Fungal melanin stimulates surfactant protein D–mediated opsonization of and host immune response to Aspergillus fumigatus spores

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Running title: SP-D binds to A. fumigatus conidial melanin

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ABSTRACT
Surfactant protein D (SP-D), a C-type lectin and pattern-recognition soluble factor, plays an important role in immune surveillance to detect and eliminate human pulmonary pathogens. SP-D has been shown to protect against infections with the most ubiquitous airborne fungal pathogen, Aspergillus fumigatus, but the fungal surface component(s) interacting with SP-D is unknown. Here, we show that SP-D binds to melanin pigment on the surface of A. fumigatus dormant spores (conidia). SP-D also exhibited an affinity to two cell-wall polysaccharides of A. fumigatus, galactomannan (GM) and galactosaminogalactan (GAG). The immunolabeling pattern of SP-D was punctate on the conidial surface and was uniform on germinating conidia, in accordance with the localization of melanin, GM, and GAG. We also found that the collagen-like domain of SP-D is involved in its interaction with melanin, whereas its carbohydrate-recognition domain recognized GM and GAG. Unlike unopsonized conidia, SP-D–opsonized conidia were phagocytosed more efficiently and stimulated the secretion of proinflammatory cytokines by human monocyte-derived macrophages. Further, SP-D−/− mice challenged intranasally with wild-type conidia or melanin ghosts (i.e. hollow melanin spheres) displayed significantly reduced proinflammatory cytokines in the lung compared with wild-type mice. In summary, SP-D binds to melanin present on the dormant A. fumigatus conidial surface, facilitates conidial phagocytosis, and stimulates the host immune response.

INTRODUCTION
Collectins are calcium-dependent C-type lectins, characterized by an N-terminal cysteine-rich region, a collagen-like domain, α-helical coiled-coil neck region and a C-terminal carbohydrate recognition domain (CRD) (1-3). The triple-helical collagen region gives rise to a homotrimeric subunit, which undergoes further oligomerization via N-terminal cysteine-rich region forming multimers (4). Their affinities toward different microbes vary depending on their degree of oligomerization as well as the density of ligands available on the microbial surface (5). Being C-type lectins, collectins primarily recognize carbohydrate moieties on the microbial surfaces as pattern recognition molecules; however, they also bind to charge-moieties on the microbial surface, leading to microbial opsonization and thus enhancing their phagocytosis (1-3). The microbial uptake via collectins also triggers killing mechanisms, including superoxide burst and pro-inflammatory immune response (6). Other members of the collectin family, mannose-
binding lectin (MBL) and Collectin-11 (CL-11) are known to activate the lectin pathway of complement activation (7,8).

Pulmonary hydrophilic surfactant proteins (SP), SP-A and SP-D, which also belong to the family of collectins, are produced by alveolar epithelial type II cells and Clara cells; their surfactant property prevents collapsing of the alveoli at the end of expiration (1-3,9). Recent findings show that SP-D is integral to innate immune response, in facilitating microbial phagocytosis through opsonization, and in modulating cellular immune response by alveolar macrophages (10). SP-D induces signalling for pro-inflammatory mediator production in alveolar macrophages by interacting with calreticulin/CD91 (11). SP-D binding to damaged/apoptotic cells is also shown to enhance their uptake and clearance by alveolar macrophages in murine model (12).

On the other hand, airborne fungal spores constitute major pulmonary pathogens in the immunocompromised individuals. Fungal cells are endowed with a cell-wall composed of different polysaccharides, the composition of which varies depending on the fungal species (13). Based on the inhibition assays, SP-D is suggested to interact with carbohydrate moieties (pathogen-associated molecular patterns, PAMPs) on fungal pathogen surfaces through its CRD (1,6). Various polysaccharides have been proposed for SP-D binding, as their monomeric units, such as mannose, maltose or glucose, could inhibit SP-D binding to different fungal species (1,14-17). β-Glucan inhibited SP-D binding to Blastomyces dermatitidis (18). Gp-120, a surface glycoprotein from Pneumocystis carinii is also shown to interact with SP-D (19).

Aspergillus fumigatus is the most ubiquitous airborne human fungal pathogen, which causes aspergillosis, allergic bronchopulmonary aspergillosis in immunocompetent individuals and life-threatening invasive aspergillosis in immunocompromised individuals (20). SP-D has been shown to bind to A. fumigatus conidia (14), however, the interacting fungal components are unknown. Here, we show that SP-D binds to A. fumigatus dormant conidial surface melanin pigment through its collagen-like domain and two other cell-wall polysaccharides of A. fumigatus, galactomannan (GM) and galactosaminogalactan (GAG) via its CRD. Opsonization by SP-D facilitated conidial phagocytosis by human monocyte-derived macrophages and stimulated the secretion of pro-inflammatory cytokines. SP-D knockout mice showed a significant down-regulation of the pro-inflammatory cytokines when challenged with conidia or melanin extracted from the dormant conidia, compared to wild-type mice, suggesting immunomodulatory potential of SP-D against inhaled conidia.

RESULTS

SP-D binds to both dormant and germinating A. fumigatus conidial surfaces

Previous study employing flow cytometry showed that SP-D binds to A. fumigatus conidia (14,21). In accordance, our data showed that SP-D binds to conidial surface in a saturable manner (Figures 1A and 1B). Although there was binding in all the pH tested (pH 4.0-8.0; mean fluorescence intensity (MFI) values, pH 4.0, 1944±147.08, pH 6.0, 2822±83.44, pH 7.4, 2162.5±71.42 and pH 8.0, 2100.5±130.81), the optimum binding was observed around pH 6.0. We were, however, interested in examining the binding pattern of SP-D, for which we used immunolabelling technique. SP-D bound to dormant conidia in a punctate manner, while binding was uniform on germinating conidia (Figure 1C). This suggested that SP-D ligands are present on both dormant and germinating A. fumigatus conidial surfaces.

Lectins are carbohydrate-specific proteins (22) and SP-D, being a C-type lectin, recognizes and binds to the carbohydrate moieties on the microbial surface in a calcium-dependent manner through its carbohydrate recognition domain (CRD) (23-25). Based on inhibition assays, glucose, maltose and mannose moieties of the fungal surface glycoconjugates are suggested to be the SP-D ligands (1,14-16). The lipid moieties of glycolipids are also shown to bind SP-D through CRD (23,24). Based on inhibition assays, glucose, maltose and mannose moieties of the fungal surface glycoconjugates are suggested to be the SP-D ligands (1,14-16). The lipid moieties of glycolipids are also shown to bind SP-D through CRD (3,26). However, in our assay, pre-incubation of SP-D with glucose maltose, mannose and lipopolysaccharide (LPS) did not inhibit the binding of SP-D to A. fumigatus dormant conidia (Figure 2), suggesting that the interaction between SP-D and dormant conidia is possibly mediated through non-glyco-/lipo-moieties.

SP-D showed affinity towards A. fumigatus conidial surface melanin pigment and two cell wall polysaccharides, galactomannan (GM) and galactosaminogalactan (GAG)
To identify the *A. fumigatus* surface/cell-wall component(s) interacting with SP-D, both conidial and mycelial cell-wall components were tested for SP-D binding, by ELISA. SP-D displayed affinity towards melanin, GM and GAG (Figure 3A) with concentration-dependency (Supplementary Figure 1). We also tested SP-A and MBL, the other two C-type lectins belonging to the collectin family, found in the lung-environment (1). SP-A did not show binding with any of the *A. fumigatus* cell wall components, while MBL showed very weak interaction with GM and GAG (Supplementary Figure 2), suggesting that selectivity of SP-D towards melanin, GM and GAG is exclusive. Pull-down and inhibition assays further confirmed specific SP-D binding to melanin, GM and GAG (Supplementary Figure 3). SP-D binding to GM/GAG was calcium-dependent, whereas its binding to melanin was calcium-independent (Figure 3B) and was not inhibited by either EDTA or EGTA (Figure 3C), suggesting the involvement of different domains of SP-D during the interaction with melanin and GM/GAG. The truncated SP-D (SP-D that only contains the CRD) showed binding only to GM and GAG (Figure 3D) but not to melanin, suggesting that CRD of SP-D is involved in the binding to the fungal cell wall polysaccharides, GM/GAG.

A truncated rhSP-D, containing only eight Gly-X-Y repeats of collagen-like domain along with the neck region and CRD but lacking N-terminal cysteine-rich region and fifty-one Gly-X-Y repeats of collagen-like domain, also showed melanin binding, suggesting that a fragment of collagen-like domain is sufficient for melanin binding. We confirmed rhSP-D binding to *A. fumigatus* conidial surface by flow cytometry, immuno-labelling and to the melanin by ELISA assay (Supplementary Figure 3). When analyzed by flow cytometry, a decrease in the rhSP-D binding with dormant conidia upon pre-incubation with melanin in a dose-dependent manner further confirmed melanin to be the SP-D ligand (Figure 3E).

GAG is synthesized during germination, and is not present in the dormant *A. fumigatus* conidial cell-wall (27). GM is found in the cell-wall of all the morphotypes of *A. fumigatus*, but masked by the surface rodlet and melanin layers in the dormant conidia (28,29). Therefore, we focused our study on melanin, as it is present on the surface of the dormant conidia, the major infective morphotype of *A. fumigatus*.

**SP-D opsonizes *A. fumigatus* conidia facilitating their phagocytosis**

Human monocyte-derived macrophages (MDMs) phagocytosed significantly higher numbers of conidia opsonized with SP-D compared to un-opsonized conidia (Figure 4A). Pre-incubation of SP-D with melanin followed by conidial opsonization resulted in a significant decrease in the conidial phagocytosis. MTT assay was also carried out to evaluate the number of rhSP-D-opsonized conidia phagocytosed by MDMs. Similar to SP-D, rhSP-D also opsonized conidia and facilitated their phagocytosis, as confirmed by flow cytometric analysis and immunolabelling assay (Supplementary Figure 4). Meanwhile, conidial opsonization by rhSP-D, that was pre-incubated with melanin, resulted in a similar degree of phagocytosis as the un-opsonized conidia (Figure 4B).

**Opsonization by SP-D reduces reactive oxygen species (ROS) quenching capacity of melanin**

*A. fumigatus* conidial melanin has been shown to quench reactive oxygen species (ROS), one of the defensive mechanisms of the human immune system (30,31). We evaluated ROS quenching property of melanin. Unopsonized melanin ghost reduced ROS production by MDMs (Figure 4C), whereas, SP-D opsonized melanin ghosts resulted in an increase in the ROS production by MDMs. Similarly, SP-D opsonized conidia caused an increase in the ROS production by MDMs compared to control MDMs (Figure 4D). Unopsonized conidia also resulted in an increase in the ROS produced compared to control MDMs. But, here it should be noted that we used metabolically active conidia unlike melanin ghost preparation. However, the ROS production by SP-D opsonized conidia was significantly higher than the ROS produced by MDMs interacted with unopsonized conidia, suggesting that SP-D opsonization reduces ROS quenching capacity of melanin.

**SP-D opsonized *A. fumigatus* conidia/melanin ghost induce pro-inflammatory cytokines secretion**

The cytokines in the co-culture supernatants of MDMs and SP-D-opsonized conidia were assessed at early and late interaction time-points. In this assay, we used para-formaldehyde (PFA)-
fixed conidia and incomplete RPMI medium (without 10% serum) to avoid any influence due to conidial germination and serum. We analyzed the mRNA level of cytokines by qPCR after 2h (early interaction) of incubation of MDMs with conidia or melanin ghosts. Upon opsonization, A. fumigatus conidia or extracted melanin ghost induced significantly higher transcripts of pro-inflammatory (tnf-α, il-6, il-8, il-1β and il-12a) and anti-inflammatory (il-10) cytokines (Figure 5A). At late interaction time (20h), MDMs cultured with SP-D-opsonized conidia produced significantly higher TNF-α, IL-6 and IL-8 than unstimulated MDMs and those co-cultured with un-opsonized conidia (Figure 5B).

We confirmed that pro-inflammatory cytokine induction was due only to the interaction of SP-D with A. fumigatus conidia/melanin ghosts as opsonin and not endotoxin contamination of SP-D. The amount of endotoxin was estimated to be 4.3 pg/μg of rhSP-D, whereas in the commercial SP-D samples used, there were no detectable amount of endotoxin. The highest amount of rhSP-D used in our study was 200 ng that corresponds to contain 0.86 pg of endotoxin. Therefore, we added MDMs with 10 pg of LPS (10-fold higher than could be present in 200 ng/mL rhSP-D); but, it failed to stimulate any cytokine secretion (Supplementary Figure 5A). Next, we compared cytokine secretion from the MDMs from three donors before and after the treatment of SP-D with polymyxin, and no difference was observed (Supplementary Figure 5B; representative cytokines, TNF-α and IL-6, are shown). Also, we cultured MDMs with SP-D alone, in the absence of conidia, that did not produce any pro-inflammatory immune response, suggesting that the observed immune response is due only to the conidial or melanin ghost opsonizing capacity of SP-D.

**SP-D knockout mice show reduced lung cytokine response when challenged with A. fumigatus conidia or extracted melanin ghosts**

Wild-type (WT) or SP-D knockout (SP-D−/−) mice were intranasally challenged with A. fumigatus conidia or melanin ghost, SP-D−/− mice showed significantly reduced cytokine transcripts in the lung homogenates during the early infection (6h) compared to the WT mice (Figure 6A and 6B). On the other hand, the melanin ghost was less immunostimulatory in both WT and SP-D−/− mice compared to conidia in WT mice (Figure 6B and 6A, respectively). Upon challenging WT mice with melanin ghost opsonized with rhSP-D, the immuno-stimulation was significantly higher than the corresponding counterpart challenged with un-opsonized melanin ghost (Figure 6B). The cytokine levels in lung homogenates showed the pattern (Figure 6C) similar to that observed with the transcripts (Figure 6B). Together, our results show that SP-D acts as an immuno-modulator, overcoming the immunological inertness of conidial melanin.

**DISCUSSION**

SP-D has been shown to bind various fungal pathogens through its CRD, as fungi are endowed with a cell-wall rich in different carbohydrate moieties (6,32). Pustulan (a β-(1,6)-glucan) inhibits SP-D binding to A. fumigatus (21); but, β-(1,6)-glucan is not a component of the A. fumigatus cell-wall (33). Hence, there is a lack of direct evidence showing the interaction of SP-D with a particular PAMPs on the surface of this fungal pathogen. Here we show, for the first time, that SP-D binds to A. fumigatus dormant conidial surface melanin pigment and two cell-wall polysaccharides, galactomannan (GM) that is masked by the surface melanin-rodlet layers in dormant conidia (28,29) and galactosaminogalactan (GAG), which is only synthesized during germination (27). The binding of GM or GAG to SP-D is mediated by its CRD in a calcium-dependent manner, while melanin binding is through the collagen-like domain of SP-D that is independent of calcium. There exists a layer of superficial rodlet and a underlying melanin layer on the dormant A. fumigatus conidial surface (28,29,34,35); however, at places, this melanin layer protrudes out of the rodlet layer, and hence the punctate binding pattern of SP-D on the dormant conidial surface. During germination, these rodlet and melanin layers are destroyed, exposing the cell wall polysaccharides, including GAG and GM, resulting in a uniform binding of SP-D.

SP-D is present in the bronchoalveolar lavage fluid (36). One of the functions attributed is its capacity to opsonize airborne pathogen facilitating their phagocytosis. In our study, SP-D efficiently opsonized A. fumigatus conidia. It is interesting to note that, not only SP-D, but rhSP-D containing only a part of collage-like domain could also opsonize conidia. A truncated SP-D containing only two collage-like domain...
repeats, neck region and CRD was less effective in agglutinating bacteria compared to native SP-D and also it failed to inhibit hemagglutinating property of Influenza A virus (37). Meanwhile, our study indicates that eight collagen-like domain repeats of rhSP-D is adequate for conidial binding.

Opsonization by SP-D modulates immune response (10). In case of A. fumigatus, conidia reach the lungs where they are encountered by the alveolar macrophages, which are responsible for the intracellular killing of the pathogen and the release of pro-inflammatory cytokines to recruit neutrophils (38). A number of in vitro and in vivo studies have been carried out to exploit the cytokine profile of the immune cells in response to A. fumigatus conidia; however, they were performed in the immune-competency or in the absence of immune factors. To examine the inflammatory response brought about by only SP-D, we assessed the production of inflammatory cytokines from human MDMs made to interact with SP-D-opsonized conidia. The cytokine in MDMs incubated with un-opsonized PFA-fixed conidia was similar to the unstimulated MDMs, confirming our earlier observation that A. fumigatus conidia are immunologically inert (28). Whereas, conidial opsonization with SP-D significantly induced the production of pro-inflammatory cytokines. In contrast, there was no significant difference in the production of the anti-inflammatory cytokines (IL-10/IL-1ra) after 20h of incubation. The il-10 was an exception, showing significant up-regulation at the early time-point. This may be due to regulation at post-transcriptional level or autoregulation of IL-10 expression, promoting inflammation to protect against infection.

In the naïve lung, SP-D interacts with Signal Regulating Protein α (SIRPα) receptors on macrophages through their CRD, suppressing pro-inflammatory cytokine production (11). But, when bound to microorganisms, SP-D interacts with calreticulin, a multifunctional protein found on the surface of macrophages, which in turn binds to the endocytic receptor CD91 and mediates the uptake of SP-D opsonized microbes (12). SP-D binds to the calreticulin and CD91 complex via its collagenous region, stimulating inflammatory response by activating NF-κB (11). Interestingly, in our study, we observed that (a) only a part of the collagen-like domain of SP-D is required for the melanin binding and (b) SP-D-melanin interaction likely promotes SP-D binds to the calreticulin/CD91, thus stimulating the secretion of pro-inflammatory cytokines. It is interesting to note that although the un-opsonized conidia were phagocytosed by the MDMs, they accounted for significantly lower numbers than the opsonized conidia and it did not trigger an inflammatory response; other studies also have similar observations (39). It would be worthy to elucidate how macrophages recognize/phagocytose un-opsonized conidia.

Upon intranasal conidial inoculation, SP-D⁻/⁻ mice showed significantly reduced cytokine transcripts in the lung homogenate compared to the wild-type (WT) mice, suggesting that SP-D possibly plays an important role in eliciting immune response against A. fumigatus. Whereas, inoculation of melanin ghost into SP-D⁻/⁻ and WT mice abolished this difference, confirming immunological inertness of melanin (29). The immune response was not completely abolished in SP-D⁻/⁻ mice exposed to conidia/melanin ghost, indicating that there exist other humoral immune components that might be able to bind melanin. However, it was shown earlier that SP-D deficient mice demonstrated higher mortality due to pulmonary aspergillosis compared to immunocompetent mice (40). In an immunosuppressed model, SP-D⁻/⁻ mice died earlier compared to WT mice upon intranasal challenging with A. fumigatus conidia (after 2-days, 43% for SP-D⁻/⁻ mice compared to 20% for WT mice), although the overall mortality on day-7 was similar in both SP-D⁻/⁻ and WT mice (57% and 60%, respectively). Manipulation of immune responses in the lungs augments antifungal immunity (41) and administration of SP-D has been shown to reduce the mortality of both WT and SP-D⁻/⁻ immunocompromised mice (40,42). Treatment with SP-D reduced mortality of both SP-D⁻/⁻ and WT mice challenged with conidia by 50% and 40%, respectively, suggesting an important role played by SP-D in clearing A. fumigatus. Administration of SP-D in the SP-D⁻/⁻ mice also modified cytokine response (40). In our study, we followed an alternative approach; the inoculation of WT mice with SP-D-opsonized melanin ghosts augmented cytokine levels compared to unopsonized melanin ghosts, confirming the direct immunomodulatory effect of SP-D. In addition, melanin has been shown to quench reactive oxygen species (ROS), one of the defensive mechanisms of the human immune system.
reactive oxygen species (50-52). However, a acidification of phagolysosome and detoxifying shielding the cell-wall PAMPs, inhibiting the fungus from the host immune defense by important virulence factor, which protects this A. fumigatus inflammatory effect in human macrophages. TNF-α is upregulated in invasive aspergillosis (IA) murine model (44) and TNF-α treatment protects mice from IA (45). In our study, there was a significant reduction in the TNF-α in the SP-D+ mice lungs upon inoculation with conidia or melanin ghost. Infecting WT mice with SP-D opsonized melanin ghost resulted in a significant increase in the lung TNF-α transcript/protein levels. IL-6 was induced in the BAL of immunocompetent mice infected intranasally with conidia (46), while IL-6-deficient mice were more susceptible to IA than wild-type mice (47). Although the cellular recruitment was not affected in IL-6-deficient mice, the fungicidal activity of the recruited phagocytes was significantly impaired (47). This indicates the importance of this cytokine in activating the recruited phagocytes into fungicidal state, and we did see an induction in the IL-6 secreted by the macrophages upon interaction with SP-D opsonized conidia. IL-8, a chemoattractant for neutrophils, was induced in dendritic cells in response to heat-killed (48) as well as swollen/germinating A. fumigatus conidia (28). However, it was unknown whether the same observation could be seen in macrophages. We observed IL-8 production by the human MDMs upon interaction with SP-D opsonized conidia. IL-12 enhances the antifungal activity of monocytes via a gamma-interferon independent pathway (49). We found an up-regulation of MDMs il-12 with rhSP-D opsonized conidia at early interaction time. Taken together, SP-D opsonized A. fumigatus conidia induces pro-inflammatory effect in human macrophages. A. fumigatus conidial surface melanin is an important virulence factor, which protects this fungus from the host immune defense by shielding the cell-wall PAMPs, inhibiting the acidification of phagolysosome and detoxifying reactive oxygen species (50-52). However, a recent study showed that A. fumigatus conidia activates platelets at least partially through conidial surface melanin, as extracted melanin ghost also showed platelet activating capacity (53). In this study, we have shown that, paradoxically, melanin serves as a PAMP, by binding to SP-D, one of the major pulmonary collectins. SP-D bound to conidia is capable of modulating cytokine production by the immune cells, stimulating the secretion of pro-/anti-inflammatory cytokines, thus playing a protective role against invading A. fumigatus as well as balancing the immune responses. In the natural context, in addition to SP-D, there are other soluble mediators of the innate immune system (including complement system, MBL, pentraxin and other collectins), playing individual or complementary role in clearing inhaled A. fumigatus conidia. However, a significantly decreased immune response in the absence of SP-D indicates that SP-D plays an important role against A. fumigatus, suggesting possible immunotherapeutic application of SP-D against A. fumigatus infection. In addition, it is worth speculating about the effect of SP-D binding to melanin in the light of various research works reporting anti-apoptotic properties of melanin.

**EXPERIMENTAL PROCEDURES**

**Strains, media and reagents:**

A. fumigatus clinical isolate CBS144-89 was used as the wild-type strain (28). They were maintained on 2% malt agar slants at ambient temperature. Conidia were harvested from 12-15 day old slants using 0.05% Tween-water, washed, re-suspended in Tween-water and filtered through 40-µm Falcon™ cell strainer (Thermo Fischer Scientific). Germinating conidia were prepared by incubating dormant conidia in Sabouraud liquid medium at 37°C for 6-6.5 h in an incubator maintained at 150 rpm, followed by collecting and washing them with water. Para-formaldehyde (PFA) fixation of conidia was performed as described earlier (28).

**Surfactant protein D**

Full length recombinant human SP-D (SP-D) was purchased from Abcam (ab152069) as well as from R&D systems (1920-SP) [375 amino acids (aa), apparent Mw, 43 kDa]. A recombinant human SP-D (rhSP-D) was expressed in Escherichia coli (42,54,55); this rhSP-D consisted of eight triplet-repeat of collagen-like domain (8 Gly-X-Y repeats; 24 aa) followed by...
46 aa of neck region and 106 aa of CRD domain (~M<sub>w</sub> of 19 kDa). A truncated SP-D (aa residues 224-375) consisting of only the CRD was purchased from Abcam (ab181961; abbreviated as SP-D-CRD, ~M<sub>w</sub> 17 kDa on SDS-PAGE), which comprised of a neck region and a CRD.

Endotoxin level in the SP-D preparation used was determined using the QCL-1000 Limulus amebocyte lysate system (BioWhittaker Inc., USA). The assay was linear over a range of 0.1–1.0 EU/ml (10 EU = 1 ng of endotoxin).

**Immunolabelling**
PFA-fixed conidia (2x10<sup>6</sup>) were incubated with heat-inactivated normal human serum (NHS; 56ºC for 30 min) for 1h. Thereafter, they were incubated with anti-human SP-D antibody (R&D Systems; Purified mouse monoclonal IgG2B Clone #292201, raised against human SP-D; 5 µg/mL dilution 1:200) and secondary anti-mouse IgG-FITC (Sigma-Aldrich; dilution 1:200) and secondary anti-mouse IgG2B Clone #292201, raised against human SP-D; 5 µg per mL, dilution 1:2000) or polyclonal antibody raised in mouse against human SP-D; 5 µg per mL, dilution 1:2000) for 1h. Quantification of SP-D binding to different cell-wall components was performed applying peroxidase conjugated anti-mouse IgG (Sigma; dilution 1:1000) together with ortho-phenylenediamine and H<sub>2</sub>O<sub>2</sub> for detection at OD<sub>492</sub>. Between the addition of SP-D primary/secondary antibodies, microtiter wells were washed at least five-times with PBS-Tween (0.5%).

**Binding assay**

(A) **Direct binding**: Performed by ELISA; 96-well Microtiter plates were coated with cell-wall components dissolved/suspended in carbonate buffer (0.1 M, pH 9.6; 25 µg/mL) overnight. Wells were blocked for 1h with PBS containing 1% BSA and then 50 ng of SP-D (in PBS-BSA with Ca<sup>2+</sup>) was added. After 1h of incubation at 37ºC, either anti-human SP-D (R&D Systems; mouse monoclonal IgG2B Clone #292201-raised against human SP-D; 5 µg per mL, dilution 1:2000) or polyclonal antibody raised in mouse against human SP-D (dilution 1:2500) was added and incubated for 1h. Quantification of SP-D binding to different cell-wall components was performed applying peroxidase conjugated anti-mouse IgG (Sigma; dilution 1:1000) together with ortho-phenylenediamine and H<sub>2</sub>O<sub>2</sub> for detection at OD<sub>492</sub>. Between the addition of SP-D primary/secondary antibodies, microtiter wells were washed at least five-times with PBS-Tween (0.5%).

(B) **Pull-down/inhibition assays**: Assays were performed by pre-incubating SP-D with cell-wall components (that showed SP-D binding) in solution, followed by centrifugation and adding the supernatants (in case of insoluble cell-wall components) or directly adding the reaction mixture to ELISA plate wells coated with respective cell-wall components.

**Preparation of the cell-wall components**
GM, GAG, chitin, RodAp and melanin ghosts were purified or prepared from conidia/mycelia as described earlier (27-29,56,57). β-(1,3)-Glucan and α-(1,3)-glucan were isolated from the alkali-insoluble (AI) and soluble (AS) cell-wall fraction, respectively, as described earlier with few modifications(57). AI-fraction was subjected to periodate oxidation for four days instead of three days and subjected to chitin deacetylation with 30% NaOH in place of 40% NaOH to obtain β-(1,3)-glucan. α-(1,3)-glucan was obtained from AS-fraction upon subjecting it to periodate oxidation-Smith degradation, followed by recombinant endo-β-(1,3)-glucanase treatment to remove residual β-(1,3)-glucan. The purity of the polysaccharides isolated was checked by gas-liquid chromatography.

**Human monocyte-derived macrophages**
Blood samples from healthy donors were obtained from Etablissement Français du Sang Saint-Louis (Paris, France) with written informed consent as per the guidelines provided by the Institutional ethics committee, Institut...
agar plate and incubated at 37ºC, the wells were collected and after dilution plated on malt buffer containing 5 mM CaCl	extsubscript{2} and incubated at 37ºC in a CO	extsubscript{2} incubator. Owing to the following incubation, the cells were washed twice with PBS and RPMI medium supplemented with 10% normal human serum (NHS) and granulocyte macrophage colony stimulating factor (GM-CSF; 10 ng/mL; Sigma-Aldrich) was added for monocyte differentiation into macrophage (MDMs). After six days, medium was discarded and the MDMs were washed with PBS and used for further experiments.

**Opsonization and phagocytosis**

**Conidial opsonization:** Conidia (1x10	extsuperscript{6}) were opsonized with SP-D (50 ng/mL) in HEPES buffer containing 5 mM CaCl	extsubscript{2} for 30 min at 37ºC. Conidia incubated with HEPES buffer alone was used as the negative control.

**Phagocytosis and colony forming units:**

Opsonized conidia (1x10	extsuperscript{6} conidia/well) were added to MDM culture in RPMI medium supplemented with 10% heat inactivated NHS and incubated at 37ºC in a CO	extsubscript{2} incubator for 1h. Supernatants were discarded and the cells were washed twice with PBS, lysed by adding 100 µL 1% TritonX-100 (30 min at 4ºC). The contents were collected and after dilution plated on malt agar plate and incubated at 37ºC for 36h followed by fungal colony counts.

**MTT assay:**

Conidia (1x10	extsuperscript{5} conidia/well) opsonized with rhSP-D (5 µg/mL) and melanin (20 µg/mL), alone or in combination, were added to MDMs and incubated for 2h in CO	extsubscript{2} incubator at 37ºC. Wells were washed with RPMI medium, MDMs were lysed with 0.5% sodium deoxycholate (Sigma-Aldrich). Then, RPMI medium was added to the wells and incubated at 37ºC for 48h to allow the conidial growth. MTT solution (150 µL; Calbiochem, 5 mg/mL in PBS) was added to each well and incubated for 4h. After washing the wells with PBS, 500 µL isopropanol in 0.04N HCl was added to dissolve the formazan (2h at 37ºC); 100 µL of the sample was transferred to another 96-well plate and absorbance was recorded at 560 nm using ELISA reader (BioTek Power Wave XS2 with Gen5 software).

**Cytokine analysis**

PFA-fixed wild-type conidia were opsonized by SP-D in a final concentration of 200 ng in 100 µL HEPES at 37ºC for 30 min. Thereafter, the opsonized conidia were washed in HEPES buffer and re-suspended in RPMI medium without serum (incomplete RPMI). The MDMs were added with 5x10	extsuperscript{6} well un-opsonized or SP-D opsonized conidia. After incubation for 20h at 37ºC in a CO	extsubscript{2} incubator, the supernatants were collected and stored at -20ºC until further analysis. The cytokines in the co-culture supernatant were quantified by DuoSet ELISA kits (R&D Systems).

**Reactive oxygen species (ROS) production**

Conidia (1x10	extsuperscript{6} per well) or melanin ghosts (corresponding to an equivalent of 1x10	extsuperscript{6} conidia) either unopsonized or opsonized with SP-D were made to interact with MDM (obtained after plating 2x10	extsuperscript{6} PBMCs per well) for 2h in a CO	extsubscript{2} incubator. Then added 100 µM dichlorodihydrofluorescein diacetate (DCF-DA; stock solution of 1 mM prepared in DMSO) and incubated further for 1h in a CO	extsubscript{2} incubator. Following, the fluorescence intensities were measured using Tecon Infinite® 200 PRO plate reader, with excitation and emission at 485 and 530 nm, respectively. The results are expressed in fluorescence intensity (58,59).

**Immune response in SP-D gene deficient mice to dormant conidia and melanin ghost**

The study was reviewed and approved by the Institutional Animal Ethics Committee (IAEC no. 17/12 of National Institute for Research in Reproductive Health, Mumbai). Animals were maintained as per the institutional guidelines for the care and use of experimental animals. Mice with targeted deletion of Sftpd (SP-D	extsuperscript{-/-}) on a Swiss black background and the wild-type (WT) mice of the same genetic background were obtained from Dr. Jefferey Whitsett, Cincinnati Children Hospital Medical Centre, USA. Both SP-D gene deficient female and male mice have reproductive defects, such as prolonged diestrus, delayed mating, significantly high pre-implantation loss, altered testicular immune milieu and sperm function (60,61), resulting in limited numbers of mice available for the study. The first and second groups were WT mice challenged with dormant conidia and melanin ghost, respectively. The third and fourth groups were SP-D	extsuperscript{-/-} mice challenged with dormant conidia and melanin ghost, respectively. The fifth group was WT mice challenged with...
melanin ghost pre-opsonized by 5 µg rhSP-D for 15 min at 37°C. Mice were administered 1x10^7 wild type dormant conidia or melanin ghost (40 µg; equivalent to 1x10^7 conidia) intranasally. After 6h, the mice were sacrificed and the lungs were harvested in ice-cold PBS and subsequently stored at -80°C for RNA isolation and lung homogenate preparation for ELISA.

**RNA isolation and real-time RT-PCR analysis**
Total RNA from human MDMs or mice lungs was extracted using RNAiso Plus (TAKARA) and quantified using Nano-spec. After determining the 260:280 ratios for the RNA quality, it was treated with DNase I (Thermo Scientific, Rockford, IL, USA; 37°C for 30 min) to remove any genomic DNA contamination. RNA (1 µg) was reverse transcribed using Superscript™ III first-strand synthesis kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions; 1 µL of cDNA was used for subsequent real-time PCRs using SYBR Green master mix (Bio-Rad). For all experiments, 18S was used for normalization. Primers for amplification of cDNAs were designed using NCBI Primer BLAST Software and further evaluated using integrated DNA technologies oligo analyzer software. Primers used for human and murine samples is provided in Supplementary Tables 1 and 2. Real-time PCR of diluted cDNA was carried out using Bio-Rad CFX 96™ Thermal Cycler machine.

**Preparation of mouse lung homogenate and cytokine assays**
Mouse lung homogenates (at 20%, w/v) were prepared in PBS of pH 7.4 (Gibco Life Technologies) containing 0.5% Triton X-100, 0.02% Sodium azide, phenylmethylsulfonyl fluoride (PMSF, 1 mM) (all from Sigma-Aldrich) and protease inhibitor cocktail mix (GE Healthcare). Homogenates were kept on ice for 30 min with intermittent vortexing. Samples were then centrifuged at 14,000x g for 20 min at 4°C. Clear supernatants were collected in fresh tubes, aliquoted and stored in -80°C until assay. Total protein was estimated using a BCA protein assay kit (Pierce Thermo Fisher) according to manufacturer’s protocol. Equal amount of protein was pooled for each group and 50 µg per well of samples were loaded in 96-well microtiter plates for ELISA. Cytokines in lung homogenates were measured by DuoSet ELISA kit (R&D Systems) according to the manufacturer’s instructions.

**Statistical analysis**
Statistical analysis of the cytokine and RT-PCR data was performed by one-way ANOVA with Fisher’s least significant difference (LSD) test in the GraphPad Prism software (version-6.07). The ‘p’ value < 0.05 was considered statistically significant.

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**Authors contributions:** VA and TM designed the study, SW, MR, VA and JB carried out the research, ED, OIG and UK contributed the materials, VA, TM, SW, MR, UK, JPL, JB and AS analyzed the results and VA and TM wrote the manuscript. All authors approved the final version of the manuscript.

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FIGURE LEGENDS

Figure 1: SP-D binds to the surface of *A. fumigatus* conidia. (A) Flow-cytometry showing binding of SP-D to dormant *A. fumigatus* conidia. (B) Flow-cytometry data showing concentration-dependent binding of SP-D to dormant conidial surface. (C) Localization of SP-D on the surface of dormant and germinating conidia by fluorescent microscopy. SP-D binds to the dormant conidial surface in a punctate manner, while there was a uniform SP-D binding on the germinating conidial surface. The data were acquired after at least four independent experiments.

Figure 2: SP-D binding to *A. fumigatus* conidia is not inhibited by simple sugars and glycolipid. (A) Flow cytometry data showing the lack of inhibition of SP-D binding to dormant *A. fumigatus* conidia upon pre-incubation of SP-D (40 ng/mL) with glucose (10 mM), maltose (10 mM), mannose (10 mM) and lipopolysaccharide (LPS; 1 µg/mL). (B) Bar-graph representing percent conidia positive for SP-D-FITC binding; mean fluorescence intensity values are presented. The data were acquired after at least four independent experiments. Pre-treatment with mannose increased SP-D binding to conidia (**p < 0.0001**), possibly due to its sticky nature compared other other sugars.

Figure 3: SP-D binds to melanin, galactomannan (GM) and galactosaminogalactan (GAG) components of the *A. fumigatus* cell-wall, whereas the truncated SP-D with carbohydrate binding domain (CRD) interacts only with GM/GAG. (A) ELISA assay showing binding of SP-D to melanin, GM and GAG. (B) SP-D binding to melanin is calcium-independent, whereas, SP-D binding to GM and GAG requires calcium (only ***p < 0.0001** are represented here). (C) Flow-cytometry and median fluorescence intensity values showing that SP-D binding to the dormant *A. fumigatus* conidia is not inhibited by the addition of EDTA, the calcium chelator (D) ELISA assay showing that SP-D-CRD binds to carbohydrates (GM and GAG) but not to melanin. (E) Flow cytometry showing inhibition of rhSP-D binding to conidia by graded concentrations of melanin ghost (2, 5 and 10 µg/mL). The data were acquired after at least three to five independent experiments.

Figure 4: SP-D opsonizes *A. fumigatus* conidia facilitating their phagocytosis by human peripheral blood monocyte derived macrophages (MDM) and SP-D opsonization reduces reactive oxygen species (ROS) quenching effect of melanin. (A) Phagocytosis of SP-D opsonized conidia [MDM+(conidia+SP-D)] was greater compared to the un-opsonized conidia (MDM+conidia). In the case of pre-incubation of SP-D with melanin before opsonization [MDM+(conidia+(SP-D+melanin))], the conidial phagocytosis was similar to that of un-opsonized conidia, suggesting that SP-D binds to conidial surface melanin. (B) MDMs were incubated with conidia, rhSP-D opsonized conidia or conidia opsonized with rhSP-D+melanin supernatant for 2h. After washing, cells were lysed and the phagocytosed conidia were allowed to grow for MTT assay. In this assay, absorbance directly correlates with the phagocytic uptake suggesting SP-D facilitates conidial phagocytosis. A significant reduction in the absorbance confirmed that melanin inhibits opsonization and thereby phagocytosis. Data for (A) and (B) represents mean ± S.D of at least three independent experiments and *p < 0.01. (C) Melanin ghosts significantly reduced ROS production by MDM compared to control MDMs, whereas SP-D opsonization reduced ROS quenching effect of melanin ghosts. (D) Similar to SP-D opsonized melanin ghosts, SP-D opsonized conidia failed to quench ROS and here, unopsonized conidia showed ROS production higher than the control MDMs as these conidia were metabolically active (alive) and not PFA-fixed. Data for (D) and (D) represents mean ± S.D of at least four independent experiments and *p < 0.01, **p < 0.001 and ***p < 0.0001.

Figure 5: Opsonization of conidia with SP-D and rhSP-D induces pro-inflammatory cytokines in human monocyte-derived macrophages (MDMs). (A) MDMs were stimulated with either conidia (Afu), melanin ghosts or conidia/melanin ghosts opsonized with rhSP-D for 2h. After washing the cells, total RNA was isolated and used for evaluation of cytokine transcripts by qRT-PCR. The transcript fold change was compared to that of the MDMs incubated with un-opsonized conidia (Afu). Data represents mean ± S.E. of three independent experiments of MDMs from three healthy donors. *
p < 0.05, ** p < 0.01, *** p < 0.001 and **** p < 0.0001. (B) MDMs were incubated with un-opsonized (Afu) or SP-D opsonized conidia for 20h and the cytokines released were measured by ELISA. Data represents mean ± S.E. of independent experiments of MDMs from eight healthy donors. * p < 0.05.

Figure 6: Deficiency of SP-D results in alleviated immune response to A. fumigatus conidia and melanin ghost in mice. (A) Transcript fold change was determined by comparison to that in WT mice challenged with dormant conidia (WT-Afu). SP-D⁻/⁻ (KO) mice challenged intranasally with dormant conidia showed reduced levels of transcripts of proinflammatory cytokines (TNF-α, IL-6, IL-8, IL-1α and IL-1β) in the lung homogenates compared to WT mice. (B) WT and SP-D⁻/⁻ mice challenged intranasally with melanin ghost extracted from A. fumigatus dormant conidia. The transcript fold change was determined by comparison to that in WT mice challenged with dormant conidia (WT-Afu) in panel (A). Levels of cytokine transcripts in WT and SP-D⁻/⁻ mice in response to melanin ghost inoculation were not significantly different. However, this response increased manifold upon inoculation of WT mice with rhSP-D opsonized melanin ghost. (C) Cytokine levels of the lung homogenates of the WT mice challenged intranasally with A. fumigatus conidia, melanin ghosts or melanin ghosts opsonized with SP-D by ELISA confirmed the transcript profiles. Data represents mean ± S.E. of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Fold change for each cytokine transcript is relative to the transcript levels of that cytokine in WT-Afu (wild type mice challenged with A. fumigatus conidia). The scale in figures 6A and 6B is relative and the values for an individual cytokine can be compared across all stimulation conditions as all these experiments were carried out simultaneously. The fold change values for cytokine transcripts of KO-Afu and KO-Mel Ghost are not significantly different, except IL-8 and IL-1α, where the KO-Mel Ghost has higher levels than KO-Afu. Nevertheless, both conidia and melanin ghost failed to stimulate cytokine secretion from SP-D⁻/⁻ mice.
Figure 1

(A) Unstained (0.2%)

(B) Median fluorescent intensity

(C) Dormant conidia

Bright-light

SP-D, 40 ng/ml (99.2%)

Fluorescent

Germinating conidia
Figure 2

(A) FITC Control (0.1%)  SP-D (97.7%)  Glucose 10 mM (99.4%)

Maltose 10 mM (95.8%)  Mannose 10 mM (94.6%)  LPS 1 µg/ml (97.9%)

(B) Median fluorescence intensity

Control  FITC-Actin  SP-D  SP-D+Glucose  SP-D+Maltose  SP-D+Mannose  SP-D+LPS

ns  ns  ***

1 µg/ml LPS significantly increased the median fluorescence intensity compared to the control and other treatments.
Figure 5

(A) tnf-α

(B) TNF-α

IL-6
Figure 6

(A) tnf-α il-6 il-8 il-1α il-1β tgf-β il-1m il-10

(B) tnf-α il-6 il-8 il-1α il-1β tgf-β il-1m il-10

(C) TNF-α (pg/ml) IL-6 (pg/ml) CXCL-1 (pg/ml) IL-10 (pg/ml)
Fungal melanin stimulates surfactant protein D-mediated opsonization of and host immune response to \textit{Aspergillus fumigatus} spores

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