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***Aspergillus fumigatus* Conidial Melanin Modulates Host Cytokine Response**

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Abstract

Melanin biopigments have been linked to fungal virulence. *Aspergillus fumigatus* conidia are melanised and are weakly immunogenic. We show that melanin pigments on the surface of resting *Aspergillus fumigatus* conidia may serve to mask pathogen-associated molecular patterns (PAMPs)-induced cytokine response. The albino conidia induced significantly more proinflammatory cytokines in human peripheral blood mononuclear cells (PBMC), as compared to melanised wild-type conidia. Blocking dectin-1 receptor, Toll-like receptor 4 or mannose receptor decreased cytokine production induced by the albino but not by the wild type conidia. Moreover, albino conidia stimulated less potently, cytokine production in PBMC isolated from an individual with defective dectin-1, compared to the stimulation of cells isolated from healthy donors. These results suggest that β -glucans, but also other stimulatory PAMPs like mannan derivatives, are exposed on conidial surface in the absence of melanin. Melanin may play a modulatory role by impeding the capability of host immune cells to respond to specific ligands on *A. fumigatus*.

Keywords

β -glucan; dectin-1; mannose receptor; mannan; melanin; modulation

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Introduction

Aspergillus fumigatus is an opportunistic pathogen that causes life-threatening invasive disease in immunocompromised hosts (Denning, 1998). Mononuclear phagocytes constitute an important component of host defence against *A. fumigatus* and also represent the precursor cell population of tissue macrophages involved in immune surveillance against invasive pulmonary aspergillosis (Simitopoulou *et al.*, 2007, Romani, 2004, Chai *et al.*, 2009). Pathogen recognition and the subsequent host immune response have been attributed to pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) on host immune cells. These PRRs recognize conserved structures on the invading fungus called pathogen-associated molecular patterns (PAMPs). Elucidation of conidial factors that contribute to pathogenicity of *Aspergillus spp.* is important. It has recently been shown that recognition of cell wall β -glucan is one of the main stimulation pathways of host defence against aspergillosis (Hohl *et al.*, 2005). In addition to the (β -and- α)-glucans, galactomannan and chitin which are the main components of the *Aspergillus* conidial cell wall, there are also melanin nodules which are localized on the exterior surface of the cell wall (Latge, 2001).

Production of melanin has been associated with survival and virulence of fungal species such as *Cryptococcus neoformans* (Kwon-Chung *et al.*, 1982, Casadevall *et al.*, 2000), *Exophiala dermatitidis* (Dixon *et al.*, 1987, Feng *et al.*, 2001) and *Aspergillus fumigatus* (Jahn *et al.*, 1997, Tsai *et al.*, 1998). *Aspergillus* strains with melanised conidia have demonstrated an increased ability to scavenge reactive oxygen species and they are protected against oxidative damage by host leukocytes (Jacobson, 2000, Jahn *et al.*, 2000). However, to date, the immunomodulatory capacity of *Aspergillus* melanin remains to be elucidated.

Since melanin is localized on the exterior surface of conidia and therefore in contact with the external milieu and the host immune system, we explored the role of melanin in modulating host response to *A. fumigatus*.

Materials and Methods

Micro-organisms

A. fumigatus strain B-5233 is a wild type strain which produces melanised bluish green conidia, and was isolated from a patient with fatal invasive aspergillosis. The strain RGD-12, which produces albino conidia devoid of melanin, was obtained by deletion of the gene *alb1* in the strain B-5233. The *alb1* gene codes for a polyketide synthase (pksP) in the 1,8-dihydroxynaphthalene (DHN)-melanin pathway involved in the biosynthesis of conidial pigment (Tsai *et al.*, 1999, Tsai *et al.*, 1998).

The strains were grown on *Aspergillus* minimal agar media for one week and resting conidia were harvested in 0.01% Tween20/PBS, washed with sterile distilled water and resuspended in Hanks' balanced saline solution (HBSS) without Ca^{2+} and Mg^{2+} (Tsai *et al.*, 1998). Conidia were heat-killed by incubation at 65 °C for 1 h (Gersuk *et al.*, 2006) and their viability was tested on malt agar. The treatment was repeated until no viable conidia were detected on malt plates. Conidia were kept at -20 °C between heat-treatments.

Heat-killed *C. albicans* blastoconidia, strain ATCC MYA-3573 (UC820), that was used as a positive control for its β -glucan-mediated stimulation of dectin-1, was characterized elsewhere (Gow *et al.*, 2007, Lehrer *et al.*, 1969). Experiments involving heat-killed *C. albicans* blastoconidia were performed in a similar manner as described above.

Extraction of melanin from *A. fumigatus* conidia

Melanin was extracted from conidia of *A. fumigatus* strain ATCC 204305, as previously described (Youngchim *et al.*, 2004). In brief, conidia were enzymatically lysed to form protoplasts. The protoplasts were incubated in the denaturant 4.0 M guanidine thiocyanate to generate dark particles, which were treated with proteinase K to remove residual proteins. The pellet was boiled in 6.0 M HCL for 1 h to obtain pure melanin. Melanin concentration was quantified by weighing the dried mass (van de Sande *et al.*, 2007).

Reagents

The Toll-like receptor (TLR)-4 antagonist *Bartonella* lipopolysaccharide (LPS) was obtained as previously described (Popa *et al.*, 2007). D-Mannose, in order to block the mannose receptor pathway (Cambi *et al.*, 2003), was purchased from Sigma Chemical Co (St. Louis, MO, USA). The dectin-1 receptor antagonist, laminarin, was kindly provided by Dr David Williams (University of Tennessee, USA).

PBMC stimulation assays

Venous blood was drawn from the cubital vein of healthy volunteers, into 10-mL EDTA tubes after informed consent. Peripheral blood mononuclear cells (PBMC) were isolated as described (Netea *et al.*, 2003). In brief, the PBMC fraction was obtained by density centrifugation on Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). Cells were washed twice in saline, counted and the number was adjusted to 5×10^6 cells/mL. A 100 μ L volume of PBMC, suspended in culture medium (RPMI 1640 DM; ICN Biomedicals, Costa Mesa, CA) supplemented with 10 μ g/mL gentamicin, 10mM L-glutamine and 10 mM pyruvate was added to 96-well plates with heat-killed conidia of *A. fumigatus* or melanin extract, in various concentrations, for 24 hours at 37°C.

For receptor-blocking experiments, laminarin (200 μ g/mL), *Bartonella* LPS (160 μ g/mL) or mannose (50mM) fractions were pre-incubated with PBMC for 1 h at 37°C prior to stimulation with *Aspergillus* conidia. The supernatants were collected at 24 hours and stored at -20°C until cytokine assay.

Cytokine assay

Interleukin-6 (IL-6), interleukin-10 (IL-10) and interferon-gamma (IFN γ) were measured by commercial ELISA kits (Pelikine Compact; Sanquin), according to the instructions of the manufacturer. Tumour necrosis factor-alpha (TNF α) was measured by another commercial ELISA kit (R&D Systems). The detection limit was 8 pg/mL for IL-6, 4.7 pg/mL for IL-10, 3.1 pg/mL for IFN γ and 40 pg/mL for TNF α .

Statistics

Each set of experiment was performed in duplicates. Results from 3 independent sets of experiments (consisting of n = 6 subjects unless otherwise specified) were pooled and analyzed using SPSS 16.0 statistical software. Data are given as mean $\pm$ standard error of the mean (SEM). Wilcoxon signed rank test was used and the level of significance was set at p < 0.05.

Results

Cytokine response to wild type (WT) melanised *A. fumigatus* and albino *A. fumigatus* conidia

Samples containing 10^5 to 10^6 *A. fumigatus* conidia/mL poorly induced cytokine production (data not shown). However, 10^7 /mL of albino conidia (defective in melanin) stimulated significantly higher cytokine productions as compared to the wild type (WT) melanised conidia of the same concentration. The albino conidia elicited a significantly stronger IL-6 response.

TNF α and IL-10 response was also significantly elevated with albino conidia at 10^7 conidia/ml. The corresponding melanised WT conidia were weakly immunogenic (Figures 1a & 1b). Even at 10^7 conidia/ml, both WT and albino *A. fumigatus* conidia were weak inducers of IFN γ with PBMC. The above representative cytokines were assayed as IL-6, TNF α and IFN γ are distinct proinflammatory mediators which represent the effector arm of the host immune response against the fungal pathogen. IL-10, on the other hand, is an anti-inflammatory cytokine which regulates and limits anti-fungal immune response (Romani, 2004).

Immunogenicity of Melanin

Given the disparity in response between albino and melanised WT conidia, we investigated the immunogenic potential of melanin extracted from *A. fumigatus* conidia in PBMC. Similar to the wild-type conidia expressing melanin on their surface, purified melanin was poorly immunogenic for proinflammatory cytokine production with various concentrations ranging up to 1 mg/ml (Table 1).

Selective inhibition of dectin-1 receptor, TLR4 and mannose receptor (MR) pathway

IL-6 and TNF α responses to conidia of 10^7 /ml from each strain were further analyzed in the presence of inhibitors that block the specific recognition pathways. Laminarin, the dectin-1 receptor antagonist, significantly reduced the IL-6 and TNF α production in response to the albino conidia, while cytokine production induced by the wild-type melanised conidia was less affected (Figure 2a & 2b). This observation indicates that β -glucans on the albino conidia play a significant role in eliciting a proinflammatory cytokine response while the stimulatory role of β -glucan in melanised conidia is less pronounced.

Bartonella LPS, a TLR4 antagonist (Popa *et al.*, 2007), similarly reduced IL-6 and TNF α production in response to the albino conidia, but not to the WT conidia (Figure 2c & 2d). It is noteworthy that the addition of mannose to block the signalling pathway mediated by the mannose receptor (MR) (Cambi *et al.*, 2003) inhibited IL-6 but not TNF α production (Figure 2e & 2f) by the albino conidia. Taken together, these observations point to the exposure of dectin-1, TLR4 and MR ligands on the surface of the melanin-deficient conidia.

Ex-vivo stimulation in Dectin-1^{-/-} PBMC

PBMC were isolated from a subject known to have a complete defect in dectin-1 receptor expression, due to a homozygous mutation leading to a premature stop codon (Tyr 238 stop) (Ferwerda G *et al.*, submitted). Cytokine production following stimulation with 10^7 conidia/ml was compared to those of three control subjects. Since β -glucans are known to be exposed on heat-killed cells of *Candida albicans* (Gow *et al.*, 2007), we used heat-killed *C. albicans* blastoconidia 10^6 /ml to stimulate PBMC pre-incubated with laminarin and validated the nonfunctional role of dectin-1 in this subject (Figure 3b & 3d). The melanin-defective albino conidia induced a lower cytokine production in cells of the dectin-1-deficient individual, compared to those of control subjects, suggesting that β -glucans are one of the components exposed on its surface of the albino conidia (Figure 3a & 3c). However, even in the absence of dectin-1, conidia from the albino strain still induced more cytokine production than the wild-type. This observation corroborates with the hypothesis that in addition to β -glucans, other PAMP components (such as mannans) are also exposed on the melanin-deficient albino conidia.

Discussion

In this study, we focused on the role of *A. fumigatus* conidial pigment in host-pathogen interactions. The airborne conidia that humans inhale are resting conidia bearing bluish green pigment on the conidial surface identified as DHN-melanin (Tsai *et al.*, 1998). This melanin

layer disappears as conidia swell to initiate germination. Since the host cells first interact with inhaled resting conidia, it is in our interest to elucidate the role of melanin in the pathobiology of *A. fumigatus*.

To our knowledge, this is the first study showing that conidial pigmentation of *A. fumigatus* modulates the host proinflammatory cytokine response. Compared to the melanised WT resting conidia, the albino resting conidia stimulated much stronger host defence mechanisms such as proinflammatory cytokine production. Our results suggest that the melanin layer shields fungal PAMPs such as β -glucan, MR- and TLR-4 ligands from recognition by host PRRs thus preventing these PAMPs from eliciting an inflammatory response. This postulation is further strengthened by our demonstration that purified melanin was a very poor inducer of cytokine production, a pre-requisite characteristic for the assumption of its 'masking' role.

The evolutionary conservation of fungal melanin points to its role beyond that of rudimentary biopigmentation. Indeed, there has been considerable evidence linking fungal melanin biosynthesis to virulence (Nosanchuk *et al.*, 2003). Melanin has been shown to protect fungi in many ways. It offers a beneficial physiologic redox buffer (Jacobson, 2000), limits complement activation (Tsai *et al.*, 1998, Tsai *et al.*, 1997), protects *Aspergillus* conidia against reactive oxygen intermediates (ROIs) released by host immune cells, and protects against killing by human monocytes (Langfelder *et al.*, 1998, Jahn *et al.*, 2000). In addition to these effects, we demonstrated in this study that melanin localized on the surface of *A. fumigatus* conidia (Latge, 2001, Rementeria *et al.*, 2005) is also able to down-regulate host immune response by shielding of fungal PAMPs from recognition by host PRRs.

Previous reports on the decreased virulence of albino *A. fumigatus* mutants (Jahn *et al.*, 1997) may partly be explained by the propensity of these mutants for an accentuated proinflammatory immune response. Wild type *A. fumigatus* conidia, which initiate human disease in immunocompromised hosts, elicit a muted proinflammatory response in its resting state. Given the ubiquity of fungal spores in the environment, it is conceivable that the host defence does not elicit an unnecessary high cytokine-mediated inflammatory cascade in the immune competent host upon inhalation of *Aspergillus* conidia, as long as other protective mechanisms are active. On the other hand, increased levels of proinflammatory cytokines like TNF α , IL-6 and IFN γ have been associated with an efficient response to *Aspergillus* infection, through induction of potent anti-fungal cellular responses to clear the pathogen (Stevens, 2006). A muted host proinflammatory response (especially with TNF α and IL-6), as seen with wild type melanised conidia, may hamper the effectiveness of the anti-fungal defence mechanisms, especially in the immunocompromised host.

Our experimental results are interpreted in light of the understanding that in the albino conidia, deletion of the *alb1* gene, and hence lack of polyketide synthase (pksP), resulted in cessation of melanin production. This was validated phenotypically by production of albino conidia instead of the greenish-blue conidia of the wild type. Nonetheless, it remains to be elucidated whether additional immunologically-active by-products are formed or silenced in the melanin-defective albino strain as a result of silencing pksP in the DHN-melanin pathway.

An additional point of interest is the identification of the fungal PAMPs which upon exposure to the albino conidia are responsible for the increased cytokine induction. Laminarin-mediated blockade of dectin-1 receptor diminished proinflammatory cytokine production induced by the albino strain. This observation points to β -glucan as being one of the PAMPs that are exposed on the surface of the non-melanised albino conidia. This has been corroborated recently by other groups, which had demonstrated a high concentration of accessible β -glucan on the conidial surface of a pksP deletant strain or on swollen conidia (Luther *et al.*, 2007, Hohl *et al.*, 2005). However, our results suggest that in addition to β -glucan, TLR4- and mannose

receptor (MR) ligands are also exposed on albino conidia. The *A. fumigatus* ligands for the above-mentioned PRRs have not been identified to date. Based on our current understanding extrapolated from *C. albicans* PAMPs (Netea *et al.*, 2008), in which *O*- and *N*-linked mannan entities serve as ligands for TLR4 and MR respectively, it is likely that galactofuranose-containing galactomannan (Latge, 2008) may be one candidate PAMP on the *A. fumigatus* conidial wall which is exposed on the albino conidia. This is further supported by our demonstration that albino conidia were still able to induce higher levels of cytokines than the WT strain in host cells defective in dectin-1. Hence, in the absence of melanin, other conidial PAMPs can potentially engage host PRRs such as TLR4 and MR, next to engagement of dectin-1 by β -glucan which had been thought to be a major ligand on swollen *A. fumigatus* conidia (Gersuk *et al.*, 2006).

In conclusion, we have demonstrated that the melanin layer plays an important role in attenuating host immune response to *A. fumigatus* conidia. This is likely to be achieved through the physical masking of the stimulatory effects of PAMPs like β -glucan and mannan-containing PAMPs located on the resting conidial surface. In addition to its other known roles of protecting the pathogen against host phagocytes (Jahn *et al.*, 2000, Langfelder *et al.*, 2003) and limiting damage by the complement system (Tsai *et al.*, 1998), melanin may have an additional role as an important modulator of cytokine response to *A. fumigatus* conidia.

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Abbreviations

α -glucan	alpha-glucan
β -glucan	beta-glucan
CLR	C-type lectin receptor
DHN	dihydroxynaphthalene
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
HBSS	Hanks' balanced saline solution
HCL	hydrochloric acid
IL-6	interleukin-6
IL-10	interleukin-10
IFN γ	interferon-gamma
LPS	lipopolysaccharide
MR	mannose receptor
PAMP	pathogen-associated molecular pattern

PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
pksP	polyketide synthase
PRR	pathogen recognition receptor
RPMI	Roswell Park Memorial Institute
ROI	reactive oxygen intermediates
SEM	standard error of the mean
TLR	Toll-like receptor
TNF α	tumour necrosis factor-alpha
Tyr	tyrosine
WT	wild type

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Figure 1.

Human PBMC response to wild-type (WT) melanised and albino *A. fumigatus* conidia (10^7 /ml). Interleukin-6 (IL-6), tumour necrosis factor alpha (TNF) (Figure 1a) and interleukin 10 (IL-10), interferon-gamma (IFN γ) levels as shown (Figure 1b). *A. fumigatus* conidia were poorly immunogenic at the concentrations of 10^5 to 10^6 /ml (data not shown). Data was pooled from 3 sets of experiments and expressed as mean $\pm$ SEM; n = 6 subjects. * p < 0.05 in respective cytokine production between WT conidia and albino conidia.

Figure 2. PBMC pre-incubated with laminarin (blocks dectin-1; Figures 2a & b), *Bartonella* LPS (blocks TLR4; Figures 2c & d) and mannose (blocks mannose receptor pathway; Figures 2e & f) and stimulated with albino and wild-type (WT) *Aspergillus* conidia. Data pooled from 3 sets of experiments and expressed as mean $\pm$ SEM, n = 6 subjects. * p < 0.05 as compared to respective control (without specified inhibitor).

Figure 3.

PBMC response to albino and wild-type (WT) strains of *A. fumigatus* conidia in dectin-1-deficient homozygous subject versus 3 control subjects. As compared to controls, the dectin-1 deficient subject displayed a trend towards less cytokine production with the albino conidia (Fig 3a & c) than WT conidia. Positive control using *Candida albicans* blastoconidia, heat-killed with exposed surface β -glucans with and without dectin-1 inhibitor, laminarin (Fig 3b & d). Data pooled from 2 sets of experiments and expressed as mean $\pm$ SEM.

Cytokines IL-6 and TNF α production to melanin extracts, dose response relationship. Data was pooled from 3 sets of experiments and expressed as mean $\pm$ SEM; n = 6 subjects.

Table 1

Melanin dry-weight	RPMI	1mg/ml	0.1 mg/ml	0.01 mg/ml	0.001 mg/ml
IL-6 pg/ml *	8	8	36 $\pm$ 19	8	8
TNF- α pg/ml #	40	40	52 $\pm$ 11	40	40

* detection limit for IL-6 assay was 8 pg/ml

detection limit for TNF- α assay was 40 pg/ml