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

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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. SPD 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, SPD-opsonized conidia were phagocytosed more efficiently and stimulated the secretion of proinflammatory cytokines by human monocytederived 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 proinflammatory 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 by facilitating microbial phagocytosis through opsonization and modulating cellular immune response by alveolar macrophages (10). SP-D induces signaling for proinflammatory 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 a murine model (12).

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 *P. carinii,* is also shown to interact with SP-D (19).

Aspergillus fumigatus is the most ubiquitous airborne human fungal pathogen, which causes aspergilloma, 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 proinflammatory 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 with 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 the conidial surface in a saturable manner (Fig. 1, *A* and *B*). Although there was binding in all the pH values tested (pH 4.0–8.0; mean fluorescence intensity 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 an immunolabeling technique. SP-D bound to dormant conidia in a punctate manner, whereas binding was uniform on germinating conidia (Fig. 1*C*). 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 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 (3, 26). However, in our assay, preincubation of SP-D with glucose, maltose, mannose and lipopolysaccharide (LPS) did not inhibit the binding of SP-D to *A. fumigatus* dormant conidia (Fig. 2), suggesting that the interaction between SP-D and dormant conidia is possibly mediated through non-glyco-/lipomoieties.

SP-D showed affinity toward A. fumigatus conidial surface melanin pigment and two cell wall polysaccharides, GM and 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 toward melanin, GM, and GAG (Fig. 3*A*) with concentration dependence (Fig. S1). 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, whereas MBL showed very weak interaction with GM and GAG (Fig. S2), suggesting that selectivity of SP-D toward melanin, GM, and GAG is exclusive. Pulldown and inhibition assays further confirmed specific SP-D binding to melanin, GM, and GAG (Fig. S3). SP-D binding to GM/GAG was calcium-dependent, whereas its binding to melanin was calcium-independent (Fig. 3*B*) and was not inhibited by either EDTA or EGTA (Fig. 3*C*), 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 (Fig. 3*D*) 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-Xaa-Yaa repeats of collagen-like domain along with the neck region and CRD but lacking the N-terminal cysteine-rich region and 51 Gly-Xaa-Yaa 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 and immunolabeling and binding to the melanin by ELISA (Fig. S3). When analyzed by flow cytometry, a decrease in the rhSP-D binding with dormant conidia upon preincubation with melanin in a dose-dependent manner further confirmed melanin to be the SP-D ligand (Fig. 3*E*).

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 is 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 with un-opsonized conidia (Fig. 4*A*). Preincubation 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 immunolabeling assay (Fig. S4). Meanwhile, conidial opsonization by rhSP-D, which was preincubated with melanin, resulted in a similar degree of phagocytosis as the un-opsonized conidia (Fig. 4*B*).

Opsonization by SP-D reduces the ROS quenching capacity of melanin

A. fumigatus conidial melanin has been shown to quench ROS, one of the defensive mechanisms of the human immune system (30, 31). We evaluated the ROS quenching property of melanin. Un-opsonized melanin ghost reduced ROS production by MDMs (Fig. 4*C*), 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 with control MDMs (Fig. 4*D*). Un-opsonized conidia also resulted in an increase in the ROS produced compared with control MDMs. 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 interacting with un-opsonized conidia, suggesting that SP-D opsonization reduces ROS quenching capacity of melanin.

SP-D–opsonized A. fumigatus conidia/melanin ghost induces proinflammatory cytokine 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 paraformaldehyde (PFA)-fixed conidia and incomplete RPMI 1640 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 proinflammatory (*tnf*-α, *il-6*, *il-8*, *il-1*β, and *il-12a*) and anti-inflammatory (*il-10*) cytokines (Fig. 5*A*). At late interaction time (20 h), 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 (Fig. 5*B*).

We confirmed that proinflammatory 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 rhSP-D, whereas in the commercial SP-D samples used, there were no detectable amounts of endotoxin. The highest amount of rhSP-D used in our study was 200 ng, which contained 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 (Fig. S5*A*). 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 (Fig. S5*B*; 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 proinflammatory 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

Wildtype (WT) or SP-D knockout (*SP-D*$^{-/-}$) mice were intranasally challenged with *A. fumigatus* conidia or melanin ghost, and *SP-D*$^{-/-}$ mice showed significantly reduced cytokine transcripts in the lung homogenates during the early infection (6 h) compared with the WT mice (Fig. 6, *A* and *B*). In contrast, the melanin ghost was less immunostimulatory in both WT and *SP-D*$^{-/-}$ mice compared with conidia in WT mice (Fig. 6, *B* and *A*, respectively). Upon challenging WT mice with melanin ghost opsonized with rhSP-D, the immunostimulation was significantly higher than the corresponding counterpart challenged with un-opsonized melanin ghost (Fig. 6*B*). The cytokine levels in lung homogenates showed a pattern (Fig. 6*C*) similar to that observed with the transcripts (Fig. 6*B*). Together, our results show that SP-D acts as an immunomodulator, 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); however, 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 PAMP 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, GM, which is masked by the surface melanin-rodlet layers in dormant conidia (28, 29), and 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, whereas melanin binding is through the collagen-like domain of SP-D that is independent of calcium. There exists a layer of superficial rodlets and an underlying melanin layer on the dormant *A. fumigatus* conidial surface (28, 29, 34, 35); however, in places this melanin layer protrudes out of the rodlet layer and hence there is 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 to it is its ability to opsonize airborne pathogens thus 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 with 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 are adequate for conidial binding.

Opsonization by SP-D modulates immune response (10). In the 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 proinflammatory 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 immunocompetency 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), although conidial opsonization with SP-D significantly induced the production of proinflammatory cytokines. In contrast, there was

no significant difference in the production of the anti-inflammatory cytokines (IL-10/IL-1ra) after 20 h of incubation. The *il-10* was an exception, showing significant up-regulation at the early time point. This may be due to regulation at a post-transcriptional level or autoregulation of IL-10 expression, promoting inflammation to protect against infection.

In the naive lung, SP-D interacts with signal regulating protein α receptors on macrophages through their CRD, suppressing proinflammatory cytokine production (11). However, 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 only a part of the collagen-like domain of SP-D is required for the melanin binding and that SP-D–melanin interaction likely promotes SP-D binding to the calreticulin/CD91, thus stimulating the secretion of proinflammatory 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 worthwhile 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 with the wildtype (WT) mice, suggesting that SP-D possibly plays an important role in eliciting immune response against *A. fumigatus*. 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 other humoral immune components exist 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 with immunocompetent mice (40). In an immunosuppressed model, *SP-D*$^{-/-}$ mice died earlier compared with WT mice upon intranasal challenging with *A. fumigatus* conidia (after 2 days, 43% for *SP-D*$^{-/-}$ mice compared with 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 with un-opsonized melanin ghosts, confirming the direct immunomodulatory effect of SP-D. In addition, melanin quenches ROS, one of the defensive mechanisms of the human immune system (30, 31), prevents phagocytosis to a certain extent (30), and also binds metals, acting as a physiological redox buffer, and thereby contributes as a sink for harmful unpaired electrons (43). However, upon opsonization by SP-D, melanin loses its ROS-quenching capacity. Together, our study suggests that SP-D acts as a pathogen recognition receptor, facilitating the *A. fumigatus* conidial recognition by the innate immune cells upon binding to the *A. fumigatus* conidial surface melanin, inhibiting ROS quenching capacity of melanin, consequentially augmenting immune responses.

TNF-α is up-regulated in the 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 *SP-D*$^{-/-}$ mouse lungs upon inoculation with conidia or melanin ghosts. 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), whereas IL-6-

deficient mice were more susceptible to IA than wildtype 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 the 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 γ-interferon independent pathway (49). We found an up-regulation of MDM *il-12* with rhSP-D–opsonized conidia at early interaction times. Taken together, SP-D–opsonized *A. fumigatus* conidia induces proinflammatory 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 ghosts 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 an 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

The *A. fumigatus* clinical isolate CBS144-89 was used as the wildtype strain (28). The isolate was maintained on 2% malt agar slants at ambient temperature. Conidia were harvested from 12- to 15-day-old slants using 0.05% Tween/water, washed, resuspended in Tween/water, and filtered through 40-μm Falcon™ cell strainer (Thermo Fisher 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. PFA fixation of conidia was performed as described earlier (28).

Surfactant protein D

Full-length recombinant human SP-D was purchased from Abcam (ab152069) as well as from R&D Systems (1920-SP) (375 amino acids (aa), apparent mass of 43 kDa). A recombinant human SP-D was expressed in *Escherichia coli* (42, 54, 55); this rhSP-D consisted of eight triple repeats of collagen-like domain (eight Gly-Xaa-Yaa repeats; 24 aa) followed by 46 aa of neck region and 106 aa of CRD domain (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, ∼17 kDa on SDS-PAGE), which is composed 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.). The assay was linear over a range of 0.1–1.0 EU/ml (10 EU = 1 ng of endotoxin).

Immunolabeling

PFA-fixed conidia (2×10^6) were incubated with heat-inactivated normal human serum (NHS; 56 °C for 30 min) for 1 h. 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; dilution 1:200). The buffer used for the assay was either PBS or HEPES buffer, both supplemented with 5 mM $CaCl_2$; between each incubation step, conidia were washed three times with the buffer. Labeled conidia were observed under a fluorescent microscope. For flow cytometry, PFA-fixed conidia (2×10^6) were washed twice with binding buffer (PBS, pH 7.4, supplemented with 1% bovine serum albumin (BSA) and 5 mM $CaCl_2$) and incubated with SP-D (1 h, 37 °C). Next, conidia were washed twice and incubated overnight with primary anti-human SP-D antibody (mouse monoclonal IgG2B clone 292201 for SP-D and rabbit polyclonal IgG, 1:50 dilution; Santa Cruz Biotechnology, Reference no. 13980 for rhSP-D) at 4 °C followed by washing and incubation with anti-rabbit fluorescein-tagged IgG (Sigma Immunochemicals F-6257 1–200 dilution for SP-D and Life Technologies, Inc., F-2765, 1:100 dilution for rhSP-D) on ice for 1 h. Conidia were fixed in 2% PFA in PBS for 5 min on ice, washed, and suspended in PBS. The samples were acquired by using LSR II (BD Biosciences), and the data were analyzed by BD FACS DIVA (BD Biosciences) and Flowjo. The data are presented as percentage of cells positive for the indicated markers or median fluorescence intensities of their expression. Inhibition/pulldown assays were performed upon preincubation of SP-D with monosaccharides, LPS, or EDTA for 15 min at 37 °C. Preincubated samples were centrifuged, and the supernatants were used for the flow-cytometric analysis.

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 alkali-soluble (AS) cell-wall fraction, respectively, as described earlier with few modifications (57). AI fraction was subjected to periodate oxidation for 4 days instead of 3 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.

Binding assay

Direct binding

Direct binding was 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 1 h with PBS containing 1% BSA, and then 50 ng of SP-D (in PBS/BSA with Ca^{2+}) was added. After 1 h of incubation at 37 °C, 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 the mouse against human SP-D (dilution 1:2500) was added and incubated for 1 h. 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_2O_2 for detection at A_{492}. Between the addition of SP-D primary/secondary antibodies, microtiter wells were washed at least five times with PBS/Tween (0.5%).

Pulldown/inhibition assays

Assays were performed by preincubating 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.

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 Pasteur (convention 12/EFS/023). The peripheral blood mononuclear cells (PBMCs) were isolated by a density-gradient separation method using Ficoll (Eurobio, France). The isolated PBMCs were resuspended in RPMI 1640 medium (Gibco), and 2×10^6 cells were seeded in each well of the 12-well microtiter plates (Nunc Labware products; Sigma). Following overnight incubation, the cells were washed twice with PBS and RPMI 1640 medium supplemented with 10% NHS and granulocyte macrophage–colony-stimulating factor (10 ng/ml; Sigma) was added for monocyte differentiation into macrophage (MDMs). After 6 days, medium was discarded, and the MDMs were washed with PBS and used for further experiments.

Opsonization and phagocytosis

Conidial opsonization

Conidia (1×10^6) were opsonized with SP-D (50 ng/ml) in HEPES buffer containing 5 mM $CaCl_2$ for 30 min at 37 °C. Conidia incubated with HEPES buffer alone was used as the negative control.

Phagocytosis and colony-forming units

Opsonized conidia (1×10^6 conidia/well) were added to MDM culture in RPMI 1640 medium supplemented with 10% heat-inactivated NHS and incubated at 37 °C in a CO_2 incubator for 1 h. Supernatants were discarded, and the cells were washed twice with PBS and lysed by adding 100 µl of 1% Triton X-100 (30 min at 4 °C). The contents were collected and after dilution plated on malt agar plate and incubated at 37 °C for 36 h followed by fungal colony counts.

MTT assay

Conidia (1×10^5 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 2 h in CO_2 incubator at 37 °C. Wells were washed with RPMI 1640 medium; MDMs were lysed with 0.5% sodium deoxycholate (Sigma). Then, RPMI 1640 medium was added to the wells and incubated at 37 °C for 48 h to allow the conidial growth. MTT solution (150 µl; Calbiochem, 5 mg/ml in PBS) was added to each well and incubated for 4 h. After washing the wells with PBS, 500 µl of isopropyl alcohol in 0.04 N HCl was added to dissolve the formazan (2 h at 37 °C); 100 µl of the sample was transferred to another 96-well plate, and absorbance was recorded at 560 nm using an ELISA reader (BioTek Power Wave XS2 with Gen5 software).

Cytokine analysis

PFA-fixed wildtype conidia were opsonized by SP-D in a final concentration of 200 ng in 100 µl of HEPES at 37 °C for 30 min. Thereafter, the opsonized conidia were washed in HEPES buffer and resuspended in RPMI 1640 medium without serum (incomplete RPMI). The MDMs were added with 5×10^5/well un-opsonized or SP-D–opsonized conidia. After incubation for 20 h at 37 °C in a CO_2 incubator, the supernatants were collected and stored at −20 °C until further analysis. The cytokines in the co-culture supernatant were quantified by DuoSet ELISA kits (R&D Systems).

ROS production

Conidia (1×10^6 per well) or melanin ghosts (corresponding to an equivalent of 1×10^6 conidia) either un-opsonized or opsonized with SP-D were made to interact with MDM (obtained after plating 2×10^6 PBMCs per well) for 2 h in a CO_2 incubator. Then we added 100 µM dichlorodihydrofluorescein diacetate (stock solution of 1 mM prepared in DMSO) and incubated further for 1 h in a CO_2 incubator. Following this, 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, India). Animals were maintained as per the institutional guidelines for the care and use of experimental animals. Mice with targeted deletion of *Sftpd* (*SP-D*$^{-/-}$) on a Swiss black background and the wildtype (WT) mice of the same genetic background were obtained from Dr. Jeffrey Whitsett, Cincinnati Children's Hospital Medical Centre. Both SP-D gene-deficient female and male mice have reproductive defects, such as prolonged distrust, delayed mating, significantly high preimplantation 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*$^{-/-}$ mice challenged with dormant conidia and melanin ghost, respectively. The fifth group was WT mice challenged with melanin ghost preopsonized by 5 µg of rhSP-D for 15 min at 37 °C. Mice were administered 1×10^7 wildtype dormant conidia or melanin ghost (40 µg; eq to 1×10^7 conidia) intranasally. After 6 h, 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 mouse lungs was extracted using RNAiso Plus (TAKARA) and quantified using nano-spectroscopy. After determining the 260:280 ratios for RNA quality, it was treated with DNase I (Thermo Fisher Scientific, Rockford, IL; 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) 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 OligoAnalyzer software. Primers used for human and murine samples are provided in Tables S1 and S2. 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, pH 7.4 (Gibco), containing 0.5% Triton X-100, 0.02% sodium azide, phenylmethylsulfonyl fluoride (1 mM) (all from Sigma), and protease inhibitor mixture (GE Healthcare). Homogenates were kept on ice for 30 min with intermittent vortexing. Samples were then centrifuged at 14,000 × *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 Scientific) according to the manufacturer's protocol. An equal amount of protein was pooled for each group, and 50 µg per well of samples was loaded into 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 were performed by one-way analysis of variance with Fisher's least significant difference test in the GraphPad Prism software (version 6.07). $p < 0.05$ was considered statistically significant.

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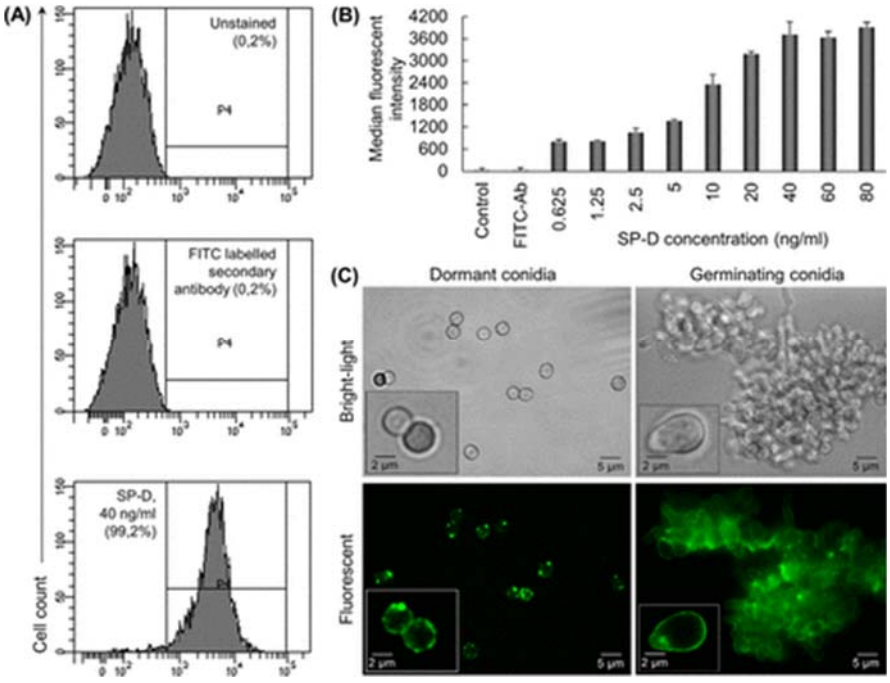

Figure 1. SP-D binds to the surface of *A. fumigatus* conidia. *A,* flow cytometry showing binding of SP-D to dormant *A. fumigatus* conidia. *B,* flow-cytometry data showing concentration-dependent binding of SP-D to dormant conidial surface. *C,* localization of SP-D on the surface of dormant and germinating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

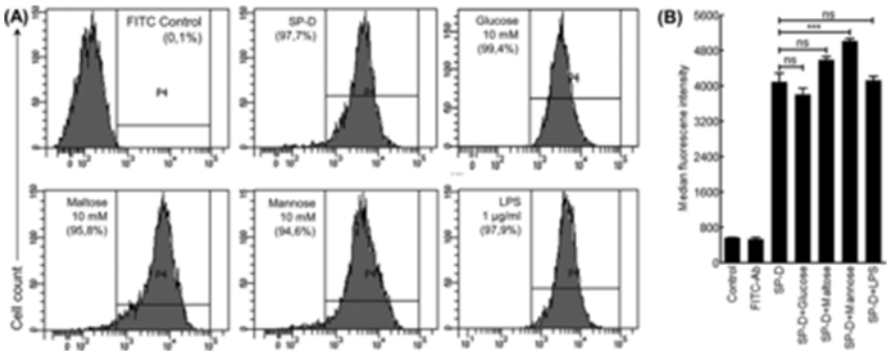

Figure 2. SP-D binding to *A. fumigatus* conidia is not inhibited by simple sugars and glycolipid. *A,* flow cytometry data showing the lack of inhibition of SP-D binding to dormant *A. fumigatus* conidia upon preincubation of SP-D (40 ng/ml) with glucose (10 mM), maltose (10 mM), mannose (10 mM), and DNA technologies oligo analyzer software 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. Pretreatment with mannose increased SP-D binding to conidia (***, $p < 0.0001$), possibly due to its sticky nature compared other sugars. *ns* means not significant.

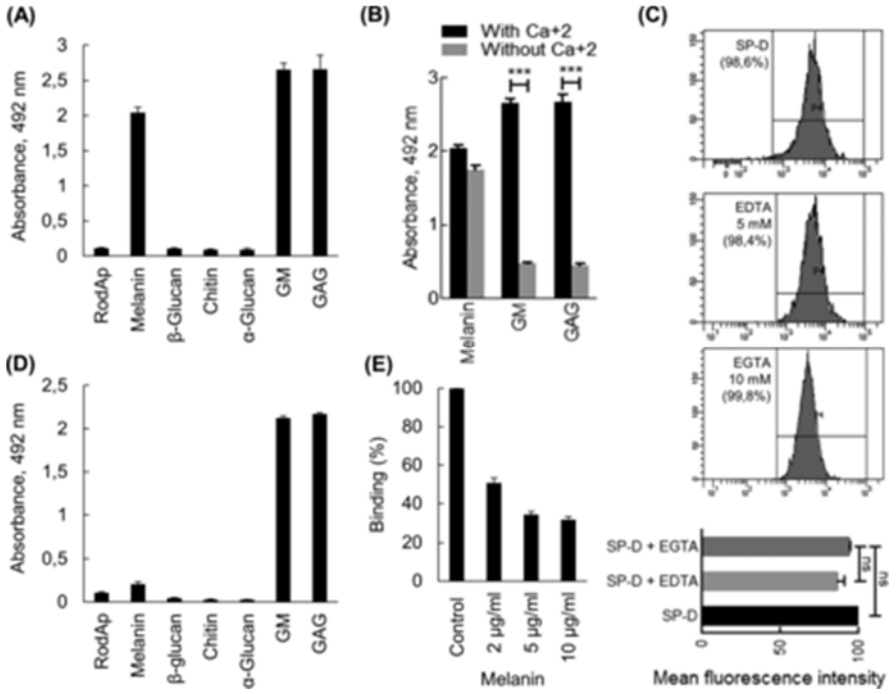

Figure 3. SP-D binds to melanin, GM, and GAG components of the *A. fumigatus* cell-wall, whereas the truncated SP-D with CRD interacts only with GM/GAG. *A,* ELISA shows binding of SP-D to melanin, GM, and GAG. *B,* SP-D binding to melanin is calcium-independent, whereas SP-D binding to GM and GAG requires calcium (only ***, $p < 0.0001$ are represented here). *C,* flow cytometry and median fluorescence intensity values show that SP-D binding to the dormant *A. fumigatus* conidia is not inhibited by the addition of EDTA or EGTA, the calcium chelators. *D,* ELISA shows that SP-D–CRD binds to carbohydrates (GM and GAG) but not to melanin. *E,* flow cytometry shows 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. *ns* means not significant.

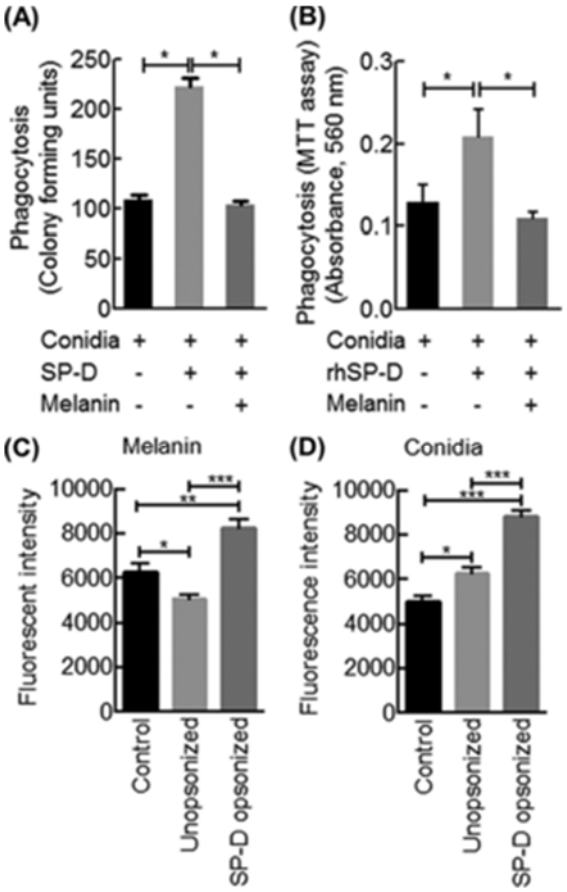

Figure 4. SP-D opsonizes *A. fumigatus* conidia facilitating their phagocytosis by human peripheral blood MDM and SP-D opsonization reduces ROS quenching effect of melanin. *A,* phagocytosis of SP-D–opsonized conidia (MDM + (conidia + SP-D)) was greater compared with the un-opsonized conidia (MDM + conidia). In the case of preincubation 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 2 h. After washing, cells were lysed, and the phagocytosed conidia were allowed to grow for MTT assay. In this assay, absorbance directly correlates with the phagocytotic 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* represent mean ± S.D. of at least three independent experiments; *, $p < 0.01$. *C,* melanin ghosts significantly reduced ROS production by MDM compared with 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 the un-opsonized conidia show ROS production higher than the control MDMs as these conidia were metabolically active (alive) and not PFA-fixed. Data for *C* and *D* represent the mean ± S.D. of at least four independent experiments; *, $p < 0.01$; **, $p < 0.001$; and ***, $p < 0.0001$.

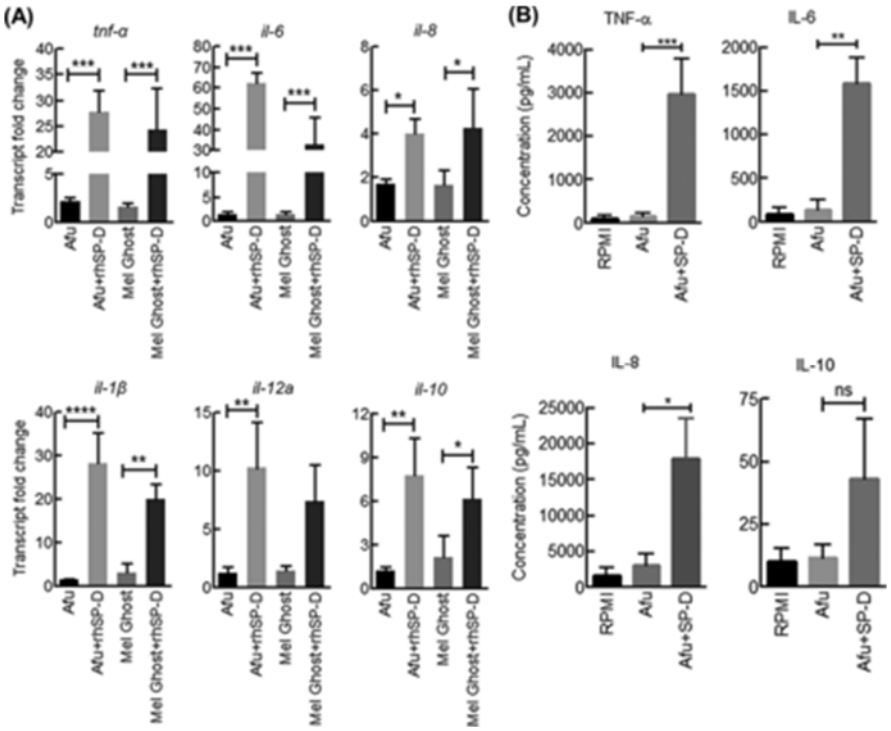

Figure 5. Opsonization of conidia with SP-D and rhSP-D induces proinflammatory cytokines in human MDMs. *A,* MDMs were stimulated with either conidia (*Afu*), melanin ghosts, or conidia/melanin ghosts opsonized with rhSP-D for 2 h. After washing the cells, total RNA was isolated and used for evaluation of cytokine transcripts by qRT-PCR. The transcript fold change was compared with that of the MDMs incubated with un-opsonized conidia (*Afu*). Data represent 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 20 h, and the cytokines released were measured by ELISA. Data represent mean ± S.E. of independent experiments of MDMs from eight healthy donors. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$; *ns* means not significant.

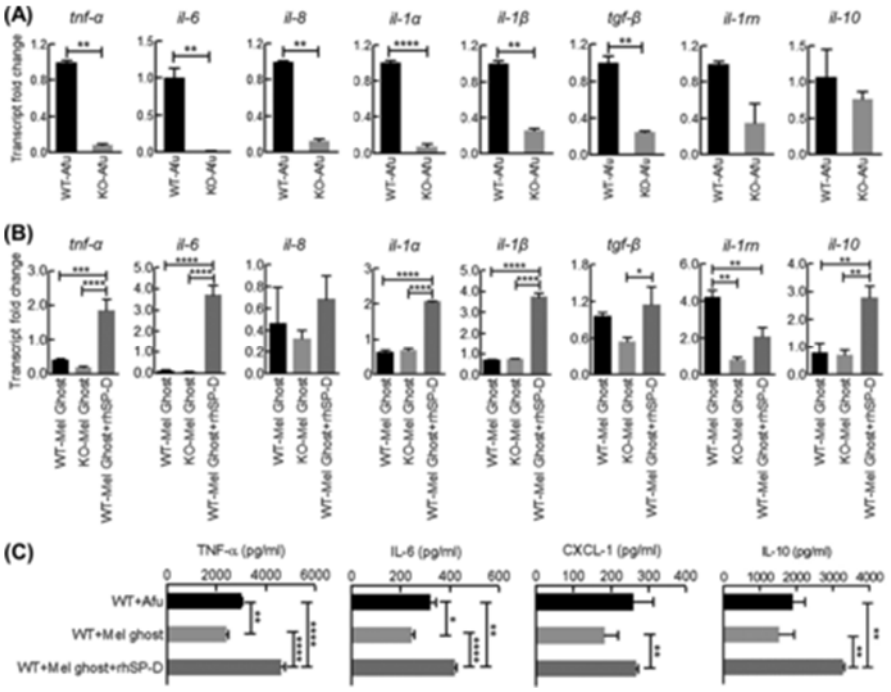

Figure 6. Deficiency of SP-D results in alleviated immune response to *A. fumigatus* conidia and melanin ghost in mice. *A,* transcript fold change was determined by comparison with that in WT mice challenged with dormant conidia (*WT-Afu*). *SP-D*$^{-/-}$ (KO) mice challenged intranasally with dormant 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 with 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 with that in WT mice challenged with dormant conidia (*WT-Afu*) in *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 represent 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 wildtype mice challenged with *A. fumigatus* conidia (*WT-Afu*). The scale in *A* and *B* 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-*A. fumigatus* and KO-melanin ghost are not significantly different, except IL-8 and IL-1α, where the KO-melanin (*KO-Mel*) ghost has higher levels than KO-*A. fumigatus*. Nevertheless, both conidia and melanin ghost failed to stimulate cytokine secretion from *SP-D*$^{-/-}$ mice.