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Surfactant protein D inhibits growth, alters cell surface polysaccharide exposure and immune activation potential of *Aspergillus fumigatus*

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ABSTRACT

Humoral immunity plays a defensive role against invading microbes. However, it has been largely overlooked with respect to *Aspergillus fumigatus*, an airborne fungal pathogen. Previously, we have demonstrated that surfactant protein D (SP-D), a major humoral component in human lung-alveoli, recognizes *A. fumigatus* conidial surface exposed melanin pigment. Through binding to melanin, SP-D opsonizes conidia, facilitates conidial phagocytosis, and induces the expression of protective pro-inflammatory cytokines in the phagocytic cells. In addition to melanin, SP-D also interacts with galactomannan (GM) and galactosaminogalactan (GAG), the cell wall polysaccharides exposed on germinating conidial surfaces. Therefore, we aimed at unravelling the biological significance of SP-D during the germination process. Here, we demonstrate that SP-D exerts direct fungistatic activity by restricting *A. fumigatus* hyphal growth. Conidial germination in the presence of SP-D significantly increased the exposure of cell wall polysaccharides chitin, α-1,3-glucan and GAG, and decreased β-1,3-glucan exposure on hyphae, but that of GM was unaltered. Hyphae grown in presence of SP-D showed positive immunolabelling for SP-D. Additionally, SP-D treated hyphae induced lower levels of pro-inflammatory cytokine, but increased IL-10 (anti-inflammatory cytokine) and IL-8 (a chemokine) secretion by human peripheral blood mononuclear cells (PBMCs), compared to control hyphae. Moreover, germ tube surface modifications due to SP-D treatment resulted in an increased hyphal susceptibility to voriconazole, an antifungal drug. It appears that SP-D exerts its anti-*A. fumigatus* functions via a range of mechanisms including hyphal growth-restriction, hyphal surface modification, masking of hyphal surface polysaccharides and thus altering hyphal immunostimulatory properties.

Introduction

Aspergillus fumigatus is an opportunistic ubiquitous airborne fungal pathogen. This mould produces numerous asexual spores (conidia) that can reach human lung alveolar space upon inhalation owing to their small size (2–3 μm in diameter) and hydrophobicity. Conidial clearance in the alveoli relies on the innate immune effector cells, mainly alveolar macrophages and recruited neutrophils (Balloy and Chignard, 2009; Margalit and Kavanagh, 2015). In individuals with compromised immune system, inhaled conidia can germinate and invade primarily the lung tissues. This can lead to invasive pulmonary aspergillosis (IPA), a fatal disease with high mortality rate (30–80%) (Baddley et al., 2010; Lortholary et al., 2011; van de Veerdonk et al., 2017; Wong et al., 2021a). Though the innate immune system consists of humoral

Abbreviations: SP-D:, Surfactant protein-D; GM:, Galactomannan; GAG:, Galactosaminogalactan; IL:, Interleukin; PBMCs:, Peripheral blood mononuclear cells.

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immunity (which includes the complement system, collectins, immunoglobulins and antimicrobial peptides), their roles in the defence against *A. fumigatus* have been underexplored (Delliere et al., 2020; Parente et al., 2020; Wong and Aimanianda, 2017). The diverse origin and functional overlap of humoral immune components warrant elucidation of their role in the host defence against *A. fumigatus*. Indeed, recent studies have demonstrated different anti-*A. fumigatus* roles played by several humoral immune components. They function as opsonins or soluble pattern recognition receptors (PRRs) recognising conidia, facilitating their phagocytosis, immunomodulation and thereby conidial clearance by innate immune system (Genster et al., 2016; Jensen et al., 2017; Rosbjerg et al., 2021; Wong et al., 2018).

One of the major humoral components in the human lung alveolar space is the surfactant protein D (SP-D). It is a multimeric *C*-type lectin belonging to the collectin family, secreted by alveolar type II cells and bronchiolar epithelial cells (Arroyo et al., 2020b). The primary function of SP-D is to reduce surface tension at the air–liquid interface of the lung-alveoli. SP-D also functions as a PRR, recognizing microbial pathogens, which leads to microbial opsonization, aggregation/agglutination and clearance by phagocytosis and inflammatory immune responses (Madan and Kishore, 2020; Wright, 2005). We showed that SP-D recognizes *A. fumigatus* conidial surface melanin pigment as a ligand, opsonizes conidia, enhances phagocytosis, induces expression of pro-inflammatory cytokines, and thus protects the host by efficiently clearing conidia (Madan et al., 1997; Wong et al., 2018). During this study, we observed that SP-D also interacted with galactomannan (GM) and galactosaminogalactan (GAG), the cell wall polysaccharides that are exposed on the *A. fumigatus* surface during germination process (Fontaine et al., 2011; Schubert et al., 2019). Therefore, we undertook the elucidation of biological significance of interaction between SP-D and *A. fumigatus* during the process of conidial germination.

Here, we demonstrate that SP-D inhibits *A. fumigatus* hyphal growth and alters the surface exposure of hyphal cell wall polysaccharides *in vitro*. Conidial germination in presence of SP-D resulted in a significant increase in the exposure of hyphal surface chitin, α-1,3-glucan, GAG, while the exposure of β-1,3-glucan was decreased. This direct growth inhibitory activity of SP-D led to a modified host immune response. There was a decreased secretion of pro-inflammatory cytokines and an increased anti-inflammatory cytokine/chemokine secretions by peripheral blood mononuclear cells (PBMCs) co-incubated with SP-D-treated hyphae, compared to untreated hyphae. Taken together, our study suggests that SP-D has a direct fungistatic effect on the growth of *A. fumigatus*, and that SP-D-induced surface modifications alter immunostimulatory potential of *A. fumigatus* hyphae.

Materials and methods

Fungal strains

A. fumigatus CBS144-89 (Thau et al., 1994), CNRMA 15.354 and *A. flavus* CI1698 (Wong et al., 2021b) were the clinical isolates used in this study. They were maintained at ambient temperature on 2% malt agar slant for 12–15 days before collecting their conidia using 0.05% Tween-80. The conidial suspension was passed through 40-μm cell strainer (ThermoScientific) to avoid any mycelial contamination.

Surfactant protein D

Plasmid encoding a signal peptide from azurocidin preproprotein and full-length human SP-D (FL-SP-D) (Met11 variant) (pcDNA3.1 plus; Genscript) was designed, transfected into EXPICHO-S cells (ThermoScientific) using ExpiFectamine according to the manufacturer's protocol, and expressed transiently. After 20 h, ExpiFectamine CHO Enhancer and ExpiCHO Feed were added to these cells and incubated at 37 °C in 8% CO_2 for 9 days with shaking. Following, the cell-free culture supernatant was collected via centrifugation. The purification of recombinant FL-SP-D from this supernatant was performed as described previously (Sorensen et al., 2009); however, Tris-buffered saline (TBS) containing 10 mM EDTA was used to elute protein. Eluted FL-SP-D was dialyzed against Tris-HCl (pH 7.5, 20 mM) containing NaCl (50 mM) and $CaCl_2$ (5 mM). The recombinant fragment of human SP-D (rfhSP-D) containing a short collagen-like stalk, neck region and carbohydrate recognition domain (CRD) was prepared according to protocol described before (Madan et al., 2021). The reason for generating rfhSP-D is that its expression yield is higher compared to FL-SP-D. Also, earlier studies indicated that this recombinant fragment has the biological activity similar to FL-SP-D (Hsieh et al., 2021; Knudsen et al., 2009; Watson et al., 2020). Using endotoxin detection kit (ThermoScientific), it was confirmed that the purified FL-SP-D/rfhSP-D preparations were devoid of any endotoxin contaminations. Recombinant FL-SP-D was also purchased from a commercial source (ab152069, Abcam), which was produced using HEK 293 expression system.

Cell wall polysaccharides extraction

Galactosaminogalactan (GAG) was purified as described previously (Gressler et al., 2019). Briefly, Brian medium (50 mL) was inoculated with $5x10^5$ conidia/mL. After 3-day incubation in an incubator maintained at 37 °C with 150 rpm, the supernatant was collected by filtration and the pH was adjusted to 3.0 by adding 12 M HCl. To the pH adjusted supernatant, two volumes of ethanol was added and stored overnight at 4 °C. The precipitate formed was then collected by centrifugation of the contents for 20 min at 5,000 g at 4 °C. The pellet obtained (GAG) was washed twice with 150 mM NaCl for 1 h under agitation, dialyzed against water, freeze-dried, and stored at ambient temperature. Galactomannan (GM) was extracted from the crude membrane preparation of *A. fumigatus*, as described earlier (Costachel et al., 2005). Purity of the extracted polysaccharides (GAG and GM) was examined by glycobiology techniques (Gressler et al., 2019).

Direct binding of galactosaminogalactan (GAG) and galactomannan (GM) to SP-D

SP-D (5 μg/mL; 100 μL/well) in 50 mM carbonate buffer (pH 9.6) was coated on 96-well microtiter plate overnight at room temperature. After blocking the coated wells with phosphate buffered saline (PBS) + 3% BSA for 1 h, GAG or GM (5 μg/mL; 100 μL/well) suspended in PBS + 3% BSA + 5 mM $CaCl_2$ was added to SP-D coated wells and incubated at room temperature for 2.5 h. After washing the wells with PBS + 0.5% Tween-20, mouse monoclonal anti-GAG antibody or rat monoclonal anti-GM antibody (1:1000 dilution in PBS + 3% BSA) were added to the wells and incubated for 1 h. Following washing with PBS + 0.5% Tween-20, the secondary antibodies (anti-mouse/anti-rat IgG conjugated to horse-radish peroxidase (HRP); 1:1000 dilution in PBS + 3% BSA) were added and incubated for 1 h. The binding was revealed by *o*-phenylenediamine dihydrochloride (Sigma) as the HRP substrate. The colour development was stopped by adding 4% H_2SO_4, and the absorbance was measured (492 nm) using Tecan Infinite 200 PRO plate-reader (Tecan Group Ltd., Switzerland). SP-D binding with deacetylated/acetylated GAG was examined following this protocol. Deacetylation or acetylation of GAG was performed following the protocols described earlier (Gressler et al., 2019).

Antifungal activity of SP-D against A. Fumigatus

To examine the antifungal activity of SP-D, $1x10^5$ conidia/mL was inoculated in RPMI + 3.45% MOPS + 2% glucose with FL-SP-D or rfhSP-D (30 μg/mL) in a 96-well microtiter plate. The control wells were devoid of SP-D. After incubation at 37 °C for 24 h, the fungal morphology was observed under light microscope (EVOS). Resazurin assay was performed to quantify the fungal growth inhibitory activity of SP-D (Clavaud et al., 2012). Briefly, $1x10^5$ conidia/mL (of *A. fumigatus*

or *A. flavus*) suspended in 1% yeast-extract medium containing 0.03 mg/mL resazurin were seeded into the 96-well microtiter plate (100 μL/well). Two-fold dilutions of rfhSP-D (ranging between 120 and 1.88 μg/mL, dissolved in 1% yeast-extract medium) were added to the corresponding wells (100 μL/well). Here, the mitochondrial respiratory chain of metabolically active *Aspergillus* conidium reduces blue nonfluorescent resazurin into red fluorescent resorufin. Accordingly, after incubating conidia with SP-D at 37 °C for 24 h, the red fluorescent resorufin formed was measured at 569 nm using Tecan Infinite 200 PRO plate-reader (Tecan Group Ltd., Switzerland). Antifungal activity of another humoral immune component, ficolin-2 (FCN-2; 5 μg/mL) (R&D Systems), was also examined against *A. fumigatus* by similar approach. The percentage of growth of the *Aspergillus* species upon treatment with rfhSP-D or FCN-2 was determined by the ratios between the absorbances of the controls (plate wells with only conidia) and SP-D/FCN-2-treated wells (conidia treated with these humoral immune components).

Kinetics of antifungal activity by SP-D

The kinetics of the growth inhibitory activity by SP-D against *A. fumigatus* was examined by time-lapse video microscopy. Conidia ($5x10^5$ per mL) were first incubated in RPMI + 3.45% MOPS + 2% glucose on a shaker at 37 °C maintained at 160 rpm for 2 h to ensure that most of the conidia were swollen. Next, this conidial suspension was transferred to the chambers of 8-well Lab-Tek chamber slide (ThermoScientific) at a final concentration of $1x10^5$ conidia/mL. For SP-D treated sample, 30 μg/mL rfhSP-D was added to the conidial suspension and mixed by pipetting. Live video microscopy was then performed using an ioLight Portable Microscope (x400 1 mm field of view, 1 μm high resolution; ioLight, Hampshire, UK). The microscope was placed in the incubator, to carry out live microscopy at steady 37 °C and 5% CO_2 conditions. Images were recorded via the integrated camera of the microscope, operated with the ioLight App (Vers.: 1.1.1.483). Settings of the ioLight App were as follows: Illumination above from sample 2, Illumination from below sample 2, HD mode ON; Time-lapse frame rate 10 min. Videos and tracking of hyphae growth and hyphal tip branching were prepared using the Fiji software (Schindelin et al., 2012). Length measurements were performed using the "Manual Tracking" plug-in (developed at Institute Curie by F. Cordeli for ImageJ Orsay, France) in Fiji. First, conidia that germinated during the time-lapse were identified and followed throughout the sequence. Only those hyphae with a clear start and endpoint located at the same focal plane were considered for the measurement of lengths. Figures were generated using Fiji software, and Adobe Photoshop (Adobe Systems Incorporated, USA).

Scanning electronic microscopy

A. fumigatus conidia were grown on glass coverslips with/without rfhSP-D (30 μg/mL in Sabouraud medium) for 9 h at 37 °C. Samples were then fixed using 2.5% glutaraldehyde in 0.1 M Cacodylate buffer (pH 7.4) overnight at 4 °C, washed for 5 min three times in 0.1 M Cacodylate buffer (pH 7.2), postfixed for 1 h in 1% osmium and rinsed with distilled water. Cells were dehydrated through a graded ethanol series followed by critical point drying with CO_2. Dried specimens were gold/palladium sputter-coated with a gun ionic evaporator PEC 682. Images were acquired using a JEOL IT700HR field emission scanning electron microscope operating at 5 kV.

Labelling of surface polysaccharides and SP-D on germ tubes after SP-D treatment

On a 8-well Lab-Tek chamber slide (ThermoScientific), $1x10^5$ conidia/mL were incubated with 30 μg/mL of rfhSP-D in Sabouraud medium at 37 °C for 9 h. The culture medium was aspirated, the germ tubes were washed with PBS and fixed in 2.5% paraformaldehyde (PFA) overnight at 4 °C. Blocking of PFA-fixed germ tubes was performed with PBS + 3% BSA for 1 h. The germ tubes were stained by Calcofluor white (CFW) for chitin, or immunolabelled with monoclonal antibodies specific for β-1,3-glucan (Matveev et al., 2019), α-1,3-glucan (Beauvais et al., 2013; Klimpel and Goldman, 1988), GAG (Fontaine et al., 2011) and GM (Stynen et al., 1992). CFW (5 μg/mL) was added to germ tubes, incubated at room temperature for 5 min, washed with PBS to remove excess of CFW, and observed under confocal microscope (LSM 700, Zeiss). For immunolabelling, respective primary antibodies (5 μg/mL in PBS-BSA) and secondary fluorescent-tagged antibodies (1:100 dilutions in PBS-BSA) were used. Between each incubation with primary and secondary antibodies, the germ tubes were washed once with PBS + BSA and then twice with PBS alone. Immunolabelling was observed under confocal microscope. The fluorescence intensities and the area of the germ tubes were evaluated by the aid of Fiji software (Schindelin et al., 2012). The fluorescence intensity normalized by the area of each germ tube was computed for the control (SP-D untreated) and the rfhSP-D-treated samples for each cell wall polysaccharide, to determine differences in their fluorescent intensities. SP-D-treated (37 °C for 9 h) and PFA-fixed germ tubes were also immunolabelled for SP-D upon sequential incubation with primary mouse monoclonal anti-SP-D antibodies (ab17781; Abcam) or polyclonal rabbit serum with anti-SP-D antibodies and secondary mouse TRITC conjugated IgG (Sigma) or rabbit Alexa Fluor 633 conjugated IgG (ThermoScientific), respectively. The positive control was SP-D untreated germ tubes that were PFA-fixed and treated with FL-SP-D (30 μg/mL), followed by labelling with respective primary and secondary antibodies. SP-D untreated and PFA-fixed germ tubes treated with only primary and secondary antibodies served as a negative control.

Assaying the cell wall permeability of SP-D-treated A. fumigatus germ tubes

Conidia ($1x10^5$/mL) were co-cultured with 30 μg/mL of rfhSP-D in Sabouraud medium at 37 °C in a 8-well Lab-Tek chamber slide (ThermoScientific) for 16 h. After aspirating the medium, the germ tubes were incubated with CFW (5 μg/mL) for 15 min at ambient temperature. Next, the medium was discarded, the germ tubes were washed with PBS (3X) to remove excess of CFW, suspended in PBS and observed under confocal microscope (LSM 700, Zeiss).

Antifungal susceptibility of SP-D treated A. fumigatus germ tubes

Conidia (1×10^5/mL; 100 μL/well) were co-cultured with 10 μg/mL of rfhSP-D in 1% yeast-extract medium in a 96-well cell culture plate at 37 °C for 9 h. Following, the wells were added with two-fold dilutions of voriconazole (1.6 to 0.001562 μg/mL; 100 μL/well) in 1% yeast-extract medium containing 0.03 mg/mL resazurin and incubated at 37 °C. Control samples were the germ tubes grown in yeast-extract medium at 37 °C for 9 h without rfhSP-D and then treated with two-fold dilutions of voriconazole. The absorbance at 569 nm (due to the conversion of resazurin into red florescent resorufin by metabolically active germ tubes) was recorded using Tecan Infinite 200 PRO plate-reader after 24 h incubation. The percent growth of *A. fumigatus* co-cultured with voriconazole and rfhSP-D was determined by the ratios between the absorbance readings of the wells treated with voriconazole and voriconazole + rfhSP-D.

Cytokines released by PBMCs co-incubated with SP-D-treated or untreated hyphae

Conidia ($1x10^4$/well) were incubated in 96-well microtiter plates with 30 μg/mL rfhSP-D or FL-SP-D for 16 h at 37 °C. Following, the culture medium was removed, hyphae were washed with PBS, and fixed in 2.5% PFA overnight at 4 °C. Control hyphae were prepared similarly in the absence of SP-D. Blood samples of healthy donors were obtained from the Etablissement Français du Sang (Paris, France) with written

informed consent as per the guidelines provided by the Institutional Ethics Committee, Institut Pasteur (convention 12/EFS/023). Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples by Ficoll density-gradient separation (Eurobio, France). PBMCs ($2x10^6$ cells/well) in complete RPMI [containing 10% pooled normal human serum (ZenBio)] were added to the fixed hyphae and incubated at 37 °C, 5% CO_2. The cell-free supernatant was collected after one and five days of co-incubation and stored at −20 °C. TNF-α, IL-1β, IL-6 (pro-inflammatory), IL-10 (an anti-inflammatory cytokine) and IL-8 (chemokine) levels in the culture supernatants were measured by using ELISA kits (DuoSet, R&D Systems).

Statistics

Statistical analyses were performed using the Prism software (version 8.2.1) by Student's *t*-test and one-way ANOVA; a *p*-value of less than 0.05 was considered significant.

Results

SP-D recognizes galactomannan (GM) and galactosaminogalactan (GAG)

Earlier, we showed that the SP-D binds to germinating morphotype of *A. fumigatus*, recognizing its cell wall polysaccharides GM and GAG as the ligands (Madan et al., 1997; Wong et al., 2018). The carbohydrate recognition domain (CRD) of SP-D was involved in the GM and GAG binding. SP-D is a multimeric protein; therefore, we checked the importance of its oligomerization in GM or GAG binding. Both FL-SP-D (capable of multivalent oligomerization) and rfhSP-D (forming only a trimer) could bind to both GM and GAG (Fig. 1A and B), although the binding capacity towards GM was lower compared to GAG. Moreover, rfhSP-D showed significantly lower binding efficiency towards both GM and GAG compared to FL-SP-D, suggesting that the degree of oligomerization of SP-D plays an important role during its interaction with GM or GAG.

Native GAG is a heteropolysaccharide composed of galactose and two aminohexoses, *N*-acetyl-galactosamine and galactosamine; the *N*-acetyl-galactosamine (degree of acetylation) accounts for 66 ± 7% (Gressler et al., 2019). Although FL-SP-D could still bind to completely deacetylated GAG, there was an increase in binding along the increasing degree of acetylation. rfhSP-D showed poor binding to deacetylated GAG, and there was a significant increase in its binding with increase in the *N*-acetyl-galactosamine content (Fig. 1C). This suggested that the increasing acetylation in GAG favors its interaction with trimeric form of human truncated SP-D.

SP-D inhibits the growth of A. fumigatus

The physiological concentration of SP-D in human alveolar fluid ranges between 50 and 90 μg/mL (van Rozendaal et al., 2000). Incubation with FL-SP-D or rfhSP-D (at a concentration of 30 μg/mL) could significantly inhibit the germination of *A. fumigatus* conidia when observed under bright-field microscope (Fig. 2A). Morphologically, SP-D-treated hyphae were shorter in length and branched compared to untreated hyphae. The hyphal growth inhibitory effect of FL-SP-D was higher compared to rfhSP-D. However, unless otherwise stated, further experiments were performed using rfhSP-D owing to the ease of producing this shorter trimeric form. The growth inhibitory activity of SP-D was not restricted to the *A. fumigatus* clinical isolate CBS 144–89. It showed growth inhibition of another clinical isolate of *A. fumigatus*, CNRMA 15.354 (Fig. 2B). We used another FL-SP-D preparation (ab152069; expressed in HEK 293 cells), which also restricted the growth of *A. fumigatus* hyphae (Fig. 2D). Altogether, these data suggested that SP-D could inhibit the fungal growth irrespective of the expression system used to produce it and independent of the *A. fumigatus* isolates.

We then quantified the growth inhibition of *A. fumigatus* with a range of rfhSP-D concentration by resazurin method (Clavaud et al., 2012). After 24 h of co-incubation, rfhSP-D at a concentration of 15 μg/mL and above resulted in 80–90% decrease in the growth of *A. fumigatus* hyphae (Fig. 2C). This growth inhibition of *A. flavus* was observed only at much higher rfhSP-D concentrations (60–120 μg/mL). Recently, we showed that the localization of GAG in these two *Aspergillus* species was different (Wong et al., 2021b), which could be the reason for differences in this growth inhibitory activity of SP-D. There was a lack of growth inhibitory effect by ficolin-2 (FCN-2), another humoral component structurally

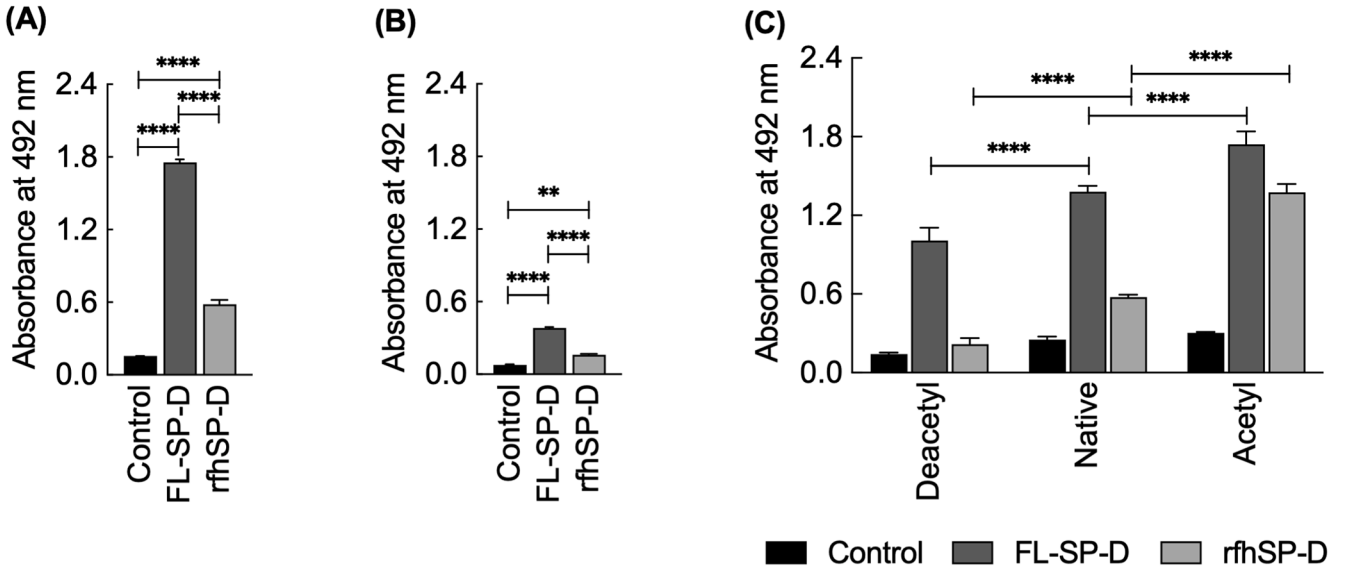

Fig. 1. SP-D binds to *A. fumigatus* cell wall polysaccharides (A) galactosaminogalactan (GAG) and (B) galactomannan (GM). FL-SP-D/rfhSP-D suspended in 50 mM carbonate buffer (pH 9.6) was coated on the microtiter plate wells (500 ng/well) and added with GAG or GM (500 ng/well) in PBS containing 3% BSA-5 mM $CaCl_2$. Interaction between polysaccharides and SP-D was examined by sequential incubation with monoclonal anti-GAG/-GM antibodies, peroxidase-conjugated secondary antibody and *O*-phenylenediamine as the substrate (with PBS washings between each step). The absorbance of the colour developed was read at 492 nm. Students' t-test was performed to evaluate the differential binding of FL-SP-D and rfhSP-D with GM/GAG (**$p<0.005$, ****$p<0.0001$). (C) Following the same protocol, the binding efficiencies of FL-SP-D and rfhSP-D with deacetylated (deacetyl), native and acetylated (Acetyl) GAGs were checked.

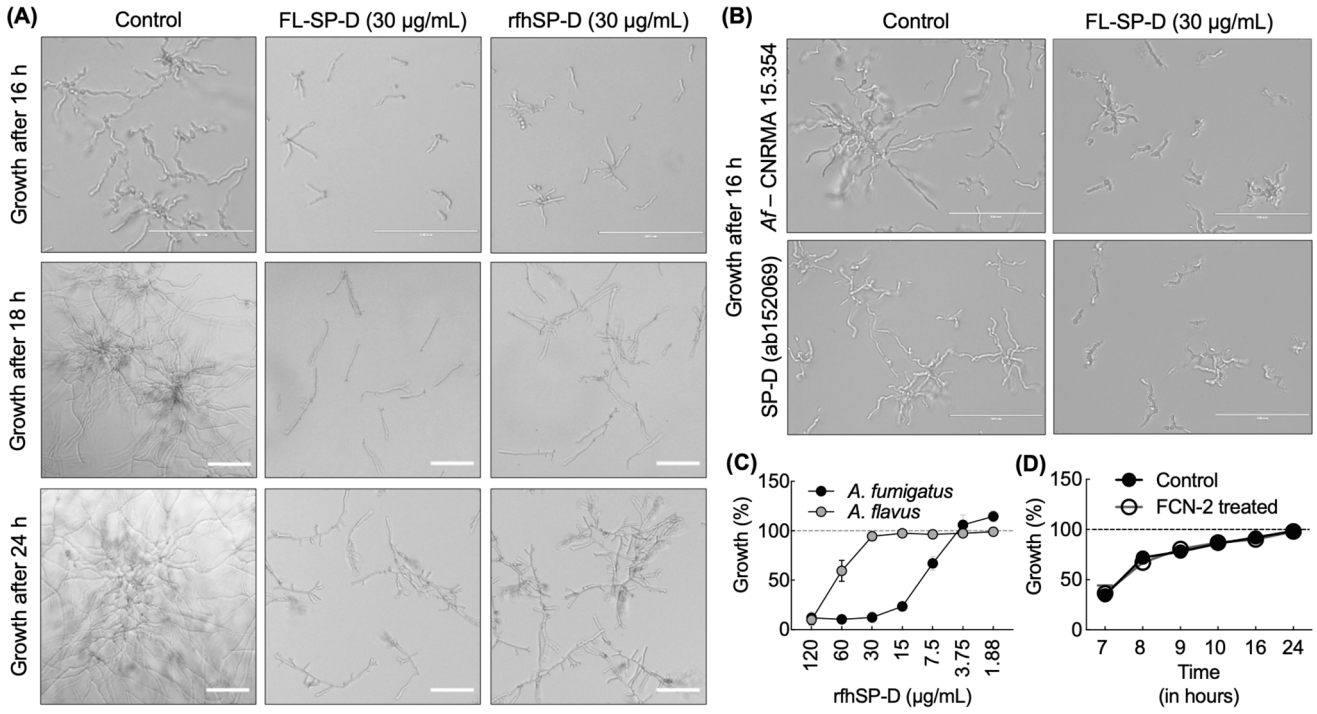

Fig. 2. Growth inhibition of *A. fumigatus* by SP-D. (A) *A. fumigatus* conidia (1x10^5/mL) were incubated with FL-SP-D/rfhSP-D (30 µg/mL) in RPMI + 3.45% MOPS + 2% glucose at 37°C. The fungal growth was monitored under light microscope after 16-24 h (scale bar = 100 µm). FL-SP-D exhibited stronger growth inhibitory effect than rfhSP-D. (B) Growth inhibitory effect of SP-D was tested against another clinical isolate of *A. fumigatus,* CNRMA 15.354 and using other source of SP-D (ab152069, Abcam) against *A. fumigatus* isolate CBS 144-89. (C) Growth inhibition by SP-D on *A. fumigatus* and *A. flavus* was examined by resazurin method using 1% yeast-extract + 2% glucose medium with a range of rfhSP-D concentration (error bars, mean ± SEM); after co-incubation of the contents at 37°C for 24 h, optical density was measured at 569 nm. (D) *A. fumigatus* growth inhibition by a humoral component, ficolin-2 (FCN2; 5 µg/mL (>physiological concentration in human alveolar fluid) by resazurin method, performed in 1% yeast-extract+2% glucose medium) was examined after 24 h of co-incubation of the contents at 37°C, and reading the optical density at 569 nm.

similar to SP-D (Holmskov et al., 2003). This suggested that the *A. fumigatus* hyphal growth inhibitory effect was specific to SP-D (Fig. 2D). Furthermore, replenishment of rfhSP-D at 8 h intervals (15 µg/mL at each interval) could maintain the growth inhibition of *A. fumigatus* (Fig. 3A).

SP-D inhibits the growth of A. fumigatus by restricting the hyphal length

Using live microscopy, we examined the kinetics of hyphal growth inhibition and the effect of rfhSP-D (30 µg/mL) on the hyphal length of *A. fumigatus* (Supplementary data; Video 1). Approximately after 3 h of incubation, conidia started to filament (Fig. 3B). The germination starting time-point was not significantly different between control (201 ± 51 min) and SP-D treated conidia (207 ± 56 min) (Fig. 3C). Starting from 6 h of incubation, the growth inhibition by rfhSP-D was effective in terms of restricting the hyphal length. The hyphal length was quantified by Fiji at the end of the growth experiment by live microscopy (8 h), with control hyphae being significantly longer (199.9 ± 81.2 µm) than SP-D-treated hyphae (56.2 ± 32.7 µm) (Fig. 3D). We also observed a difference in the hyphal tip branching upon SP-D treatment: 28.9% of the SP-D-treated hyphae showed branching (number of conidia quantified, n = 90; three independent experiments) compared to 4.3% in the control sample (n = 93, from three independent experiments). A representative live microscopy video is presented (Supplementary data; Video 2). Taken together, the major effect of rfhSP-D on *A. fumigatus* appears to be in restricting the hyphal length and leading to branching of hyphal tips, rather than suppressing or delaying the breaking of conidial dormancy.

SP-D alters A. fumigatus germ tube surface morphology and polysaccharide exposure

We further examined the surface morphology of the SP-D-treated germ tubes by scanning electronic microscopy (SEM). The SEM images revealed that the control *A. fumigatus* germ tubes were longer with few branches, while SP-D-treated germ tubes were presented with shorter length and some of them with branched tips (Fig. 4). Germ tube branching was also quantified using SEM images: branching in SP-D-treated germ tubes was 36.4% (number of hyphae counted, n = 32), while 5.9% of the untreated germ tubes showed branching (n = 17). The high magnification SEM images (at 15,000x magnification) indicated that the untreated (control) germ tubes had a smoother surface. SP-D treatment resulted in germ tubes showing rougher/deformed cell surface compared to untreated (control) germ tubes. The SEM images further confirmed and extended our live imaging observations that the untreated germ tubes were longer with a smooth surface compared to a rough surface and with more branched tips in the rfhSP-D-treated germ tubes.

Altered morphology of the *A. fumigatus* germ tubes upon SP-D treatment led us to examine surface exposure of the different cell wall polysaccharides. SP-D-treated germ tubes were shorter in length and hyperbranched; therefore, the area of the germ tubes was computed for both untreated and SP-D-treated germ tubes and used to normalize the fluorescence intensity. Quantification of fluorescent intensities indicated that SP-D-treated germ tubes showed significantly lower exposure of β-1,3-glucan and higher chitin, α-1,3-glucan, and GAG exposures on their surfaces compared to the germ tubes grown in the absence of SP-D (Fig. 5). The abundance of GM on the SP-D-treated germ tube surfaces was comparable to that of untreated germ tubes (Fig. 5).

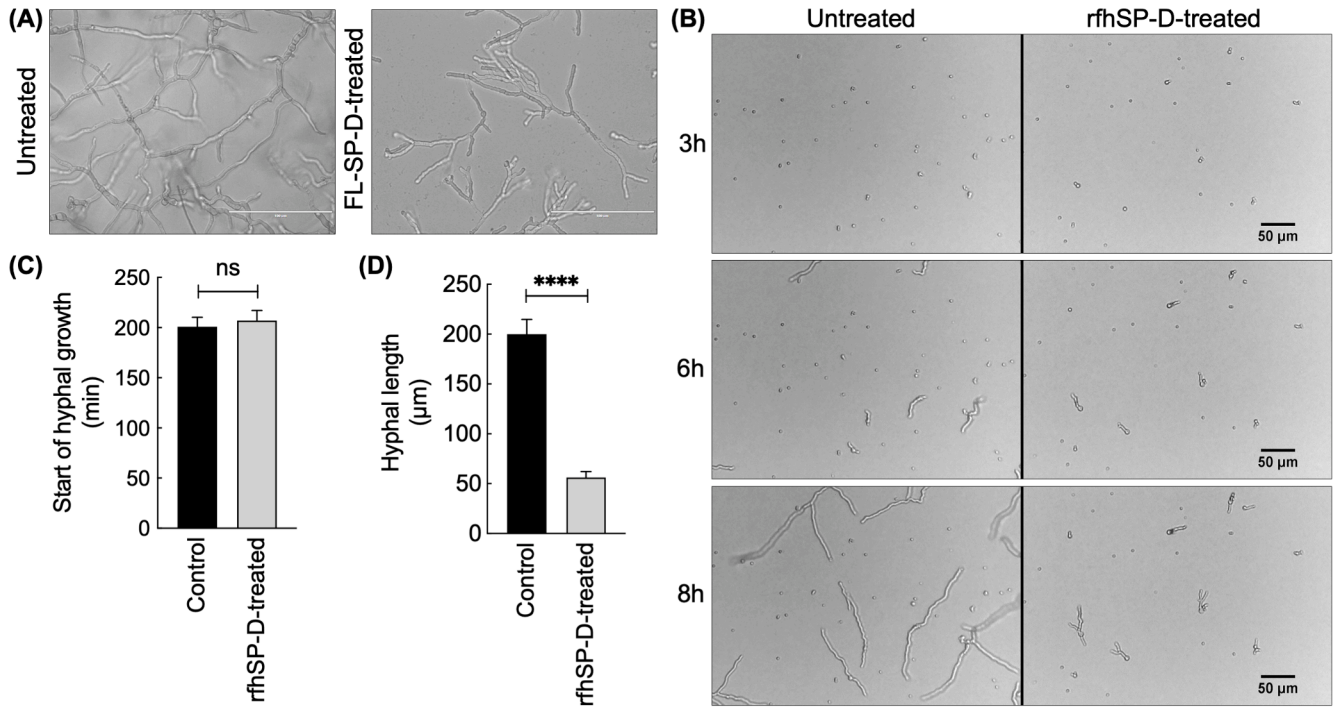

Fig. 3. Growth inhibition of *A. fumigatus* by SP-D upon replenishment, and kinetics of growth inhibition. (A) rfhSP-D (15 μg/mL) was added to conidia ($1x10^5$/mL) in RPMI + 3.45% MOPS + 2% glucose and incubated at 37°C. At 8 h-intervals, culture medium was aspirated, fresh medium with rfhSP-D (10 μg/mL) was added, incubation continued and the growth at 24 h was monitored under light microscope (scale bar = 100 μm). (B) Screen shots of live microscopy at 3 h, 6 h and 8 h of co-incubation of conidia and rfhSP-D. Starting from 6 h, the hyphal length of untreated control was longer than rfhSP-D-treated hyphae. (C) The hyphal length at 8 h was quantified by Fiji. The hyphal germination starting time was not significantly different between control (201 ± 51 min) and rfhSP-D (207 ± 56 min) treated conidia. (D) The control hyphae were significantly longer (199.9 ± 81.2 μm) than the rfhSP-D-treated hyphae (56.2 ± 32.7 μm) at the end of the incubation (8 h). Quantification was performed for six independent experiments (number of quantified conidia = 60). The bars and the error bars indicate the means and standard error of the mean, respectively. Students' t-test was performed to examine the difference between control and rfhSP-D-treated samples (ns – not significant, ****$p<0.0001$).

SP-D treatment modulates the cytokine expressions against A. fumigatus

The combined effect of SP-D on *A. fumigatus* including inhibition of hyphal growth and modulation of cell surface exposure of polysaccharides could lead to an altered immune response. We, therefore, examined the stimulation of cytokine secretion by co-incubating PBMCs with fixed hyphae grown with/without rfhSP-D or FL-SP-D. After one day and five days of co-incubation, the levels of pro-/anti-inflammatory cytokines (TNF-α, IL-6, IL-1β and IL-10) and chemokine (IL-8) were evaluated in the cell-free culture supernatants. Hyphae obtained after FL-SP-D or rfhSP-D treatment induced significantly lower levels of the pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) as compared to the untreated germ tubes after one day and five days of co-culturing with PBMCs (Fig. 6). Hyphae treated with FL-SP-D induced significantly lower cytokine secretions compared to those induced by rfhSP-D-treated hyphae. On the other hand, FL-SP-D and rfhSP-D treated germ tubes significantly increased the secretion of IL-10, an anti-inflammatory cytokine, after both one day and five days of co-culturing with an increase in the level from one day to five days (Fig. 6). Whereas IL-8 secretion was significantly higher after one day of co-culturing with PBMCs for FL-SP-D/rfhSP-D treated germ tubes compared to untreated germ tubes (Fig. 6), suggesting immunomodulatory effect by SP-D.

PBMCs were also stimulated with SP-D-untreated germ tubes after 10 h of growth, which had lengths comparable to the germ tubes obtained after 16 h of growth in presence of SP-D. This was performed to study the influence of fungal mass on immunostimulatory capacity, as, SP-D treatment resulted in a decreased fungal mass. After one day, the culture supernatants of PBMCs stimulated with 10 h-grown untreated germ tubes contained 6601.35 ± 527.95 pg/mL of TNF-α and 9006.94 ± 777.78 pg/mL of IL-6. Supernatants of 16 h-grown untreated germ tubes and PBMCs co-cultures had 7588.03 ± 1140.41 pg/mL of TNF-α and 10769.79 ± 2914.29 pg/mL of IL-6 (Fig. 6). Although slightly lower, these pro-inflammatory cytokine secretions by PBMCs stimulated with 10 h-untreated germ tubes were not significantly different compared to PBMCs stimulated with 16 h-untreated germ tubes. This suggests that the reduced fungal mass of *A. fumigatus* due to SP-D treatment could be one of the minor factors contributing to reduced pro-inflammatory immune response.

SP-D is deposited on the germ tube surfaces

We sought to check if SP-D added initially to the conidial culture remained bound to the germ tubes. There was a uniform positive immunolabelling for SP-D on the surface of germ tubes grown in the presence of FL-SP-D/rfhSP-D for 9 h (Fig. 7A), suggesting the deposition of SP-D on germ tube surfaces. On the other hand, when germ tubes, grown in the absence of SP-D, were subsequently treated with SP-D and immunolabelled, there was the labelling of the germ tubes but not the conidial head (marked with arrow; Fig. 7A). SP-D recognizes conidial surface-exposed melanin pigment as well as germ tube surfaces GM/GAG as the ligands (Wong et al., 2018). Therefore, SP-D added to the conidial culture can interact with all these three cell surface ligands. On the other hand, SP-D added to the germ tubes can only bind to the surface exposed GM and GAG. There was also positive immunolabelling for SP-D on the germ tubes grown in the presence of SP-D for 16 h (Fig. 7B).

SP-D-treated germ tubes are more susceptible to antifungals

Modification in the cell surfaces and the exposure of cell wall polysaccharides upon SP-D treatment allowed us to test their cell wall permeability and susceptibility to antifungal treatment in comparison

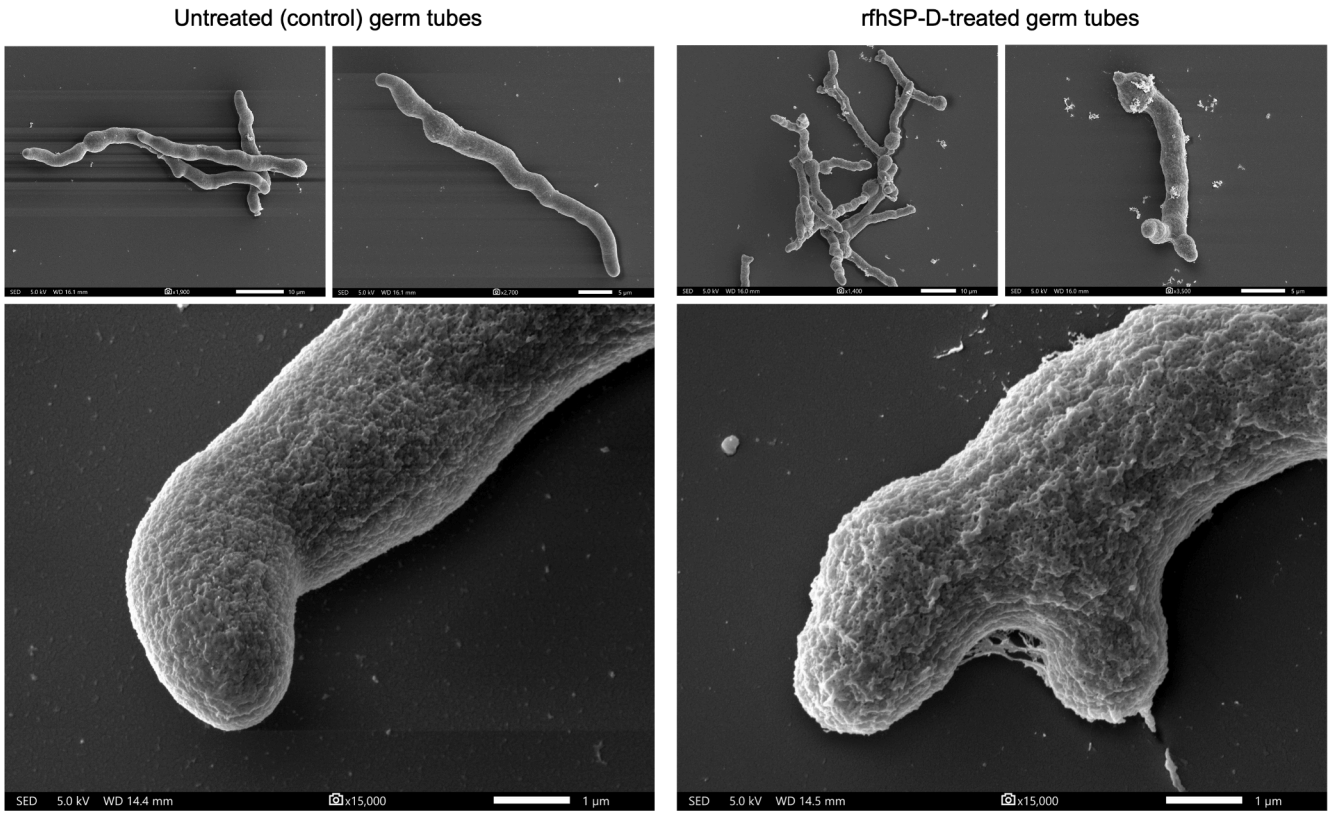

Fig. 4. Scanning Electronic Microscopic images of rfhSP-D treated *A. fumigatus* germ tubes. Control (untreated) germ tubes were longer with smooth surfaces, while rfhSP-D-treated germ tubes were comparatively shorter with rougher surfaces and branched hyphal tips; a closer examination of the surface showed more disorganized architecture compared to untreated germ tube.

with untreated germ tubes. We first examined the difference in the permeability of the untreated and SP-D-treated germ tubes. Upon incubation with CFW for a longer duration (15 min instead of 5 min as in Fig. 5), there was fluorescence in the cytoplasmic compartment of the SP-D-treated germ tubes unlike untreated germ tubes (Fig. 8A). This suggested that SP-D treatment indeed alters the germ tube cell wall permeability. Secondly, upon sequential incubation of conidia with SP-D (for 9 h) and voriconazole (an antifungal drug; for furthermore 3 h), there was a significant decrease in the growth (measured by metabolic activity) compared to control germ tubes incubated with voriconazole. This assay suggested that SP-D treatment facilitated the antifungal activity of voriconazole (Fig. 8B).

Discussion

In this study, we demonstrate that SP-D, a *C*-type lectin belonging to the family of collectins and one of the major humoral immune components in the lung alveoli, has a direct growth inhibitory effect on *A. fumigatus*. We used three different fungal culture media in our study (RPMI + MOPS + glucose, yeast-extract and Sabouraud). There was hyphal growth inhibition regardless of the medium used, suggesting that the observed effect by SP-D is independent of the fungal culture environment. FL-SP-D showed higher GM and GAG binding efficiency compared to rfhSP-D [which is composed of a short collagen-like stalk, neck, and carbohydrate recognition domain (CRD) regions]. FL-SP-D has the native dodecameric structure with collagen-like stalks, whereas rfhSP-D forms only a trimeric structure. It suggests that the oligomeric status of SP-D is important for binding efficiency with GAG and GM. The degree of oligomerization of SP-D was shown to be proportional to its *C*-type lectin activity. SP-D with higher degree of oligomerization is more efficient in binding to and aggregation of microbial pathogens (Arroyo et al., 2020a; Zhang et al., 2001). The difference in the *A. fumigatus* growth inhibitory abilities of FL-SP-D and rfhSP-D was also consistently observed in this study. Nevertheless, we mainly used rfhSP-D for our study given the ease of rfhSP-D production. The fact that the trimeric rfhSP-D can also exert anti-*Aspergillus* effect highlights the importance of functional modularity within the CRD region (Kaur et al., 2018a; Kaur et al., 2018b; Madan et al., 2001).

SP-D has been shown to inhibit the growth of Gram-negative bacteria and fungal pathogens such as *Candida albicans* and *Histoplasma capsulatum* (Madan and Kishore, 2020; McCormack et al., 2003; van Rozendaal et al., 2000; Wu et al., 2003). The possible direct effect of SP-D on *A. fumigatus* has never been explored. In this study, we demonstrate that SP-D exhibits fungistatic activity against *A. fumigatus* in a concentration-dependent manner. Replenishment of SP-D could sustain the growth inhibition of *A. fumigatus*. Administration of exogenous SP-D has been demonstrated to increase the survival of immunocompromised mice with IPA induced by intranasal inoculation of *A. fumigatus* conidia (Madan et al., 2001; Singh et al., 2009). The data herein suggested that the direct fungistatic activity of SP-D on *A. fumigatus* could be associated with one of its protective mechanisms *in vivo*.

Previously, we showed that the *A. fumigatus* cell wall melanin, GM, and GAG are the ligands of SP-D (Wong et al., 2018). Here, we observed that the growth inhibitory effect of SP-D was more profound against *A. fumigatus* compared to *A. flavus*. *A. fumigatus* is the most ubiquitous *Aspergillus* species responsible for IPA, followed by *A. flavus* (Sugui et al., 2015). While melanin and GAG are present in the cell wall of both *A. fumigatus* and *A. flavus*, the GAG of *A. flavus* is not exposed on the hyphal surface as in *A. fumigatus* (Wong et al., 2021b). Furthermore, melanin pigments produced by these two *Aspergillus* species are different. *A. fumigatus* produces 1,8-dihydroxynaphthalene-melanin, whereas 3,4-dihydroxyphenylalanine-melanin is produced by *A. flavus* (Pal et al., 2013). Therefore, we could hypothesize that the differences in the localization/composition of these cell wall PAMPs lead to the

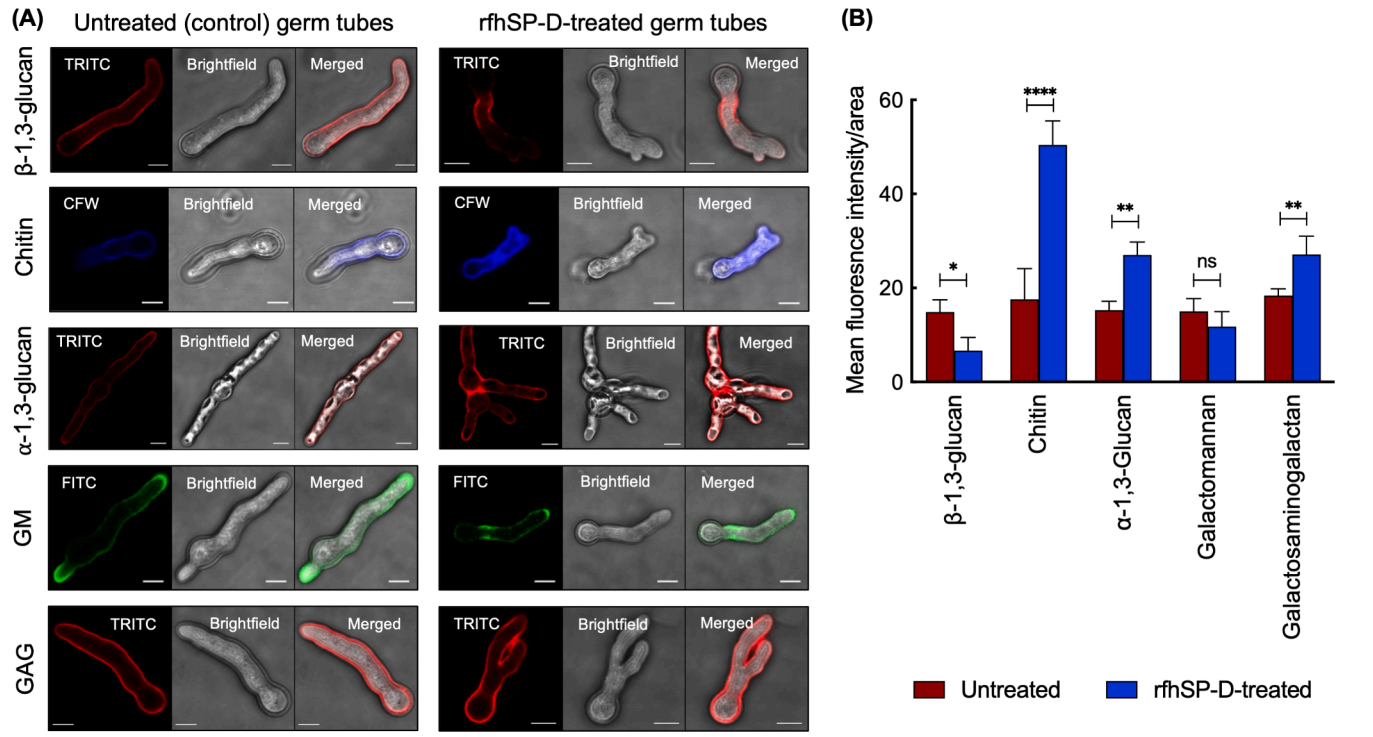

Fig. 5. Immunolabelling of the cell wall polysaccharides of rfhSP-D-treated *A. fumigatus* germ tubes. (A) Control or rfhSP-D-treated germ tubes were prepared on a 8-well Lab-Tek chamber slide in RPMI+3.45% MOPS+2% glucose medium by incubating at 37°C for 9 h. The germ tubes were then fixed in 2.5% PFA overnight at 4°C and immunolabelled by respective monoclonal antibodies specific for β-1,3-glucan, α-1,3-glucan, GAG, GM or stained with Calcofluor white for chitin (scale bar = 5 μm). (B) The mean fluorescence intensity (MFI) and area of the germ tubes were determined using the Fiji software. MFI per area of each germ tube was presented as bars graph, and at least ten germ tubes were measured for the calculation of their fluorescent intensities. Students' t-test was performed to examine the statistical difference between the control and rfhSP-D-treated germ tubes (ns – not significant, *$p<0.05$, **$p<0.01$, ****$p<0.001$).

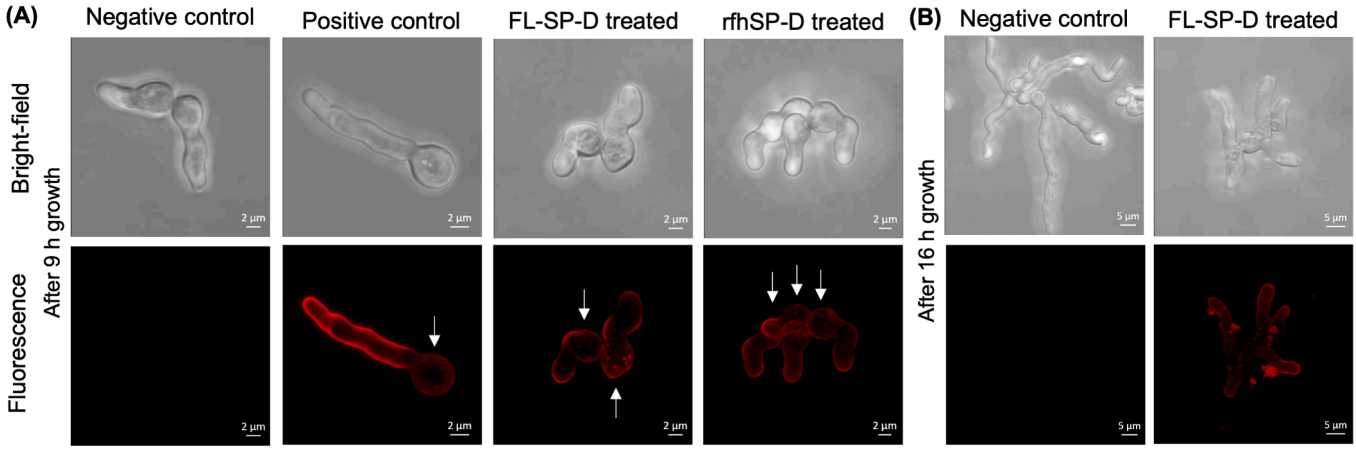

Fig. 6. SP-D gets deposited on the *A. fumigatus* germ tubes/hyphae during the course of germination. *A. fumigatus* germ tubes were prepared on a 8-well Lab-Tek chamber slide well by incubating conidia ($1x10^5$/mL, 200 μL/well) in RPMI + 3.45% MOPS + 2% glucose with/without FL-SP-D or rfhSP-D (30 μg/mL) at 37°C for 9-16 h. SP-D treated germ-tubes were then PFA fixed and immunolabelled upon sequential incubation with primary rabbit polyclonal anti-SP-D antibodies (serum) or mouse monoclonal anti-SP-D antibodies and secondary rabbit Alexa Fluor 633 conjugated IgG or TRITC conjugated mouse IgG, respectively. (A) and (B) are immunolabelling of 9 h and 16 h grown germ tubes for SP-D. Positive control: SP-D untreated germ tubes that were PFA fixed and treated with FL-SP-D (30 μg/mL) followed by immunolabelling with primary and secondary antibodies. Negative control: SP-D untreated germ tubes, PFA fixed and treated with primary and secondary antibodies.

differential growth inhibitory effect of SP-D on the two *Aspergillus* species. Although IPA due to non-*fumigatus Aspergillus* species is less common, these species show varying susceptibility to different antifungal drugs (Gheith et al., 2014; Pinto et al., 2018; Torres et al., 2003). Therefore, elucidation of growth inhibitory effect of SP-D on non-*fumigatus Aspergillus* species is warranted.

In this study, we observed that SP-D inhibits the germination of *A. fumigatus* mainly through limiting its hyphal growth, similar to its effect on another hypha producing fungal pathogen, *Candida albicans* (van Rozendaal et al., 2000). Due to restricted hyphal growth and branched morphology of the hyphae after SP-D treatment, we hypothesized that SP-D influenced the surface architecture of *A. fumigatus* germ tubes. Indeed, we observed that SP-D treatment alters the surface exposure of the different cell wall polysaccharides of the germ tubes. SP-

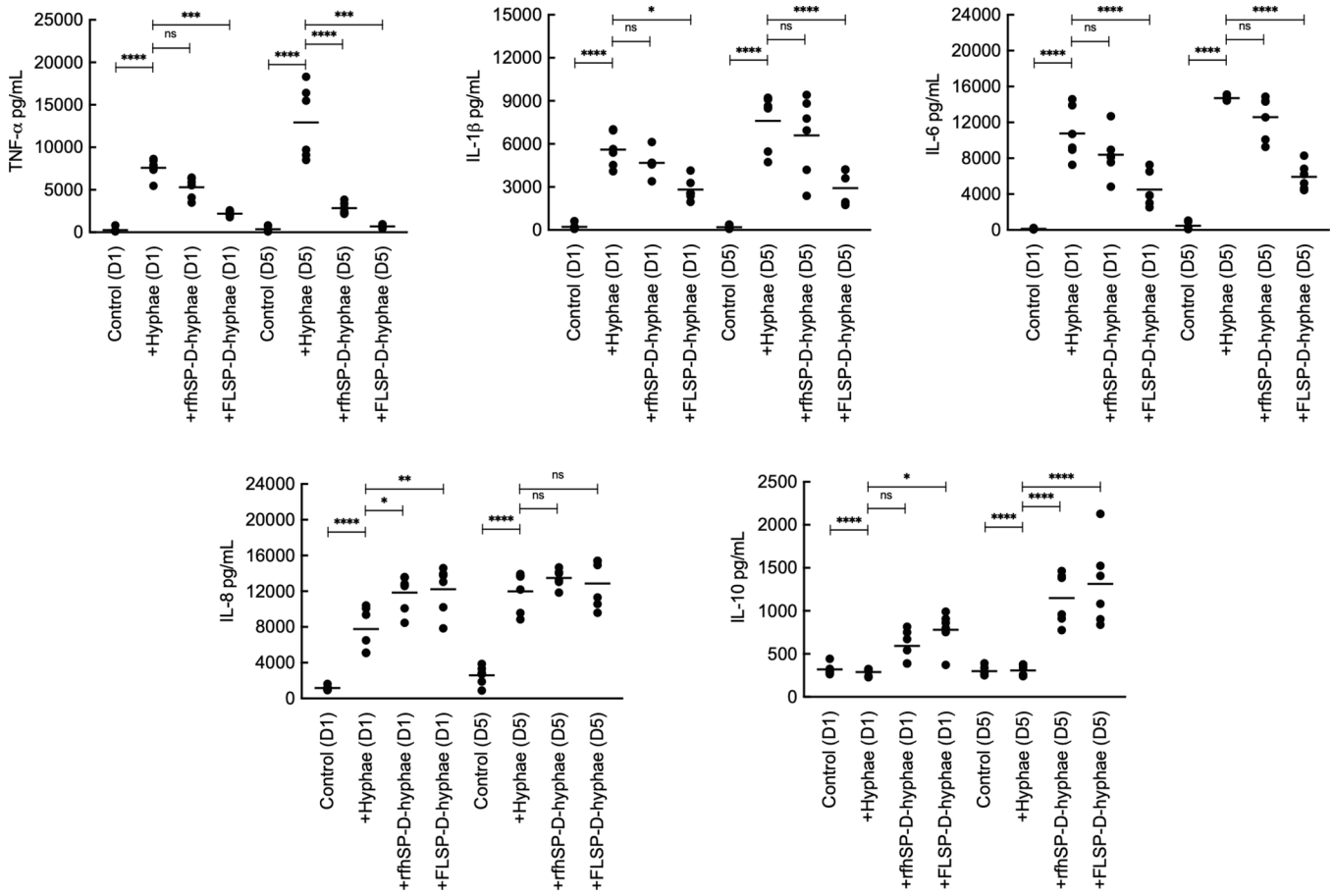

Fig. 7. SP-D treatment modifies immune stimulatory capacity of *A. fumigatus* hyphae. Conidia ($1x10^4$/well) were incubated with 30 μg/mL rfhSP-D or FL-SP-D for 16 h; the culture medium was removed, the hyphae were washed and fixed in 2.5% PFA. Control hyphae were prepared in the absence of SP-D. Human PBMCs ($2x10^6$ cells/well; n=6) were added to the fixed hyphae in RPMI with 10% normal human serum. After incubation at 37°C with 5% CO_2 for 1-day (D1) and 5-days (D5), cell-free supernatants were collected for measuring the cytokines. One-way ANOVA with Tukey's multiple comparisons test was performed for statistics (ns - not significant; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

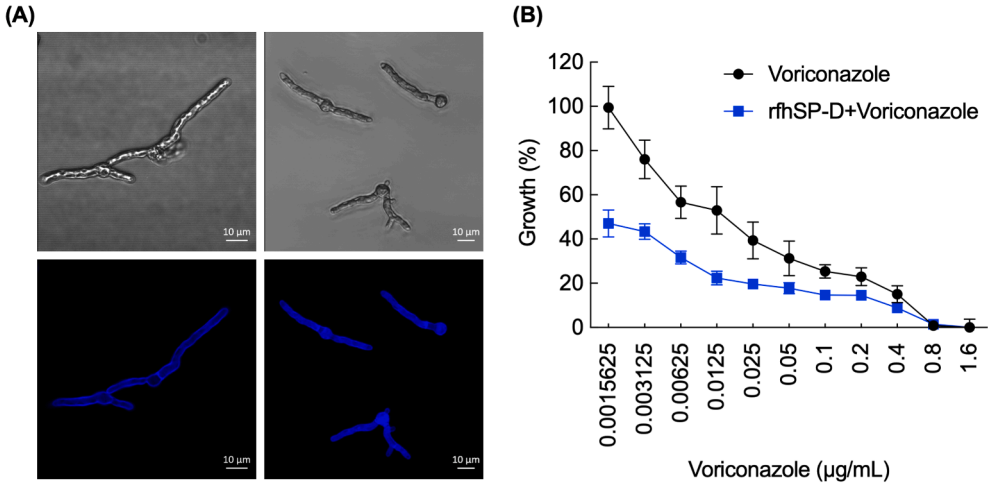

Fig. 8. (A) Incubation with SP-D increases the permeability of *A. fumigatus* germ tubes. Control and rfhSP-D-treated (30 μg/mL) germ tubes were prepared on 8-well Lab-Tek chamber slide well in 1% yeast-extract medium at 37°C for 16 h. Following, they were stained with Calcofluor white (5 μg/mL) for 15 min, washed with and suspended in PBS and observe under confocal microscope. (B) SP-D treatment facilitated the antifungal efficacy of voriconazole towards *A. fumigatus*. Control and rfhSP-D-treated (10 μg/mL) germ tubes were prepared in 1% yeast-extract medium at 37°C for 9 h and then treated with voriconazole (at two-fold dilutions, between 1.6-0.0015625 μg/mL) in 1% yeast-extract medium containing resazurin (0.03%). After incubating the contents at 37°C for 24 h, the optical densities were measured at 569 nm using a Tecan plate reader. The percent growth inhibition of voriconazole and rfhSP-D + voriconazole treated hyphae was calculated considering optical density of both rfhSP-D and voriconazole untreated hyphal growth as 100%.

D-treated germ tubes showed increased permeability to CFW. Defect in the β-glucan layer has been suggested to increase the porosity of the fungal cell wall (Birkaya et al., 2009). There was altered β-glucan exposure upon SP-D treatment, which could explain the increased permeability of SP-D-treated hyphae of *A. fumigatus*. Higher permeability indicates a disrupted cell wall integrity, and thus, could be more

accessible to antifungal drug function (Birkaya et al., 2009; Gow and Hube, 2012). In accordance, we show that SP-D treatment facilitates the efficacy of antifungal drug, voriconazole that has its target in the fungal cytoplasm (Geissel et al., 2018). This observation warrants investigation of antifungal susceptibility profiles of azole-resistant clinical isolates of *A. fumigatus* in presence of SP-D.

The major components of fungal cell wall are the polysaccharides that serve as pathogen-associated molecular patterns (PAMPs) recognized by host PRRs (Garcia-Rubio et al., 2020; Latgé, 2010). Due to differential exposure of the cell wall polysaccharides between SP-D-treated and untreated germ tubes of *A. fumigatus*, we sought to investigate its impact on host immune response. To limit immune response due to fungal metabolic activity, untreated/SP-D-treated hyphae were inactivated before co-incubating them with PBMCs. As PBMCs are a mixture of innate and adaptive immune cells, cell-free supernatants were collected on day-one and five days after the co-incubation. There was a significant decrease in the secretion of pro-inflammatory cytokines from PBMCs incubated with *A. fumigatus* hyphae treated with SP-D, compared to untreated hyphae, after one day and five days of co-culturing. FL-SP-D-treated hyphae induced even lower level of cytokines when compared to those induced by the rfhSP-D-treated hyphae. This could be due to the relatively higher growth inhibitory effect of FL-SP-D compared to rfhSP-D. Here, it is important to note that SP-D-treated germ tubes were shorter in length due to the restricted growth. This leads to the argument that a lower fungal mass interacting with PBMCs would be responsible for decreased stimulation of pro-inflammatory cytokine secretions. However, compared to untreated germ tubes, SP-D-treated germ tubes resulted in increased secretions of IL-10 (an anti-inflammatory cytokine) and IL-8 (a chemokine) by PBMCs. This suggests that the lower fungal mass, altered surface exposure of cell wall polysaccharides and masking of cell wall polysaccharide PAMPs upon SP-D treatment together contribute to the altered immunomodulatory effect of hyphae, compared to control hyphae.

Pro-inflammatory cytokines are crucial in anti-*Aspergillus* activity (Cenci et al., 1997; Stevens, 2006). Previously, we have demonstrated that SP-D exerts a pro-inflammatory immune response against conidial morphotype of *A. fumigatus*, resulting in their clearance (Wong et al., 2018). However, aspergillosis could also occur in the settings of excess inflammation that could directly lead to host tissue damage, as seen in chronic granulomatous disease patients (Carvalho et al., 2012; Segal and Romani, 2009). Therefore, there is a need for a balanced immune response against inhaled *A. fumigatus* conidia. In healthy individuals, resident alveolar macrophages are the phagocytes clearing inhaled conidia. However, some conidia may escape from macrophages and initiate germination, exposing their cell wall PAMPs that can stimulate an excessive pro-inflammatory immune response (Pinto et al., 2008; Snarr et al., 2017). In this context, our study adds up another anti-*Aspergillus* mechanism by SP-D via altering the exposure of cell wall PAMPs during conidial germination. In our study, SP-D treatment decreased exposure of β-(1,3)-glucan on the *A. fumigatus* germ tubes. While β-(1,3)-glucan stimulates pro-inflammatory cytokine secretion (Steele et al., 2005). α-(1,3)-Glucan, chitin and GAG exposures were increased on the germ tube surfaces upon SP-D treatment; these cell wall polysaccharides have an anti-inflammatory properties (Becker, et al., 2016; Gressler et al., 2019; Stephen-Victor et al., 2017). Together, our previous and present studies allow us to propose that SP-D exerts morphotype specific anti-*A. fumigatus* mechanisms.

An opportunistic pathogen like *A. fumigatus* can establish infection when the host defense system is suppressed or compromised. Our recent study comparing the proteomics of bronchoalveolar lavage fluid (BALF) from control and *A. fumigatus*-infected and colonized hosts indicated that SP-D was one of the key humoral immune components depleted in the infected and colonized BALF (Delliere et al., 2021). One of the reasons for this depletion could be due to the degradation of SP-D. *Pseudomonas aeruginosa*, a Gram-negative bacteria, was shown to secrete an elastase that can degrade SP-D, abrogating its immunological functions (Alcorn and Wright, 2004). We and others have described that *A. fumigatus* also secretes an aspartic protease Alp1p and a metalloprotease Mep1p, which can degrade a number of the humoral immune components (Behnsen et al., 2010; Shende et al., 2018). We observed that, although not vigorously, Mep1p is also capable of degrading SP-D. Whether secretion of these proteases prevails during infection, needs to be validated. However, the detection of anti-Mep1p antibodies in the sera of patients suffering from aspergilloma supports the potential secretion of this protease during *A. fumigatus* infection (Monod et al., 1993). On the other hand, intranasal administration of SP-D could increase the survival of immunocompromised mice with IPA (Madan et al., 2001; Singh et al., 2009). In this present study, our observation that the replenishment of SP-D during germination process can sustain growth inhibition of *A. fumigatus* suggests that the exogenous administration of SP-D in *A. fumigatus* infected or colonized patients could be of potential therapeutic benefit, which needs to be validated.

Conclusion

Our study demonstrates a direct fungistatic activity of SP-D *in vitro*, and that it alters as well as masks the exposure of cell wall polysaccharide of *A. fumigatus* germ tubes/hyphae. These combined effects of SP-D on *A. fumigatus* modulate host immune response by modifying the secretion of inflammatory cytokines and chemokines by immune cells encountering the fungus. This is the first report ravelling a direct growth inhibitory activity by SP-D, a humoral immune component. In here, although SP-D-fungal interaction appears to be a physical phenomenon, it cannot be ruled out that SP-D may elicit transcriptomic changes leading to hyphal growth inhibition and polysaccharide reorganization in the cell wall. Such hypothesis needs to be validated, and currently we are exploring the mechanisms underlying growth inhibitory activity of SP-D against *A. fumigatus*. Nonetheless, our present study highlights the importance of investigating possible direct antifungal activities of other relevant humoral components against *A. fumigatus*, to exploit their therapeutic benefits.

CRediT authorship contribution statement

Sarah Sze Wah Wong: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – original draft. **Sarah Dellière:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Natalia Schiefermeier-Mach:** Formal analysis, Methodology, Visualization, Writing – review & editing. **Lukas Lechner:** Methodology, Visualization, Writing – review & editing. **Susanne Perkhofer:** Formal analysis, Methodology, Visualization, Writing – review & editing. **Perrine Bomme:** Methodology, Visualization, Writing – review & editing. **Thierry Fontaine:** Methodology, Writing – review & editing. **Anders G. Schlosser:** Resources, Writing – review & editing. **Grith L. Sorensen:** Resources, Writing – review & editing. **Taruna Madan:** Supervision, Writing – review & editing. **Uday Kishore:** Conceptualization, Investigation, Methodology, Supervision, Writing – review & editing. **Vishukumar Aimanianda:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tcsw.2022.100072.

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