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Surface structure characterization of *Aspergillus fumigatus* conidia mutated in the melanin synthesis pathway and their human cellular immune response

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Running title: Melanin mutants of *Aspergillus fumigatus* and immune response

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Abstract

In *Aspergillus fumigatus*, the conidial surface contains dihydroxynaphthalene (DHN)-melanin. Six-clustered gene products have been identified that mediate sequential catalysis of DHN-melanin biosynthesis. Melanin thus produced is known to be a virulence factor, protecting the fungus from the host defense mechanisms. In the present study, individual deletion of the genes involved in the initial three steps of melanin biosynthesis resulted in an altered conidial surface with masked surface rodlet layer, leaky cell wall allowing the deposition of proteins on the cell surface and exposing the, otherwise masked, cell wall polysaccharides at the surface. Melanin as such was immunologically inert; however, deletion mutant conidia with modified surfaces could activate human dendritic cells and the subsequent cytokine production in contrast to the wild-type conidia. Cell surface defects were rectified in the conidia mutated in downstream melanin biosynthetic pathway and maximum immune inertness was observed upon synthesis of vermelone onwards. These observations suggest that though melanin as such is an immunologically inert material, it confers virulence by facilitating proper formation of the *A. fumigatus* conidial surface.

Introduction

Melanin is a pigment that exists from humans to plants and has several functions including resistance against environmental stress such as UV light and oxidizing agents (1, 2). In air-borne fungal spores, melanin helps in invasion of the host (3, 4) and contributes to the virulence of fungal pathogens (5, 6). Fungi produce different types of melanin: dihydroxynaphthalene (DHN) melanin, pyomelanin and DOPA-melanin. *Aspergillus fumigatus* produces the pigment DHN-melanin, responsible for the characteristic gray-green color of conidia. *A. fumigatus* is also able to produce a brownish pigment, pyomelanin, as an alternative melanin (7). Pyomelanin is produced
via degradation of L-tyrosine with homogentisic acid (HGA) as the main intermediate. On the other hand, *Cryptococcus neoformans* and *Paracoccidioides brasiliensis* synthesize DOPA-melanin (8). Production of melanin has been associated with the survival of the fungal species in the host (8, 9). DHN-melanin is hydrophobic and negatively charged, which modulates the binding capacity of conidia to host fibronectin and laminin present in the lungs (10). DHN-melanin is also essential for the proper assembly of cell wall layers in *A. fumigatus*. Pyomelanin was shown to protect the fungus from host defense mechanism, i.e., reactive oxygen intermediates, and hence considered to be protecting the fungus against immune effector cells during infection (11). DOPA-melanin contributes to host death, fungal burden and dissemination (8).

Genes responsible for the synthesis of DHN-melanin in *A. fumigatus* belong to a 19-kb cluster located on chromosome 2. Six genes have been identified in this cluster and their functions were elucidated (Fig. 1) (4-6, 12-14). The *PKSP* (*ALBI; AFUA_2G17600*) is the first gene of the pathway and codes for a polyketide synthase, which is responsible for catalyzing the synthesis of the heptaketide naphthopyrone from acetyl-CoA and malonyl-CoA. The heptaketide is then shortened by hydrolysis, reduction and dehydration by Ayg1p (*AFUA_2G17550*, *Arp2p* (*AFUA_2G17560*) and *Arp1p* (*AFUA_2G17580*) respectively. The generated product 1,3,6,8-tetrahydroxynaphthalene (THN) is reduced again by Arp2p and the resulting vermelone is oxidized by the copper oxidase Abr1p (*AFUA_2G17540*) to form the 1,8 DHN melanin, which is polymerized by the laccase Abr2p (*AFUA_2G17530*) (12, 15).

The role of the conidial melanin in the *A. fumigatus* virulence has been studied by using either melanin ghosts or the pigment-less mutant wherein *PKSP* gene, which encodes protein involved
in the first step of melanin biosynthesis, has been deleted (4, 9, 16-19). These reports demonstrated that melanin protects the conidia against reactive oxygen species (ROS), masks the recognition of various *A. fumigatus* pattern-associated molecular patterns (PAMPs), inhibits macrophage apoptosis and phagolysosome fusion and attenuates the host immune response. All these functions of melanin contribute to increased survival of conidia in the macrophages and to promote the dissemination of *A. fumigatus* within the host.

However, the importance of melanin in the organization of the *A. fumigatus* conidial cell wall, structural organization of the conidial surface due to the lack of melanin or in the presence of melanin intermediates and effect of melanin intermediate biosynthetic gene deletion on the activation of host immune cells are still unknown. In this study, by using melanin mutants that are deleted in each of the genes of the melanin synthesis pathway, we analyzed the surface structure of conidia by biochemical and biophysical methods and explored the immune-modulatory role of these conidia on human dendritic cells (DCs), the professional antigen presenting cells (APC) that act as sentinels of the immune system. We demonstrate for the first time that until the scytalone precursor was synthesized by Arp2p, the first three melanin biosynthetic gene deletion mutants (ΔpksP, ΔaygI and Δarp2) induce the maturation of DCs and cytokine production. Upon vermelone biosynthesis after dehydration of the scytalone by Arp1p and reduction by Arp2, the subsequent mutants (Δabr1 and Δabr2) behaved like wild-type (WT) conidia, losing their capacity to prime the maturation of DCs and cytokine production. The Δarp1 conidia having scytalone but not vermelone on their surface were able to induce only a weak maturation of DCs. Further, we found that activation of DCs by ΔpksP, ΔaygI and Δarp2 conidia was in part due to amorphous proteinaceous conidial surface with patchy rodlets or surface exposed other cell wall component. Δarp1 conidia phenotype was intermediate between Δarp2
and Δabr1, whereas Δabr1, Δabr2 and WT conidia lack such material and have conidial surfaces covered with rodlets, which contribute for the masking of conidia recognition by the innate immune cells.

1. Material and Methods

2.1. Fungal strains and culture conditions

The melanin precursor strains ΔpksP, Δayg1, Δarp2, Δarp1, Δabr1, Δabr2 and the wild-type (WT) strain B5233 have been maintained in silica gel at J. Kwon-Chung’s laboratory in NIH until use (6, 14, 15). All strains were cultivated on malt-agar (2%) medium at ambient temperature for at least 15-days before collecting the resting conidia. Conidia were harvested from the culture medium using 0.05% Tween-20 in water. Conidial suspensions were filtered on BD Falcons (BD Biosciences) to remove any mycelium. For immunolabeling and DC experiments, resting conidia were fixed with paraformaldehyde (PFA)–fixed (2.5% (v/v) PFA in PBS) for overnight at 4°C. The fixed conidia were subsequently washed three times with 0.1 M NH₄Cl and once with PBS-0.1% Tween-20.

2.2. Melanin extraction

The isolation of melanin from the WT conidia was performed as previously described (20, 21). After growing the fungus on malt-agar medium for 15-days at ambient temperature, conidia of each strain were collected in an aqueous 0.05% Tween-water. Briefly, Conidia were treated with a combination of proteolytic (proteinase K, Sigma) and glycohydrolytic (Glucanex, Novo) enzymes, denaturant (guanidine thiocyanate) and hot, concentrated HCl (6 M). This treatment resulted in an electron-dense layer similar in size and shape to the original conidial melanin layer without underlying cell components for which reason they were called “melanin ghosts” (20).
2.3. *Extraction of the alkali soluble (AS) polysaccharide fraction from conidia*

Conidia were disrupted with 0.5 mm diameter glass beads in a FastPrep (MP Biomedicals). The conidial cell wall fraction was recovered by centrifugation, washed with water then freeze-dried. Dried cell wall fraction was boiled 50 mM Tris-HCl pH 7.4 containing 50 mM EDTA, 2% SDS and 40 mM β-mercaptoethanol (10 min, twice) to get rid of proteins and extensively washed with water so as to have cell wall polysaccharides. From the latter, the AS-fraction was extracted as described earlier (22).

2.4. *Extraction of conidial surface RodA protein (RodAp) involved in the rodlet formation*

The RodAp was extracted from the spore surface by incubating $10^9$ dry conidia with 48% (v/v) hydrofluoric acid (HF) for 72 h at 4°C (23). The contents were centrifuged (10,000 g, 10 min) and the supernatant obtained was dried under N$_2$. The dried material was reconstituted in H$_2$O and an aliquot was subjected to 15% (w/v) SDS-PAGE analysis and visualized by silver nitrate staining.

2.5. *Analysis of proteins on the conidial surface*

Conidia were incubated in 0.5 M NaCl solution for 2 h at room temperature at a ratio of $10^{10}$ conidia per ml. The NaCl supernatant was recovered after centrifugation and directly subjected to 10% SDS-PAGE (w/v). 2D-gel electrophoretic analysis of the NaCl extract was carried out as described previously with slight modifications (24, 25). A total amount of 50-100 µg protein was loaded onto IPG strips (11 cm, pH 3-7; GE Healthcare Life Sciences) by in-gel rehydration. After equilibration of the IPG strips, SDS-gel electrophoresis was carried out using Criterion AnykD TGX Stain-Free precast gels (Bio-RAD). Proteins were visualised by UV light and colloidal Coomassie staining (Candiano2004). After scanning, gel images were analysed with the software
Delta 2D 4.3. (Decodon). Protein spots were excised and analysed by mass spectrometry using an ultrafleXtreme MALDI-TOF/TOF device (Bruker Daltonics) as described (26).

2.6. Analysis of carbohydrate on the conidial surface by fluorescence microscopy

The mannose moieties of glycoproteins on the resting conidial surface were labeled with ConA-FITC (Sigma) at 0.1 mg/ml concentrations after incubating the resting conidia for 1 h at 37°C in 0.1 M carbonate buffer pH 9.6 containing 0.1% Tween-20. The hexosamines were labeled with WGA-FITC at 0.1 mg/ml concentrations upon incubating the resting conidia for 1 h at the room temperature in PBS containing 0.1% Tween-20. For immunolabeling, PFA-fixed conidia were incubated with different antibodies as described previously (27). β-(1,3)-glucan was labeled with Dectin-1 (Fc-dectin1 6 µg/ml) followed by FITC conjugated GaHu-Fab2-human IgG (15 µg/ml, a kind gift from G. Brown, University of Aberdeen, Aberdeen, UK) (28).

2.7. Antibodies and reagents for human immunology

Recombinant human (rh) GM-CSF and IL-4 were from Miltenyi Biotec (France). Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (MAbs) to CD80; phycoerythrin (PE)-conjugated MAbs to CD83 and CD86 were from BD Biosciences (France) and PE-conjugated MAb to CD40 was from Becton Dickinson (France). Anti Thr202/Tyr204 phospho-ERK1/2, anti-Thr180/Tyr182 phospho-p38 MAPK, antibodies were purchased from Cell Signaling Technology (USA). Anti-β-actin antibody (AC-15) was from Sigma-Aldrich (USA).

2.8. Generation and culture of human dendritic cells

Monocyte-derived DCs were generated as previously described (29, 30). Immature DCs (0.5x10^6 cells/well/ml) were cultured in the presence of GM-CSF and IL-4 (cytokines) alone; with
cytokines and PFA-fixed conidia of wild type or melanin mutants (1:1 ratio); or cytokines and 1 μg of melanin extracts; or cytokines and 1 μg of alkali soluble polysaccharide fraction of \( A. fumigatus \) cell wall (positive control); or cytokines and NaCl extracts from \( 0.75 \times 10^9 \) conidia for 48 hours. Cells were harvested and cell-free supernatants were stored at -80°C for cytokine analysis. Cells were labeled with fluorochrome-conjugated MAbS for surface marker analysis by using LSR II flow cytometry (BD Biosciences). Five thousand events were recorded for each sample and data were analyzed by BD FACS DIVA software (BD Biosciences).

2.9. Mixed lymphocyte reaction

CD4+ T cells were isolated from peripheral blood mononuclear cells of healthy donors using CD4 micro-beads (Miltenyi Biotec). DCs were washed extensively and were co-cultured with \( 1 \times 10^5 \) responder CD4+ T cells at DC:T cell ratios of 1:10, 1:20, 1:40 and 1:80. After 4 days, either cells were harvested and cell-free supernatants were stored at -80°C for cytokine analysis or cells were pulsed for 16-18 h with \( 0.5 \mu\text{Ci} \) of \(^{3}\text{H}\) thymidine. Radioactive incorporation was measured by standard liquid scintillation counting. The proliferation of cells was measured as counts per minute (cpm) (mean ± SEM of quadruplicate values) after subtracting values of responder T cell cultures alone.

2.10. Measurement of cytokines

Cytokines were quantified in cell-free culture supernatants using BD CBA Human Inflammation kit and Human Th1/Th2 kits (BD Biosciences).

2.11. Statistical analysis
Two-sided, Student-t-test was used for the statistical analysis. P<0.05 was considered as significant (*P<0.05, **P<0.01).

2.12. **Immunoblotting**

Immunoblotting was performed as described previously (31). DCs were washed with ice-cold PBS in RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 1%NP-40, 0.25%Na-deoxycholate, 150mM NaCl, 1mM EDTA, 1mM PMSF, 1 µg/ml each aprotinin, leupeptin, pepstatin, 1mM Na$_3$VO$_4$, 1mM NaF). Equal amounts of proteins from the total cell lysates were subjected to SDS-PAGE followed by transfer of proteins to polyvinylidene difluoride membranes. Membranes were blocked in TBST buffer (0.02 M Tris-HCl (pH 7.5), 0.15 M NaCl and 0.1% Tween 20) containing 5% non-fat dried milk and investigated with a primary antibody for overnight at 4°C. After washing with TBST, membranes were incubated with HRP-conjugated secondary antibody (Jackson immunologicals, USA). The blots were then developed with an enhanced chemiluminescence detection system (Perkin Elmer, USA) as per manufacturer’s instructions.

2.13. **Analysis of the conidial surface by atomic force microscopy (AFM)**

Conidial surfaces were analyzed by AFM, using a Multimode VIII instrument (Bruker, Santa Barbara, CA). Sample immobilization was achieved by mechanically trapping living conidia into porous polycarbonate membranes (it4ip SA, Belgium). After filtering a concentrated suspension of cells, the membrane was rinsed with deionized water, carefully cut and attached to a metallic puck using double-sided tape. Then the mounted sample was transferred to the AFM liquid cell while avoid dewetting. Imagings were performed in contact mode under minimal applied force, using oxide-sharpened microfabricated Si$_3$N$_4$ cantilevers (MSCT, Bruker) with a nominal spring constant of 0.01 N/m. Force measurements were carried out by chemical force microscopy.
(CFM) (32, 33) using gold tips (OMCL-TR4, Olympus, Tokyo, Japan), coated with hydrophobic thiols. To do so, cleaned tips were immersed for 12 h in 1 mM solutions of HS(CH$_2$)$_{11}$CH$_3$ (Sigma) in ethanol, rinsed and dried with N$_2$ prior to use. The cantilevers spring constants were measured by the thermal noise method (Picoforce, Bruker). Force curves were analyzed in order to determine the adhesion force between the conidia and the AFM tip. These adhesion forces were plotted as bright pixels, brighter colors indicating larger adhesion values. Data were processed using the commercial Nanoscope analysis (Bruker) and MATLAB softwares (The MathWorks, Natick, MA). For each strain, several images were taken on different conidia and force measurements were obtained in duplicate using different tips.

3. Results

3.1. *A. fumigatus* conidia from ΔpkSP, Δaygl and Δarp2 induce maturation and activation of human dendritic cells: Conidia from individual melanin biosynthetic pathways gene deletion mutants were used to study the maturation and activation of DCs. Melanin-mutant conidia deficient in the early steps of the biosynthetic pathway (ΔpkSP, Δaygl and Δarp2) induced maturation of DCs as demonstrated by the significantly enhanced expression of CD83 and co-stimulatory molecules CD86, CD80 and CD40 (Fig. 2) (34, 35). The ΔpkSP, Δaygl and Δarp2 conidia also stimulated a panel of DC-cytokines such as TNF-α, IL-1β, IL-6 and IL-10 (Fig. 3). Upon vermelone biosynthesis, the subsequent downstream melanin biosynthetic pathway mutant conidia (Δabr1, Δabr2) behaved like the WT strain, becoming immunologically inert (Fig. 2, 3). The Δarp1 conidia presented an intermediate phenotype, as they induced only modest changes in the expression of co-stimulatory molecules of DCs and secretion of DC-cytokines (Figs. 2, 3).
3.2. **Melanin does not stimulate DC maturation:** As shown in the Figure 4, Melanin ghosts from WT conidia did not induce DC maturation (Fig 4A). We further verified the lack of activation of DCs by analyzing the intracellular signaling pathways and ability of DCs to induce T cell proliferation and cytokines (Fig. 4B-D). We show that melanin ghost failed to phosphorylate p38 MAPK and ERK thus confirming the immunological inert nature of melanin (Fig. 4B). The lack of activation of DCs by WT ghost extract was also reflected in the inability of WT ghost extract-treated DCs to promote T cell proliferation (Fig. 4C) and T cell cytokines IL-2, IFN-γ and IL-5 (Fig. 4D). On the contrary, the AS polysaccharide fraction (rich in α(1,3)-glucan) of the *A. fumigatus* cell wall, used as a positive control, induced maturation of DCs, activation of intracellular signaling pathways and promoted DC-mediated T cell proliferation and cytokine production (Fig. 4A-D). Taken together, these data indicated that conidial surface melanin, either *in situ* or in the extracted form (melanin ghosts), failed to stimulate DC activation and cytokine production.

3.3. **The surface rodlet layer is masked by an amorphous hydrophilic layer:** To investigate if the structural modifications in the ΔpksP, Δayg1, Δarp2 and Δarp1 conidia could be responsible for stimulating the DCs, conidial surfaces were imaged by atomic force microscopy (AFM) (36, 37) (Fig. 5A-O). Rather than using an incident beam as in classical microscopy, AFM probes the small forces acting on the sample surface (37). Three-dimensional images are generated in buffer by scanning a sharp tip over the cell surface while sensing the interaction force between the tip and the surface. Originally invented for topographic imaging, AFM has evolved into a multifunctional molecular toolkit, enabling researchers not only to observe structural details of cells to near molecular resolution (36, 38), but also to measure their
biophysical properties and interactions (39-41). In contrast to the WT conidia that are covered with rodlet structure (30, 42) (Fig. 5M-O), the ΔpksP mutant conidial surface was amorphous without organized rodlet structure (Fig. 5A-C). Some patches of organized rodlet layers were observed on the Δayg1 conidial surface (Fig. 5D-F) and their percentage increased on the Δarp2 conidia (Fig. 5G-I), to finally cover almost all the surface of Δarp1 conidia (Fig. 5J-L). However, the rodlet layer on Δarp1 conidia appeared to be less compact and less organized than that of the WT conidia (Fig. 5M and O). The mutants of further down-stream melanin biosynthetic pathway genes, Δabr1 and Δabr2, presented organized and compact rodlets on the entire surface of their conidia, similar to WT conidia (data not shown).

To investigate if RodAp (responsible for the formation of rodlet layer on the conidial surface) is still present on the ΔpksP mutant conidial surface, all the mutant conidia as well as the WT conidia were treated with HF (30). RodAp could be extracted from the ΔpksP, other melanin mutants and the WT conidia. Their SDS-PAGE profiles (Fig. 6) showed that two bands of RodAp classically seen in the HF extracts of conidia were present in all the melanin pathway mutants and WT conidia (30). A band at 18 kDa could also be observed in ΔpksP, Δayg1 and Δarp2 HF extracts (Fig. 6). Mass spectrometry (MS) and MS/MS analysis showed that this 18 kDa protein corresponds to the Aspf1 antigen, suggesting the loose architecture of rodlets in these mutants. These data confirmed AFM observations that the RodAp were present but hidden by an amorphous material on the surface of ΔpksP, Δayg1 and Δarp2 mutant conidia.

Because, the presence of this amorphous material covers the hydrophobic rodlets, we asked whether the observed surface changes correlated with differences in the conidial hydrophobic adhesive properties. To understand this, we mapped and quantified the nanoscale adhesion
properties of WT and Δpk
sP mutant conidia by AFM with hydrophobic tips. The presence of this
unorganized material on the Δpk
sP mutant conidial surface was associated with a dramatic
reduction in their conidial surface hydrophobicity (Fig. 7). For the WT, force-distance curves
recorded across the cell surface revealed large adhesion forces, ranging from 0.2 to 6 nN (Figs.
7M-O). In contrast, structural changes in Δpk
sP conidia caused profound modifications in the cell
surface physico-chemical properties (Figs. 7A-C). Force-distance curves and force maps showed
the absence of adhesion forces over the entire surface of the Δpk
sP mutant conidia, indicating
that this mutant is hydrophilic. The adhesion force of the other mutants of the melanin pathway
increased with the rank of the mutant in the pathway, from a low adhesion with the Δpk
sP and
Δayg1 conidia (Figs. 7A-F), to a maximal adhesion with WT conidia (Figs. 7M-O). The low
adhesion capacities of the Δpk
sP, Δayg1 and Δarp2 conidia indicated a modification of the cell
surface hydrophobicity that could have influenced conidial recognition by DCs.

3.4 Proteins are present in the amorphous hydrophilic layer of Δpk
sP and Δayg1 mutant conidia: We then investigated the chemical nature of the amorphous layer present on the surface
of Δpk
sP, Δayg1 and Δarp2 mutant conidia. A strong labeling with ConA was observed only
with the Δpk
sP conidia, suggesting that its surface layer is rich in glyco-conjugates. However,
labeling with ConA was either low or negative in other melanin mutants including Δayg1 and
Δarp2, and WT conidia (Fig. 8; data not shown). The surface amorphous material could be
extracted by incubating mutant conidia (mutants for the initial steps of melanin biosynthesis) with
0.5 M NaCl for 2 h and they were positive for protein test, suggesting the presence of glyco-
proteins in this conidial surface amorphous material. As shown in Figure 9, the amount of
proteins present in the extract was very high in Δpk
sP mutant followed by Δayg1. Extract from
Δpk
sP contained 53 µg of proteins per 3x10^9 conidia whereas Δayg1 conidial extract contained
12 µg of proteins. The surface extracts of other mutants conidia i.e., Δarp2 and Δarp1 contained 3.8 µg proteins while in Δabr1, Δabr2 and WT extracts, the amount of proteins was too low to detect. These results thus indicate that smaller amounts of proteins on the surface layer of Δayg1, Δarp2 and Δarp1 conidia reflected in the low or negative ConA-FITC staining of these conidia. Extracted protein mixture of ΔpksP was the subjected to proteomic analysis. Forty-two proteins were identified in the extract and *in-silico* analysis of these proteins by SigPred (http://www.cbs.dtu.dk/services/SignalP/) and CADRE (http://www.cadre-genomes.org.uk/Aspergillus_fumigatus/) revealed that all of them had a signal peptide (Table 1). Extracellular proteins, normally secreted during the vegetative growth of *A. fumigatus*, such as Cat1p, Exg1p and ExoG2p, Aspf1p, ChiB1 were identified in the NaCl extract of ΔpksP resting conidia (43-46). Nine of forty-one proteins were glycosylhydrolases. RodAp was also identified in the NaCl extract. Other proteins, such as proteasome components, translation elongation factors, pyruvate dehydrogenases, adenosine deaminase, protein disulfide isomerase normally found in intracellular compartments, were present in very low amounts as they were identified only once or twice in the proteomic survey.

In order to determine whether the proteins present on the surface of ΔpksP and Δayg1 conidia are responsible for the activation of DC by these mutant conidia, we incubated DCs with the NaCl extract of ΔpksP, Δayg1, Δarp2, Δarp1 and WT mutant conidia. As expected, NaCl extract of ΔpksP and Δayg1 induced maturation of DCs, whereas the NaCl extracts of Δarp2, Δarp1 and WT did not (Fig. 10A-C). These results demonstrated that the surface protein layer was responsible at least in part for the induction of DC maturation following incubation of cells with resting conidia of ΔpksP and Δayg1. However, the amount of proteins present on the surface of Δarp2 and Δarp1 was too low to stimulate DC cells (Fig. 10). Although NaCl extracts from the
ΔpksP and Δayg1 mutant conidial surfaces induced maturation of the DCs based on the phenotype analysis of cells (Fig. 10), they did not induce production of the cytokines such as IL-1β, IL-10 or IL-6 (data not shown). The level of production of above cytokines was on par with control DCs. These data suggest that signals provided by NaCl extracts of ΔpksP and Δayg1 mutant conidia were not sufficient to induce functional activation of the DCs. On the other hand, ΔpksP, Δayg1 and Δarp2 mutant conidia induce various DC cytokines, which could be due to the exposure of cell wall polysaccharides on their conidial surfaces.

3.5 Glucosamine-containing components are exposed at the ΔpksP, Δayg1 and Δarp2 conidial surfaces: To check if any structural cell wall modification occurred and was responsible for DC activation by ΔpksP, Δayg1 and Δarp2 conidia, we labeled mutant and WT conidia with the β-(1,3)-glucan receptor Dectin-1 and with the glucosamine (GlcN) recognizing lectin wheat-germ agglutinin (WGA). Mutants and WT conidia did not bind to Dectin-1 (data not shown), suggesting that β-(1,3)-glucans were not exposed at the conidial surfaces. However, ΔpksP, Δayg1 and Δarp2 were positive for WGA-FITC (Fig. 11), whereas Δabr1, Δabr2 and WT conidia were 3 to 9% WGA-FITC positive (Fig. 11). In line with partial stimulation of DCs, Δarp1 presented an intermediate phenotype: about 40% conidia were WGA-positive. These results suggested that conidia with uniform exposure of glucosamine-containing components on the surface (in cases of ΔpksP, Δayg1 and Δarp2 mutant conidia) were able to induce DC activation whereas conidia with low level of exposure of GlcN-content on the surface did not stimulate DC cells (Δabr1, Δabr2 and WT). When GlcN-exposure was intermediate as in Δarp1 conidia, such conidia were able to induce partial activation of DCs.
Consequently, the absence of melanin or at least of the intermediate scytalone increased permeability of the conidia to secreted proteins which otherwise secreted normally during conidial germination, and exposed the GlcN-polymers on the conidial surfaces. Proteins and GlcN-polymers were responsible for the DCs activation following incubation of cells with ΔpksP, Δayg1 and Δarp2 conidia. The Δabr1 and Δabr2 conidia were immunologically inert like their WT counterparts whereas Δarp1 presented an intermediate phenotype.

4. Discussion

In fungal biology, melanin pigments are attributed with a variety of beneficial function including protection against exogenous stress, UV-irradiation and host defense mechanisms including reactive oxygen species, lytic enzymes and antimicrobial peptides (47) and hence considered to be one of the fungal virulence factors (48). Melanin, being extracellular, also contributes to the fungal spore structure (5). In A. fumigatus, the melanin biosynthesis is reported to require a cluster of six genes (6, 13, 14). In the present study, for the first time we show the effect of deletion of individual genes from the melanin biosynthetic gene cluster on the respective conidial surface morphology and the consequent immune responses to the mutant conidia. The mutant conidia with deletion in one of the first three genes of the melanin pathway, ΔpksP, Δayg1 and Δarp2, stimulated the maturation and elicited production of IL-6, IL-1β and TNF-α and IL-10 cytokines by DCs, whereas Δabr1, Δabr2 and WT conidia were not immunogenic. The Δarp1 mutant presented an intermediate phenotype as it induced partial activation of DCs. Melanin as such were immunologically inert as the melanin ghost extracted from the WT conidia failed to activate DCs. These results suggested that the surface of ΔpksP, Δayg1, Δarp2 resting
conidia are covered with specific common compounds which are rare in Δarp1 and are responsible for the maturation of DCs.

The major obvious phenotypes of the ΔpksP Δayg1 and Δarp2 resting conidia were the absence or very few patches of rodlets at the surface. Although the rodlets that immune-silence the resting conidia were present in the above mutant conidia, the masking of rodlets by an amorphous and hydrophilic layer enabled resting conidia to be immunogenic (24, 49). A few patches of amorphous and hydrophilic layer could also be observed on Δarp1 conidial surface. When downstream genes of the DHN-melanin pathway were deleted, the appearance of the rodlets and the hydrophobicity of the conidia increased. Previous studies demonstrated that deletion of PKSP and ARP2 correlated with a decreased ability to bind laminin on the conidial surface (10). This result is explained by the low hydrophobicity of the conidia, which reduced the electronegative charge, required for laminin binding.

The amorphous and hydrophilic layer is composed of GlcN-containing components and deposited ConA-positive proteins mostly in ΔpksP. These proteins were analyzed in the ΔpksP mutant and their identification showed that they are usually secreted during vegetative growth. Most hydrolases (such as β-1,3-glucosidases, β-N-acetylhexosaminidase, mannosidase), catalase, AspF1, Asp-hemolysin, and chitinase found in the amorphous surface layer of the resting ΔpksP conidia were usually identified during mycelial growth in a protein-based medium (43-46). Their presence on the surface of the resting conidia of ΔpksP and Δayg1 is explained by modifications of the ionic strength of the hydrophobin layer resulting from the absence of melanin or at least the YWA1, a melanin biosynthetic intermediate which is synthesized by the combined activities of PksPp and Ayg1p. The easy removal of these hydrophilic glycoproteins by 0.5 M NaCl suggested
that they adhered to the conidial cell wall through electrostatic binding. These surface proteins in
the conidial amorphous layers were responsible for the DC maturation and cytokine production.
Interestingly, cell wall structural modifications resulting from the absence of α-(1,3) glucan as in
*A. fumigatus Δags1Δags2Δags3 (Δags)* mutant also gave the similar conidial phenotype, a
hydrophilic protein layer on the surface of the conidia which stimulated the host defense
reactions (24). However, the composition of proteins in this amorphous layer in the triple Δags
and ΔpksP deletion mutants was not the same, suggesting defined cell wall permeability defects
due to the deletion of different cell wall component biosynthetic genes. When the melanin
synthesis pathway was blocked more downstream by gene deletion, fewer proteins were able to
cross the conidial cell wall. Of note, DC cytokine production was less for ΔpksP than for Δayg1
and Δarp2 mutant conidia (Fig. 3). This result suggests that glycoproteins were less stimulatory
than GlcN-residues present on the surfaces of the ΔpksP, Δayg1 and Δarp2 mutant conidia, and
that these GlcN-residues are less exposed on ΔpksP mutant surface due to the higher amount of
glycoproteins in this mutant.

The presence of GlcN-residues on the surfaces of the ΔpksP Δayg1 and Δarp2 mutant conidia
could be explained by the unmasking of cell wall chitin due to the absence of melanin. The
hexosamines present in the conidial cell wall were composed of long chains of fibrillar water
insoluble chitin, amorphous and soluble chitin oligosaccharides (containing 10-15 N-acetyl-
glucosamine) and deacetylated chitin (chitosan) (Beauvais et al, unpublished results). Previous
studies on different sized chitin polymers have shown that >70 µm chitin polymers were
immunologically inert with murine macrophages, while both intermediate-sized (40–70 µm) and
<40 µm chitin polymers stimulated TNF elaboration by macrophages (50), and only <40 µm
chitin polymers induced IL-10 production. Small particles of chitosan were even better
macrophage immune-stimulators. Chitosan of less than 20 µm elicited the most IL-1β from bone marrow-derived macrophages (50). Although several chitin-binding proteins have been identified in mammalian cells, no chitin receptor had thus far been identified so far. A recent study on recognition of innate immune cells by *Candida albicans* chitin revealed that though there was no direct dectin-1 and chitin binding, chitin was capable of blocking dectin-1 mediated immune responses (51). Similarly, these small and/or deacetylated chitins were likely unmasked on the surface of the first three melanin mutants and were responsible for the DC maturation. Such an unmasking phenomenon was reported in chitin synthase mutants ΔcsmA and ΔcsmB, wherein the deletion of two of the chitin synthase genes (CSMA and CSMB) in *A. fumigatus* resulted in the increased exposure of WGA-positive components on the conidial surfaces (42). The ΔcsmA conidial surface was also amorphous and ConA-positive; however, ConA-positive materials were not glycoproteins, rather due to the exposure of mannan-containing polymers. This further confirms the differential permeability defects due to the deletion of specific cell wall component biosynthetic genes.

Chai and co-workers (9) also demonstrated that melanin purified from WT conidia was also poorly immunogenic for the stimulation of cytokines by peripheral blood mononuclear cells. On the other hand, as seen in our study, ΔpksP conidia elicited significantly higher cytokine production, such as IL-10, IL-6 and TNF-α. They found that blockage of dectin-1 with laminarin reduced the cytokine production in response to ΔpksP conidia, which could be correlated with WGA-FITC positivity of ΔpksP, Δayg1 and Δarp2 mutant conidia and the observations that chitin can influence dectin-1 mediated immune responses (51). Jahn and co-workers (52) observed that macrophage phagocytosis and intracellular killing was significantly higher with ΔpksP conidia than WT conidia. Thywissen and co-workers (18) showed that inside the
phagolysosome, WT conidial DHN-melanin was responsible for the inhibition of the phagolysosomal acidification of mouse and human macrophages and neutrophils. The $\Delta pksP$ conidia, in contrast, were located in an acidic environment in the phagolysosome, which coincides with more effective killing of these conidia. The percentage of $\Delta ayg1$, $\Delta arp2$ and $\Delta abr2$ conidia were lower in acidified-phagolysosomes than $\Delta pksP$ conidia, but higher compared to the WT conidia. These results show that melanin intermediates formed by the downstream biosynthetic pathway increase the conidial protection. However, the final product, the DHN-melanin, was important for the maximal protection as it facilitates the formation of a complete surface rodlet layer that hides conidia from their immediate recognition by the immune system.

5. Conclusions

The absence of at least the scytalone intermediate of DHN melanin is responsible for structural and chemical modifications of the cell surface, which will have an obvious impact on the immune response of the host towards the corresponding mutant. Our results also show that melanin is essential to acquire the right surface properties with precise charge and hydrophobicity that are necessary to have immunologically inert conidia due to an exposed rodlet layer.

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References:


Figures legends

**Figure 1:** Melanin biosynthetic pathway – schematic representation.

**Figure 2:** Effect of melanin biosynthetic pathways mutant conidia on the maturation of human dendritic cells. Immature DCs (0.5x10^6 cells/ml) were cultured in the presence of cytokines GM-CSF and IL-4 (Ctr DC) or with cytokines and WT conidia or melanin biosynthetic pathways mutant conidia (ΔpksP, Δaygl, Δarp2, Δarp1, Δarb1, Δarb2) at 1:1 ratio for 48 hours. The percentage expression of (A) CD83, (B) CD86, and mean fluorescence intensities (MFI) of (C) CD80 and (D) CD40 were analyzed by flow cytometry. Data (mean ± SEM) are from four to five donors. The level of statistical significance is indicated (*P<0.05, **P<0.01).

**Figure 3:** Induction of dendritic cell cytokines by the melanin biosynthetic pathways mutant conidia. Immature DCs (0.5x10^6 cells/ml) were cultured in the presence of cytokines GM-CSF and IL-4 (Ctr DC) or with cytokines and WT conidia or melanin biosynthetic pathways mutant conidia (ΔpksP, Δaygl, Δarp2, Δarp1, Δarb1, Δarb2) at 1:1 ratio for 48 hours. The cell-free culture supernatants were analyzed for the secretion of (A) TNF-α, (B) IL-1β, (C) IL-6 and (D) IL-10. Data (mean ± SEM) are from four donors and were presented as pg/ml. The level of statistical significance is indicated (*P<0.05, **P<0.01).

**Figure 4:** Effect of dihydroxynaphthalene (DHN) melanin on the phenotype, intra-cellular signaling pathways and T cell stimulatory abilities of dendritic cells. (A) Immature DCs (0.5x10^6 cells/ml) were cultured in the presence of cytokines GM-CSF and IL-4 (negative control :Ctr-DC) or with cytokines and 1 μg of DHN melanin from WT conidia (Melanin-DC) or 1 μg of
alkali soluble polysaccharide fraction of *A. fumigatus* cell wall (AS-DC; positive control) for 48 hours. The percentage expression of CD83, CD86, and mean fluorescence intensities (MFI) of CD80 were analyzed by flow cytometry. Data (mean ± SEM) are from four donors. (B) DCs were treated with 1 μg of DHN melanin from WT conidia (left panels) or 1 μg of alkali soluble polysaccharide fraction of *A. fumigatus* cell wall (AS; positive control) (right panels) for indicated time points. The phosphorylation of ERK1/2 (pERK1/2) and p38 MAPK (pp38) was analyzed by immunoblotting. Unstimulated condition is represented by *Med*. (C) DCs were cultured in the presence of cytokines GM-CSF and IL-4 (negative control: Ctr-DC) or cytokines and 1 μg of melanin extracts from WT conidia or cytokines and 1 μg of alkali-soluble (AS) fraction (positive control: AS-DC) for 48 hours. Following extensive washing, DCs were co-cultured with CD4+ T cells at various DC:T cell ratios. The T cell proliferation was quantified by (³H) thymidine incorporation and values are presented as counts per minute (cpm). (D) The CD4+ T cell cytokines IL-2, IFN-γ and IL-5 in the above DC-T cell co-cultures were quantified and presented as pg/ml. Data (mean ± SEM) are from four donors. The level of statistical significance is indicated (*P<0.05, **P<0.01).

**Figure 5:** AFM imaging reveals that the loss of melanin correlates with the lack of exposed rodlet layer. AFM deflection images of the surface of ΔpksP (A-C), Δayg1 (D-F), Δarp2 (G-I), Δarp1 (J-L) and WT (M-O) conidia recorded in deionized water at low (A,D,G,J,M), medium (B,E,H,K,N) and high (C,F,I,L,O) resolutions. Black labels R and A indicate regions made of rodlets and amorphous materials, respectively.
Figure 6: SDS-PAGE (15% gel) profile of the RodAp extracted from the WT and melanin biosynthetic pathway mutant conidial surfaces using hydrofluoric acid (HF). Protein bands were revealed by silver staining. RodAp* represents degraded form of RodAp due to HF-treatment.

Figure 7: Structural modifications influence the conidial surface hydrophobicity. AFM deflection images (A,D,G,J,M) recorded in deionized water, together with adhesion force maps (x-y: 1 μm x 1 μm; z-range: 3 nN) (B,E,H,K,N) and corresponding adhesion histograms (n=1024) (C,F,I,L,O) recorded with hydrophobic tips on the surface of ΔpksP (A-C), Δayg1 (D-F), Δarp2 (G-I), Δarp1 (J-L) and WT (M-O).

Figure 8: ConA-FITC labeling of ΔpksP, Δayg1 and WT strain resting conidia. Note the increase in the ConA labeling on the ΔpksP mutant conidial surface compared to Wt and Δayg1 conidia. Scale bar: 10 μm.

Figure 9: NaCl extracted proteins from the surface of mutant and WT resting conidia. SDS-PAGE (10% gel) of proteins extracted after 2 h incubation of resting conidia in 0.5 M NaCl.

Figure 10: Effect of NaCl extracts from the surface of mutant and WT resting conidia on the maturation of dendritic cells. Immature DCs were cultured in the presence of cytokines GM-CSF and IL-4 (Ctr DC) or cytokines and NaCl-extracts from the surface of WT resting conidia or mutant conidia (ΔpksP, Δayg1, Δarp2, Δarp1) for 48 hours. The percentage expression of (A) CD86, and mean fluorescence intensities (MFI) of (B) CD80 and (C) CD40 were analyzed by flow cytometry. Data (mean ± SEM) are from four donors. The level of statistical significance is indicated (*P<0.05, **P<0.01).
Figure 11: WGA-FITC labeling of mutant and WT resting conidia. Note the decreasing number of ConA-positive conidia of the first mutants of the melanin pathway to last one. Scale bar: 10 µm.
**Table 1:** Identification of the proteins extracted from the Δ*pksP* mutant conidial surface.

Identification was done by MS/MS and MS with a mascot score above a threshold of 54.

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Figure 1

\[
\text{Acetyl CoA + Malonyl CoA} \quad \xrightarrow{\text{PksP}} \quad \text{Polyketide synthase}
\]

\[
\text{YWA1} \quad \xrightarrow{\text{Ayg1}} \quad \text{Hydrolase}
\]

\[
1,3,6,8\text{-THN} \quad \xrightarrow{\text{Arp2}} \quad \text{Reductase}
\]

\[
\text{Scytalone} \quad \xrightarrow{\text{Arp1}} \quad \text{Dehydratase}
\]

\[
1,3,8\text{-THN} \quad \xrightarrow{\text{Arp2}} \quad \text{Reductase}
\]

\[
\text{Vermelone} \quad \xrightarrow{\text{Abr1}} \quad \text{Oxidase}
\]

\[
1,8\text{-DHN} \quad \xrightarrow{\text{Abr2}} \quad \text{Laccase}
\]

\[
\text{Melanin}
\]
Figure 2

A. CD83

B. CD86

C. CD80

D. CD40
Figure 3

A

B

C

D
Figure 4

A

CD83

CD86

CD80

% positive cells

MFI

B

+ melanin

15 Min
30 Min
60 Min

Mod

pERK 1/2

pp38

β-actin

C

IFN-γ

IL-2

IL-5

DC: T cell ratio

1/10
1/20
1/40
1/80

D
Figure 5

ΔpksP

D FE

1 µm 100 nm 200 nm

Δayg1

A
R

1 µm 100 nm 200 nm

Δarp2

J LK

1 µm 100 nm 200 nm

Δarp1

A
R

1 µm 100 nm 200 nm

WT

W
Figure 6
Figure 7

A CB

$\Delta \psi_{\text{k3p}}$

$\Delta \psi_{\text{yg1}}$

$\Delta \psi_{\text{a2p}}$

$\Delta \psi_{\text{a1p}}$

WT
Figure 11

\[
\begin{array}{cc}
\Delta \text{pksP} & \Delta \text{pksP} \\
\Delta \text{ayg}1 & \Delta \text{ayg}1 \\
\Delta \text{arp}2 & \Delta \text{arp}2 \\
\Delta \text{arp}1 & \Delta \text{arp}1 \\
\Delta \text{abr}1 & \Delta \text{abr}1 \\
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