Fig. 1.** Binding of SP-D to *C. albicans* is  $\text{Ca}^{2+}$ -dependent. A) Flow cytometry depicting the % of maximum fluorescence of Alexa647 labeled SP-D bound to *C. albicans* either alone, in the presence of 10  $\mu\text{g}/\text{ml}$  mannan or 5 mM EDTA. B–D) Confocal imaging of SP-D (red) binding to *C. albicans* yeasts or hyphae either B) alone, C) in the presence of 10  $\mu\text{g}/\text{ml}$  mannan or D) in the presence of 5 mM EDTA. Representative images are shown from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

a quadruple band beam splitter for the 647 nm laser line.

### 2.6.2. Confocal imaging and fungal growth measurements at the Calu-3 cell surface

Live imaging was used to observe the localization of SP-D during fungal infection of Calu-3 cells. Briefly, the internal chamber of a transwell containing polarized Calu-3 cells was transferred to a glass bottom chamber (35-mm culture dishes (FluoroDish; WPI, Sarasota, FL). This chamber was positioned inside a 37 °C, 5%  $\text{CO}_2$ , incubation chamber of a Nikon A1R microscope. After focusing, cells were infected and treatment was carried out as described in the previous section. Visualization of A647-SP-D was done using the 645 nm (300 mW) MPBC laser. Fungal growth was also assessed by live imaging. Calu-3 cells that were previously infected by *C. albicans* or *A. fumigatus* were visualized after the start of hyphal growth, when hyphae were long enough to be measured (3 h for *C. albicans* and 8 h for *A. fumigatus*). Calcofluor (SIGMA, St. Louis, MO, USA), at a concentration of 0.003% was used to stain hyphae before imaging. Visualization was done with a 405 nm (100 mW) Coherent Violet Cube laser. The same laser was used for visualization of Hoechst stained neutrophils (Molecular Probes). Measurements and image analyses were done using ImageJ/Fiji software ([www.fiji.sc](http://www.fiji.sc)).

Localization of tight junction protein zonula occludens-1 (ZO-1), was used to determine tight junction formation by Calu-3 cells. Calu-3 cells were fixed with 4% paraformaldehyde for 30 min at RT, washed once with PBS and once with 1% BSA (SIGMA, St. Louis, MO, USA) in HEPES supp. This was followed by an incubation with a rabbit anti ZO-1 antibody (1:100; Invitrogen) overnight at 4 °C A secondary goat anti-rabbit antibody (1:10,000) fluorescently labeled with A488 (Invitrogen) was used for detection.

### 2.7. Cytokine measurements

For experiments where cytokine concentrations were measured, no wash after fungal infection and SP-D treatment was done. Two hundred  $\mu\text{l}$  of basal medium was collected every 4 h and replaced with fresh medium. Collected basal medium was frozen at  $-20$  °C until the analysis was performed. The concentrations of IL-6 and IL-8 were measured

using human IL-6 and IL-8 DuoSet ELISA kits (R & D Systems, Minneapolis, MN).

### 2.8. Antifungal activity of neutrophils at the Calu-3 cell surface

Calu-3 cells were treated with SP-D as in previous experiments. Addition of  $2 \times 10^6$  (Madsen et al., 2000) neutrophils/well was done after 3 h or 6 h of infection by *C. albicans* or *A. fumigatus*, respectively. Effects of neutrophils (either killing or fungal growth inhibition) were assessed after an incubation of 4 h at 37 °C, 5%  $\text{CO}_2$ . For assessing fungal clearance by neutrophils the same procedure for measuring fungal association (described in the previous section) was used. Briefly, surviving fungal cells at the surface of Calu-3 cells were plated and colonies were counted. As a reference, conidia/yeasts from both fungal strains were grown without epithelial cells and treated with neutrophils immediately after germination started. If imaging of neutrophils was required, these cells were pre-incubated with Hoechst stain for 30 min before addition to the Calu-3 apical surface. Hyphal growth was also measured as described in the confocal section above.

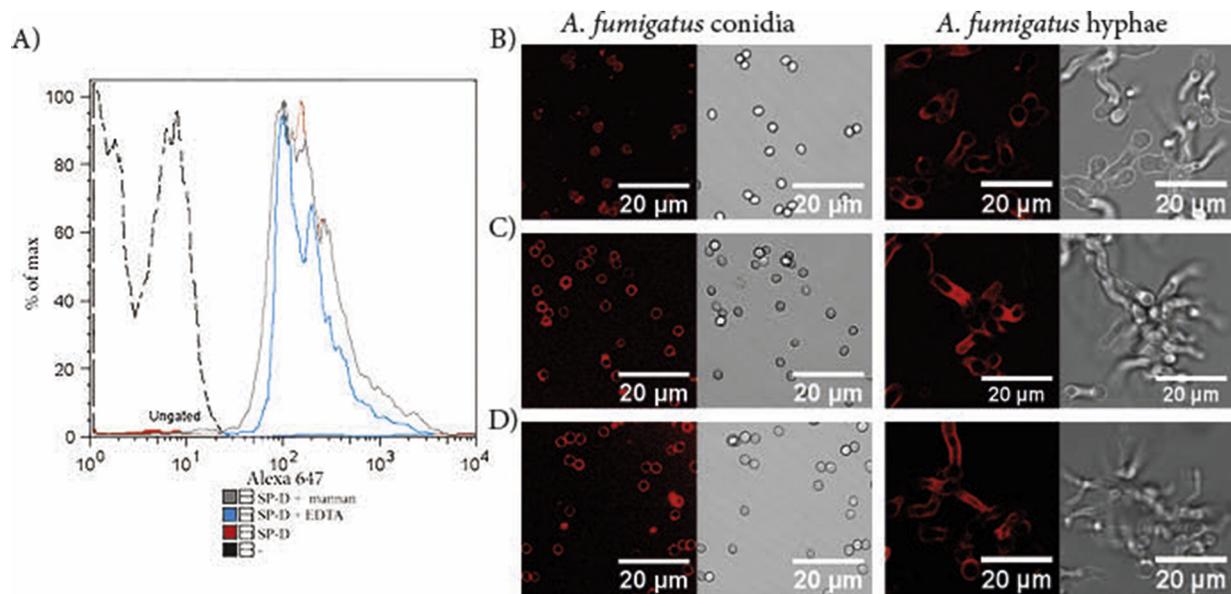
### 2.9. Statistical analysis

GraphPad Prism Software (GraphPad Software Inc., La Jolla, United States) was used for statistical analysis. Differences were analyzed using the student's unpaired test, (two-tailed P value) or ONE-WAY ANOVA. P-values of  $\leq 0.05$  were considered significant,  $\leq 0.01$  very significant and  $\leq 0.001$  as highly significant. Analysis of internalization and internalization blockers values was performed using IBM SPSS Statistics for Windows, Version 22.0. Values were scored as in or out and treated as binary data. Pearson Chi-square test was used evaluating significance with p-values  $\leq 0.05$ .

## 3. Results

### 3.1. SP-D binding to the cell surface of *C. albicans* and *A. fumigatus*

In order to track the localization of SP-D during live imaging at the surface of Calu-3 cells, SP-D was labeled with Alexa 647. Since labeling



**Fig. 2.** Binding of SP-D to *A. fumigatus* is not  $\text{Ca}^{2+}$ -dependent. A) Flow cytometry depicting the % of maximum fluorescence of Alexa647 labeled SP-D bound to *A. fumigatus* either alone, in the presence of 10  $\mu\text{g}/\text{ml}$  mannose or 5 mM EDTA. B–D) Confocal imaging of SP-D (red) binding to *A. fumigatus* resting spores or hyphae either B) alone, C) in the presence of 10  $\mu\text{g}/\text{ml}$  mannose or D) in the presence of 5 mM EDTA. Representative images are shown from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

can potentially affect its activity, the labeled protein was tested for its binding properties using live cell imaging. For both fungi, two fungal stages that are usually encountered during infection were used: the yeast and hyphae morphologies for *C. albicans* and the resting conidia and hyphae for *A. fumigatus*. Labeled molecules were still able to bind fungal cells (Fig. S1). Using flow cytometry and live-cell imaging it was found that SP-D binds to *C. albicans* yeast and hyphal forms in a strictly  $\text{Ca}^{2+}$ -dependent manner (Fig. 1). Interestingly, binding of SP-D to *A. fumigatus* was not  $\text{Ca}^{2+}$ -dependent under these conditions. Flow cytometric analysis showed that SP-D still bound to conidia (Fig. 2A). The latter was more clearly visible by live imaging, where no loss of fluorescence at the surface of the fungi was detected (Fig. 2B–D).

There are two published studies describing the binding of SP-D to *A. fumigatus* conidia (Madan et al., 1997; Allen et al., 2001). Both studies pointed towards a strict  $\text{Ca}^{2+}$ -dependent interaction of SP-D with the conidial surface. To verify whether the different experimental settings could account for the disparities in our results, we executed experiments with the conditions and methods described by (Madan et al., 1997). Indeed, the addition of 1% BSA blocked most of the  $\text{Ca}^{2+}$ -independent binding that was observed by flow cytometry in our previous experiments (Fig. S2).

### 3.2. SP-D binding to the cell surface of *C. albicans* and *A. fumigatus* at the apical surface of Calu-3 cells

To test if SP-D can recognize fungal surfaces in an environment similar to the one found at the lung inner surface, an *in vitro* system that mimics the lung lining was developed. In order to achieve this, lung epithelial cells (Calu-3 cell line) were grown at an air-liquid interface. Under these conditions the cells are capable to polarize and to secrete proteins such as mucins to its apical surface (Fig. S3). In this system, it was observed that SP-D strongly binds to *C. albicans* yeasts and *A. fumigatus* conidia immediately after addition (Fig. 3). SP-D also was found to bind to cell debris at the Calu-3 surface, observed in the control. The specificity of this interaction after fungal germination was similar (2 h for *C. albicans* and 8 h for *A. fumigatus*) (Fig. 3).

### 3.3. SP-D binding affects fungal interaction with Calu-3 cells

After the initial contact with fungal intruders, SP-D-coated fungal cells are likely to interact with the epithelial lining. To test if this SP-D coat affects fungal interaction with the epithelium, three processes were measured: 1) binding of fungi to the surface of Calu-3 cells, 2) fungal growth (hyphal length), and 3) cytokine secretion by Calu-3 cells.

*C. albicans* and *A. fumigatus* bound significantly less to the Calu-3 surface when SP-D was present (Fig. 4A–B). Blocking of this binding seems to be a characteristic of the SP-D multimeric structure, since pre-incubation with CRD trimeric heads even increased the association of *C. albicans* to Calu-3 cells as compared to the control.

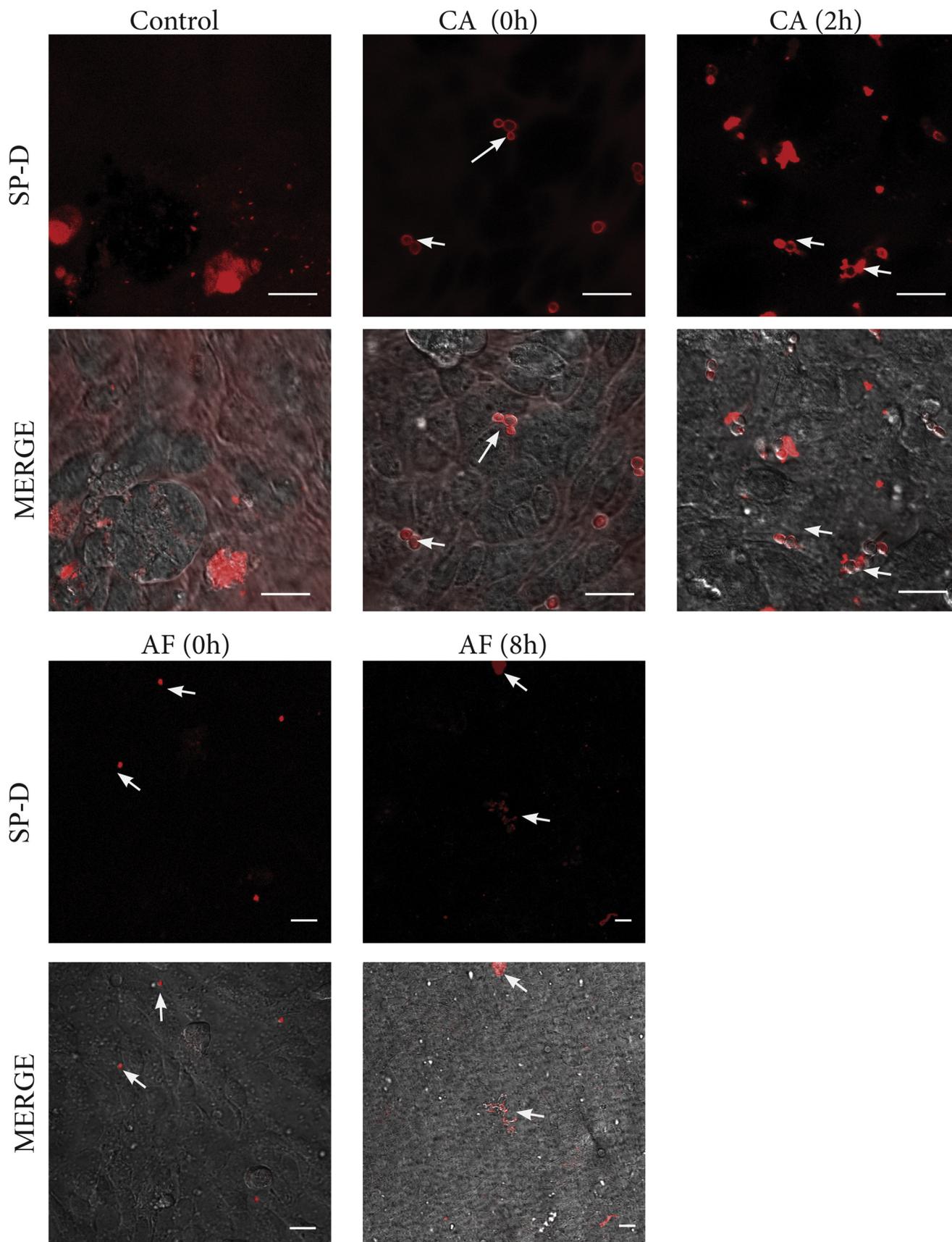
The effect of SP-D on fungal growth could only be measured for *A. fumigatus* since hyphal growth was slower than that of *C. albicans*, and did not produce aggregates at the apical surface of the Calu-3 cells. Addition of SP-D to *A. fumigatus* resulted in a significant decrease in hyphal length 8 h after infection (Fig. 4C).

The third activity that was measured was the effect of opsonized fungal cells on cytokine production. Four cytokines that are produced by Calu-3 cells were measured: IL-8, IL-6, IL-1 $\beta$  and IL-10. Only IL-8 and IL-6 production was significantly increased after infection with either *C. albicans* or *A. fumigatus* (Fig. 5A and B). During *C. albicans* infection, no significant effect of SP-D was observed on levels of IL-8 while for IL-6 there was a slight decrease in IL-6 production when SP-D was present but this did not reach statistical significance production ( $p = 0.0523$ ). Similarly, no significant effect of SP-D was observed after 24 h of *A. fumigatus* infection of Calu-3 cells.

### 3.4. Neutrophil activity is affected by SP-D coating of fungal surfaces

One of the major defenses against fungal infections are neutrophils. In fact, fungi cannot be cleared effectively without them (Feldmesser, 2006). Therefore, the effect of SP-D on neutrophils that are present at the lung lining and encounter fungal intruders, was determined. Three characteristics of neutrophil activation were measured: 1) Fungal clearance at the Calu-3 surface, 2) Oxidative burst and 3) Inhibition of hyphal growth.

Confocal images showed that binding of fluorescently labeled neutrophils to invading fungi and the Calu-3 surface was increased by SP-D



**Fig. 3.** Confocal microscopy of SP-D binding to *C. albicans* (CA) and *A. fumigatus* (AF) at the surface of Calu-3 cells. SP-D was added to the surface of Calu-3 cells before infection with *C. albicans* or *A. fumigatus*. Confocal images were taken at the beginning of infection and after fungi started forming hyphae. SP-D (red) binds to cell debris (control panels) and to the surface of *C. albicans* and *A. fumigatus* (white arrows, fungi were identified by their distinctive morphology). Second and fourth row shows merge of red fluorescence and differential contrast (DIC) channels to visualize Calu-3 cells. White bars are 20  $\mu\text{m}$ . Representative pictures of at least three independent experiments are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

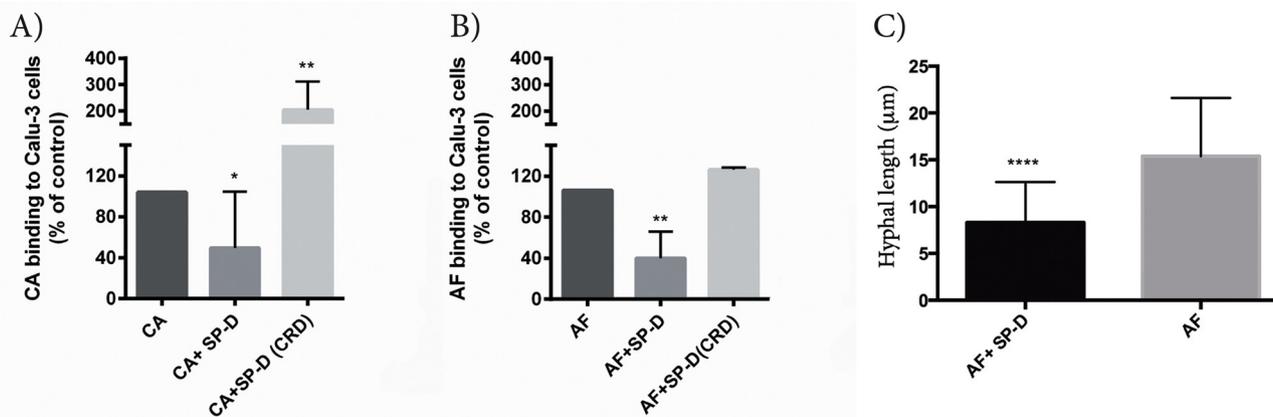


Fig. 4. Effect of preincubation with SP-D oligomer/CRD (20 µg/ml) on fungal binding. A) Effect of SP-D on *C. albicans* binding to Calu-3 cells. B) Effect of SP-D on *A. fumigatus* binding to Calu-3 cells. C) Effects of SP-D on hyphal length of *A. fumigatus* during Calu-3 infection. Measurements from three independent experiments are shown as mean values with standard deviation. \*, \*\* and \*\*\*\* indicate  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$ , respectively.

(Fig. 6). The increase in neutrophils was more visible for *A. fumigatus* infection (Fig. 6B). Furthermore, fungal infection was decreased by neutrophils in the presence of SP-D for both *C. albicans* and *A. fumigatus* (Fig. 7A and B). Neutrophils alone reduced the infection by 90%, but when SP-D was present a further reduction in infection was observed. The CRD heads of SP-D, however, did not have an effect, indicating that either the multimerization of SP-D or its collagen domain is important for eliciting this activity.

To find out if neutrophil activity changes in the presence of SP-D, the oxidative burst and the neutrophilic killing capacity were tested in the absence of Calu-3 cells. The oxidative burst of neutrophils decreased when they were in contact with free SP-D oligomers, but no effect was seen when CRD heads were used (Fig. 8A). In contrast, when *C. albicans* interacted with neutrophils, the oxidative burst increased sharply and the presence of SP-D in this case had no significant effect on the *C. albicans*-induced oxidative burst (Fig. 8B). No effect on the oxidative burst was observed if neutrophils were incubated with *A. fumigatus* conidia (Fig. 8C).

Finally, during the period of co-incubation of neutrophils and fungi it was observed that fungal growth was more effectively inhibited when SP-D was present. This is similar to what was observed for the incubation of Calu-3 cells with *A. fumigatus*. Especially in the case of *A. fumigatus*, SP-D significantly decreased hyphal growth (Fig. 9A). For *C. albicans* the effects were smaller and not significant (Fig. 9B).

#### 4. Discussion

In this study an *in vitro* system mimicking the lung lining environment was developed. This system consisted of Calu-3 epithelial cells at an air/liquid interface that allowed them to polarize and produce a stratified fluid layer composed of an aqueous phase and a gel phase (also called the mucus layer). This fluid has a similar  $\text{Ca}^{2+}$  concentration and a similar pH to what is found at the lung lining (Grainger et al., 2006). Additionally, Calu-3 cells secrete, to their apical surface, multiple soluble molecules such as antimicrobial peptides. To this system, the host defense molecule, SP-D was added in order to study its role against the fungal pathogens *C. albicans* and *A. fumigatus*. To make this environment more realistic and to discover other host defense interactions, neutrophils were also included and the effect of SP-D was subsequently assessed. An overview of all our findings can be found in Table 1.

In our current study the recognition of fungal cells by SP-D was initially tested in a buffer with pH, salt and divalent concentrations similar to those encountered at the lung lining. SP-D binds to the surface of both *C. albicans* yeast and pseudohyphae, and this binding was inhibited in the presence of EDTA and the polysaccharide mannan.

In contrast to *C. albicans*, *A. fumigatus* enters the lung as conidia, highly resistant structures, which are not metabolically active. The surface of these conidia differs strongly from that of *C. albicans* yeasts by possessing a coat of hydrophobic proteins called hydrophobins. This different coat could potentially result in different interactions of *A. fumigatus* with SP-D. In addition, the hyphal form of *A. fumigatus* differs strongly in cell wall composition with respect to conidia and, importantly, represents the active state of fungi during infection (Paris et al., 2003; Latge, 2007). The interaction of SP-D with *A. fumigatus* conidia and hyphae was first tested in buffer only. Strikingly, SP-D bound even when  $\text{Ca}^{2+}$  was not present and neither EDTA nor mannan could block this interaction. However,  $\text{Ca}^{2+}$ -independent binding was almost completely blocked by the addition of 1% BSA. At the surface of Calu-3 cells a low amount of FCS is present, due to its use as a component of the basal growth medium, thus  $\text{Ca}^{2+}$ -independent binding might not be relevant in this setting.

Association of fungi with Calu-3 cells was reduced in the presence of oligomeric SP-D. It is known that SP-D can aggregate *C. albicans* and *A. fumigatus* (Madan et al., 1997; van Rozendaal et al., 2001). Thus, it could be that aggregation of fungal cells helps to block their association with the epithelium and facilitates their removal. Another reason for the observed decrease in fungal association to Calu-3 cells could be a decrease in fungal recognition by Toll-like receptors and lectin receptors at the epithelial surface (Cheng et al., 2000; Figueiredo et al., 2011; Netea et al., 2006). Fungal cells coated with trimeric or oligomeric SP-D may mask polysaccharide ligands that are usually recognized by the receptors, thus decreasing adhesion. Curiously, an increase in adhering fungi to Calu-3 cells was observed after addition of the CRD domain of SP-D. This observation is hard to explain but could mean that CRDs are also being recognized by receptors at the epithelium. Whether this observation has any value in an *in vivo* setting is highly unlikely since only oligomeric and trimeric structures of SP-D are secreted into the lung lining, but not trimeric CRDs (Haagsman and Diemel, 2001).

To test the immunomodulatory effect of SP-D during fungal infection, Calu-3 derived cytokine measurements were done at 24 h post-infection. At this time point *A. fumigatus* infection caused a modest increase in IL-8 and IL-6, whereas *C. albicans* infection increased these two cytokines significantly. This is probably related to the fact that *C. albicans* is growing faster than *A. fumigatus* for which it takes 8 h before germination starts. It is also known that *A. fumigatus* can block apoptosis of epithelial cells and that it decreases TNF $\alpha$  production (Berkova et al., 2006). The effect of SP-D was only evident for *C. albicans*, where IL-6 levels were decreased by 20%. IL-6 is a cytokine secreted during *C. albicans* infection by activation through Dectin-1, and it is also linked to cell damage (Netea et al., 2008; Yu et al., 2002). This suggests that

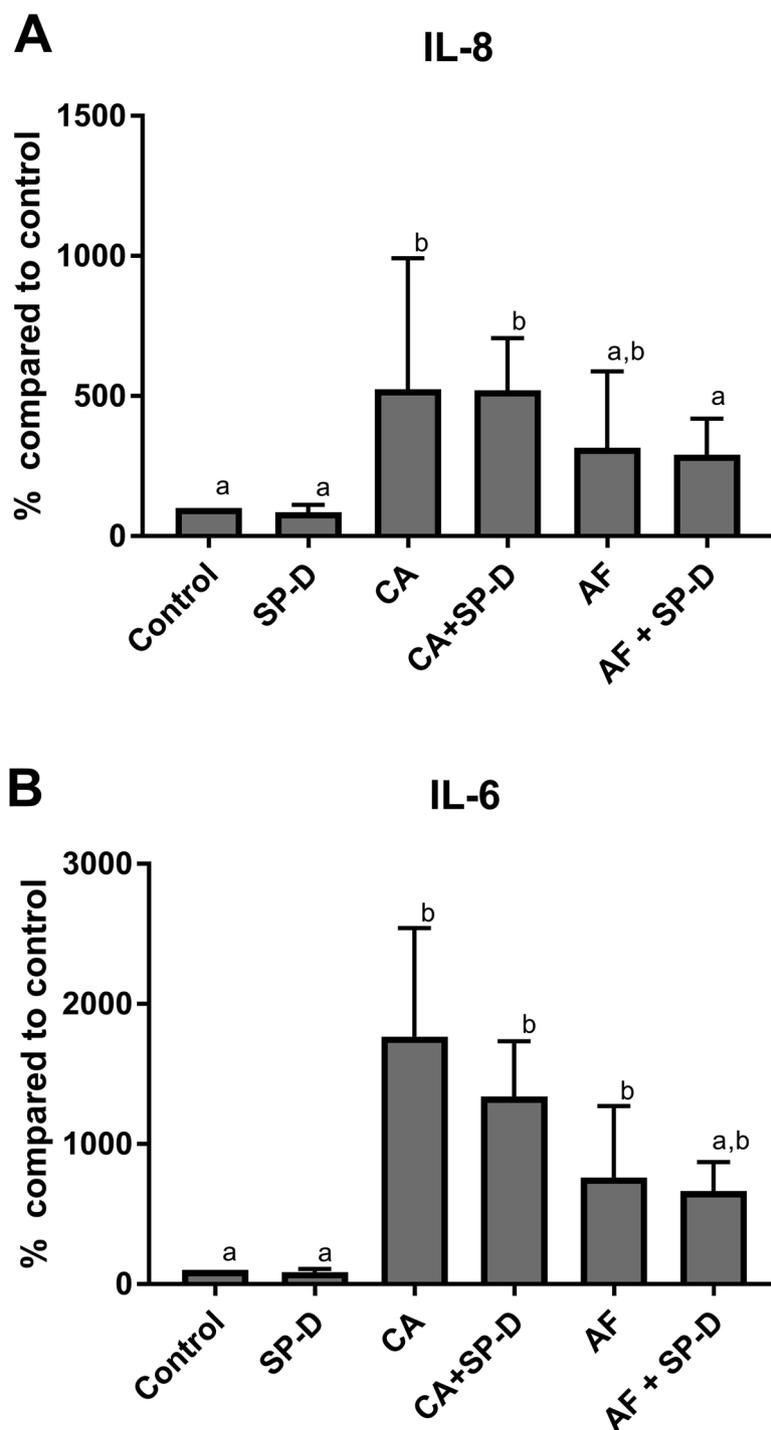


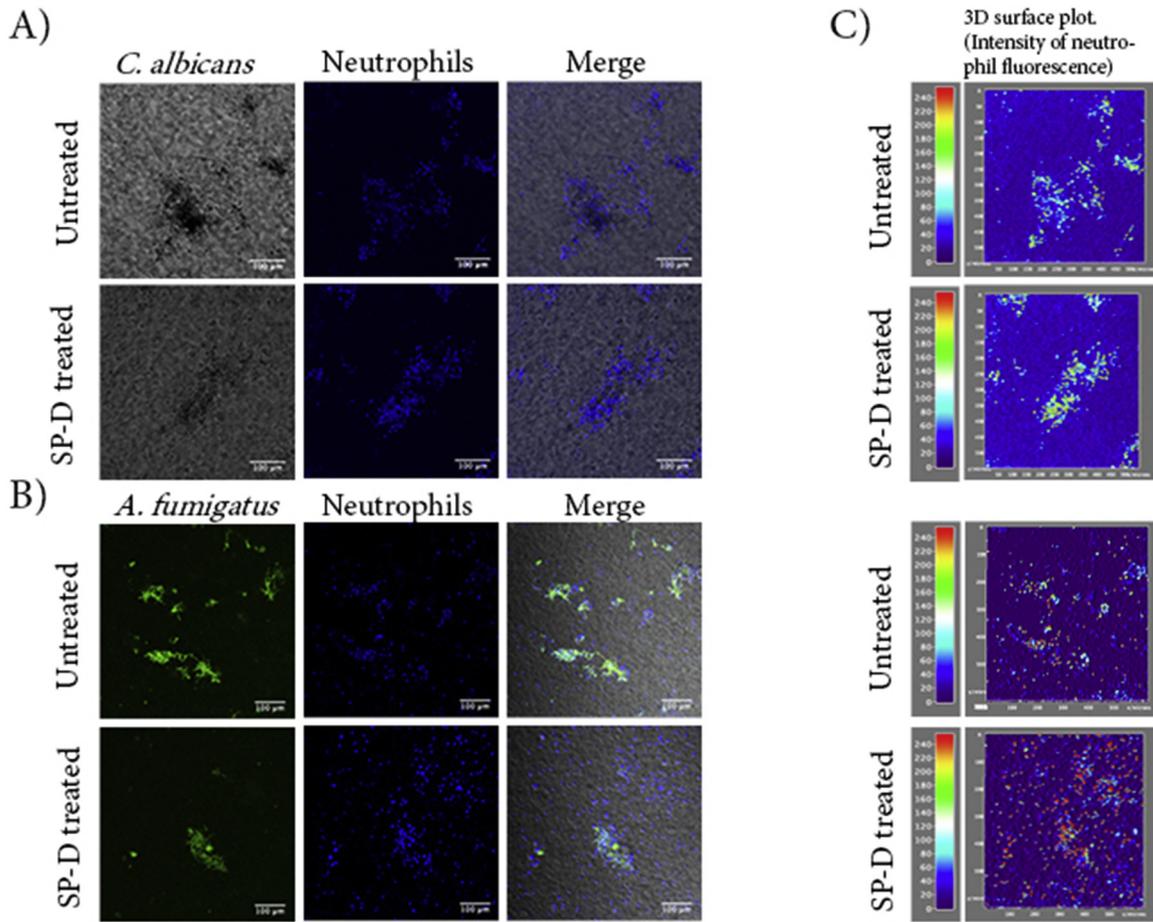
Fig. 5. Cytokine production after 24 h by Calu-3 cells upon fungal infection.

A) Expression of IL-8 upon *C. albicans* (CA) or *A. fumigatus* (AF) infection in the presence or absence of SP-D. B) Expression of IL-6 upon fungal infection in the presence or absence of SP-D. Data from 10 independent experiments are shown as mean values with standard deviations. Data points which are significantly different from each other are denoted by different letters ( $p < 0.05$ ).

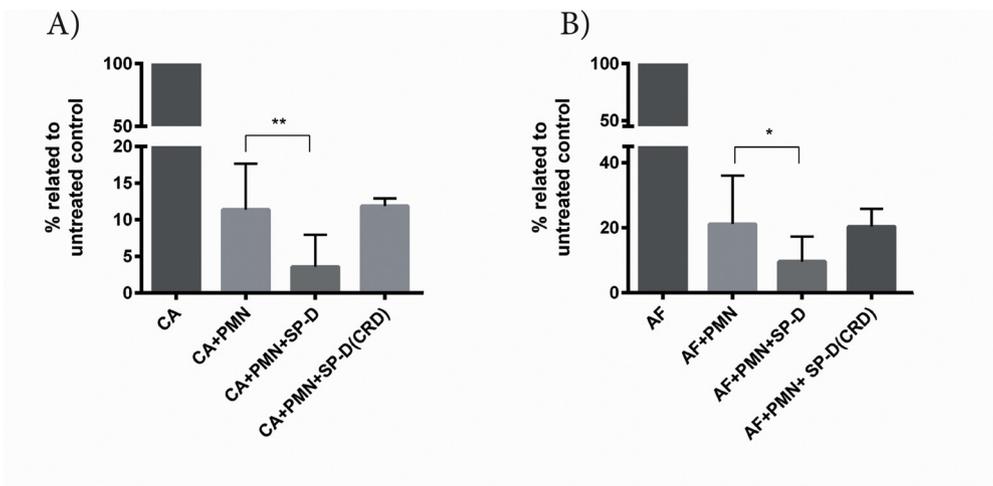
SP-D may block the binding of  $\beta$ -glucans present in the cell wall of *C. albicans* to this receptor.

The effect of SP-D during neutrophil fungal clearance at the Calu-3 apical environment was also tested. The first finding was an increased number of neutrophils at Calu-3 infected sites when SP-D was present. Several reasons could explain this finding: increased adhesion, increased chemotaxis and increased activation of neutrophils by SP-D (Madan et al., 1997). We tested these options in a more simplified setting having only neutrophils and SP-D present. It was found that

neither an increase in adhesion nor chemotaxis was exerted by SP-D (data not shown). However, a different effect could occur when SP-D is bound to fungi since only the collagenous region of SP-D would be available for interaction with neutrophils and epithelial cells. For example, the Leukocyte-associated Ig-like receptor-1 (LAIR-1) has been described to interact with the collagen domain of SP-D, resulting in inhibition of reactive oxygen species by neutrophils (Olde Nordkamp et al., 2014). Additionally, the presence of SP-D could aid neutrophils to clear the fungal infection and to decrease fungal growth. These results



**Fig. 6.** Binding of neutrophils to fungal cells at the epithelial surface. A) Calu-3 infection with *C. albicans*. First row without SP-D and second row with SP-D present. First three columns show: fungi light contrast, Hoechst labeled neutrophils (blue) and a merged picture for both channels. B) Calu-3 infection with *A. fumigatus*, first row without SP-D and second row with SP-D present. First three columns show: A488-labeled fungi (green), Hoechst labeled neutrophils (blue) and a merged picture for both channels. C) 3D surface-plots showing the intensity of Hoechst (number of neutrophils) of the images in A and B. Different colors represent different intensity according to the scale on the left of the images. SP-D treatment increased the intensity of neutrophil fluorescence indicating a higher number of these cells binding to the fungi. Representative pictures of three independent experiments are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

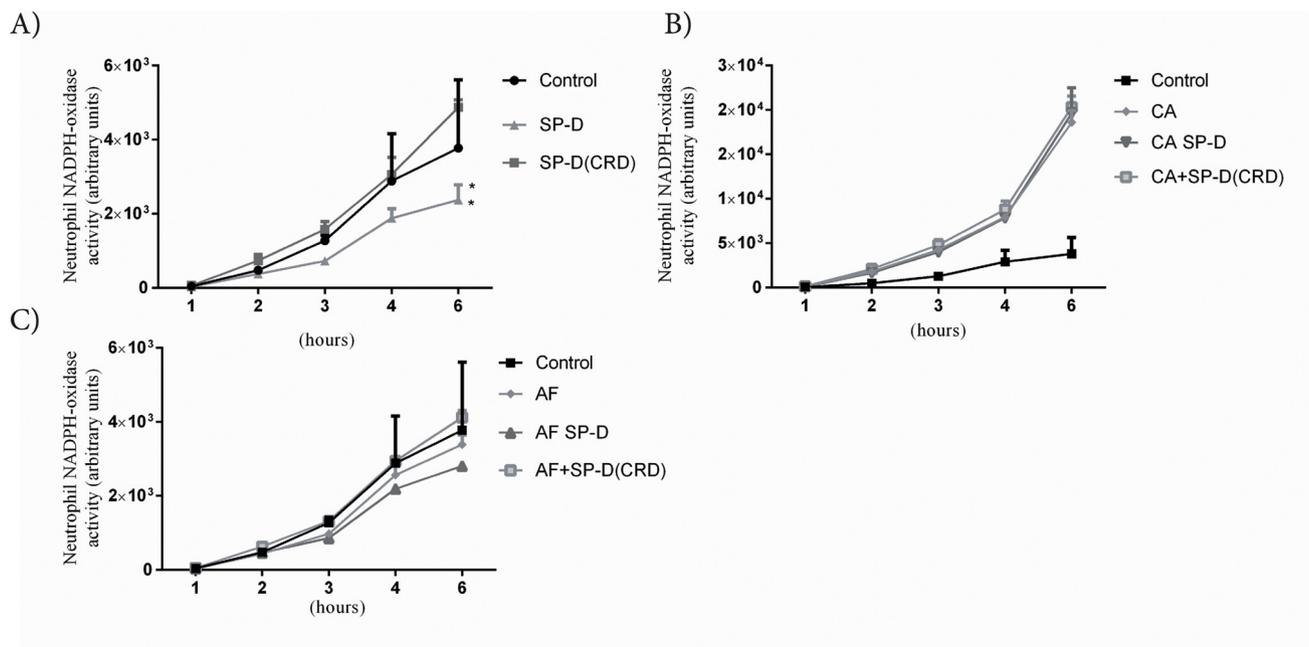


**Fig. 7.** Clearance of fungal infection from Calu-3 cells surface by neutrophils (PMN) in presence and absence of SP-D. PMNs ( $2 \times 10^6$  neutrophils/well) were added after 3 and 6 h to Calu-3 cells infected by A) *C. albicans* (CA) or B) *A. fumigatus* (AF). After 4 h, surviving fungi at the surface of the Calu-3 cells were counted. Data are means and standard deviations of three independent experiments. \* and \*\* indicate  $p < 0.05$  and  $p < 0.01$ , respectively.

are important and support the conclusion that SP-D contributes to clearance of fungal pathogens at the lung epithelium.

As with all studies, our experiments are associated with some limitations. With respect to the model system, Calu-3 is a cell line that is derived from a lung adenocarcinoma which resembles epithelial cells in

many characteristics. However, a single cell type does not completely reflect the situation *in vivo* and it is unclear to what extent the predictive value of the model is affected by this. An interesting option, albeit with its own limitations, would be the use of NHBE cells which potentially reflects better the situation of the conducting airways. In



**Fig. 8.** Effect of SP-D oligomer/CRD (20 µg/ml) on the neutrophil NADPH-oxidase activity upon 6 h of infection with fungi. A) Controls showed a decrease in neutrophil oxidase activity due to the oligomeric fractions of SP-D. B) Effect of *C. albicans* infection on oxidase activity. C) Effect of *A. fumigatus* infection on oxidase activity. Shown is Mean Fluorescence Intensity of three independent experiments, bars represent standard deviation. \* represents significant difference (P < 0.05).

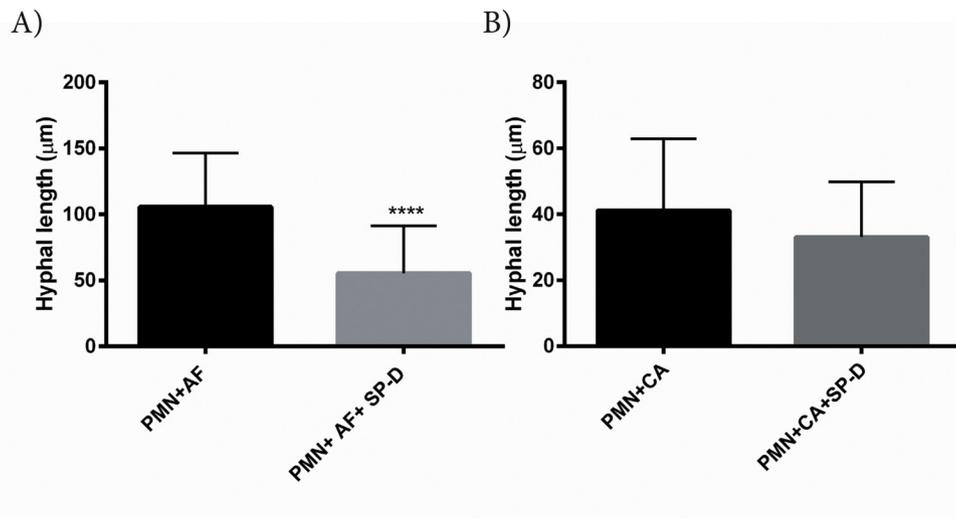
addition, although produced cytokines are usually directed towards the underlying basolateral tissue, including determination of the apical secretion of cytokines would have provided a more complete image of this aspect of the study. Finally, the lung fluid contains multiple immune molecules that affect each other's activity, that were not yet included in the model system. Most notably Surfactant Protein A (SP-A), which could behave similarly to SP-D, or for example host defence peptides which can also have an impact on immune functionality, should eventually be included in the system to really determine the potency of SP-D in fungal clearing. However, overall the described results clearly provide a first step towards improving the complexity of the system to represent the lung environment

Altogether, this study shows the potential of our system to mimic the lung lining fluid in many aspects, and provides novel insights into the function and activity of innate immune molecules and/or innate immune cells. Additional factors can be considered to be included in future experiments, such as alveolar macrophages, pulmonary surfactant, or other innate immune molecules. This will enable us to study the

**Table 1**

Effects of SP-D during *C. albicans* and *A. fumigatus* infection of Calu-3 cells, in the presence or absence of neutrophils.

	SP-D (Oligomer)	SP-D (CRD)
<i>C. albicans</i>	<ul style="list-style-type: none"> <li>● Binds fungal surface</li> <li>● Decreases binding to Calu-3 cells</li> <li>● Decreases hyphal growth</li> <li>● Increases neutrophil binding to infected sites</li> </ul>	<ul style="list-style-type: none"> <li>● Binds fungal surface</li> <li>● Increases binding to Calu-3 cells</li> </ul>
<i>A. fumigatus</i>	<ul style="list-style-type: none"> <li>● Increases neutrophil clearance</li> <li>● Binds fungal surface</li> <li>● Decreases binding to Calu-3 cells</li> <li>● Decreases growth</li> <li>● Increases neutrophil binding to infected sites</li> <li>● Increases neutrophil clearance</li> </ul>	<ul style="list-style-type: none"> <li>● Binds fungal surface</li> </ul>



**Fig. 9.** Effect of SP-D oligomer (20 µg/ml) on growth of fungal hyphae during co-incubation with neutrophils. A) *A. fumigatus* growth in the presence or absence of SP-D. B) *C. albicans* growth in the presence or absence of SP-D. Data shown are average of more than 80 measurements from two independent experiments. Bars represent standard deviation. \*\*\*\* represents highly significant difference (P < 0.0001).

fungal or bacterial interactions with the lung epithelium in a more realistic and reliable manner.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.molimm.2018.12.003>.

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