Aspergillus Melanin Potently Blocks Human Airway Epithelium Mediated Inflammation

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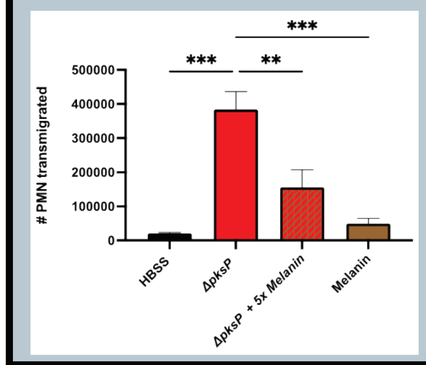
ABSTRACT

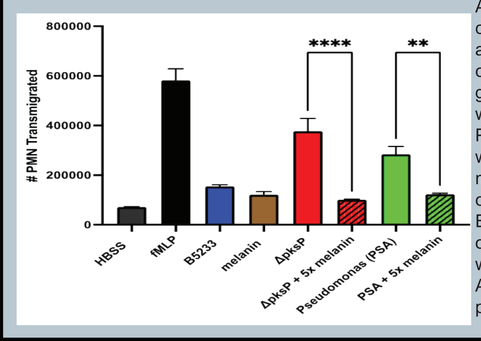
Most fungal pathogens, including Aspergillus fumigatus, are found in the environment and humans are exposed through the inhalation of fungal conidia. Conidia that remain in our lungs after exhalation are deposited onto the respiratory epithelium, particularly within terminal small airways. Clearance of fungal pathogens is multifactorial, relying upon mechanical means (clearance through the action of cilia and mucous) and through cell-mediated immunity, particularly via recruitment of innate immune cells to sites of infection. The airway epithelial is not just a passive barrier, but an active participant that is able to communicate with the immune system and trigger immune responses. However, the role of human airway epithelial cells (HAEC) in orchestrating protective or maladaptive immune responses is poorly understood. Initiation of a host-response depends upon recognition of the dynamic, multi-layered carbohydrate rich fungal cell wall. In addition to the typical carbohydrate components, most fungal conidia, including Aspergillus fumigatus, also contain a melanin layer that provides protection against environmental stresses, but it's role in modulating the airway drive immune response has not been explored. Using both a human muco-epithelial cell line, NCI-H292, and a fully pseudostratified primary Human airway epithelial cell model grown at air-liquid interface, we demonstrate that A. fumigatus melanin potently inhibits transmigration of neutrophils across the airway epithelium. Aspergillus melanin blocks the production of pro-inflammatory neutrophil chemoattractant, CXCL8 (IL-8) and CXCL1 (Gro-alpha) by selectively targeting apical secretion into the airways, resulting in a failure to generate a chemokine gradient that triggers neutrophil influx (or efflux) into the airways. Our results demonstrate that melanin actively down-regulates airway epithelial mediated pro-inflammatory responses toward Aspergillus and reveals a new strategy by which aspergillus subverts the immune system.

A. fumigatus Melanin Ghosts Suppress Transepithelial PMN Migration



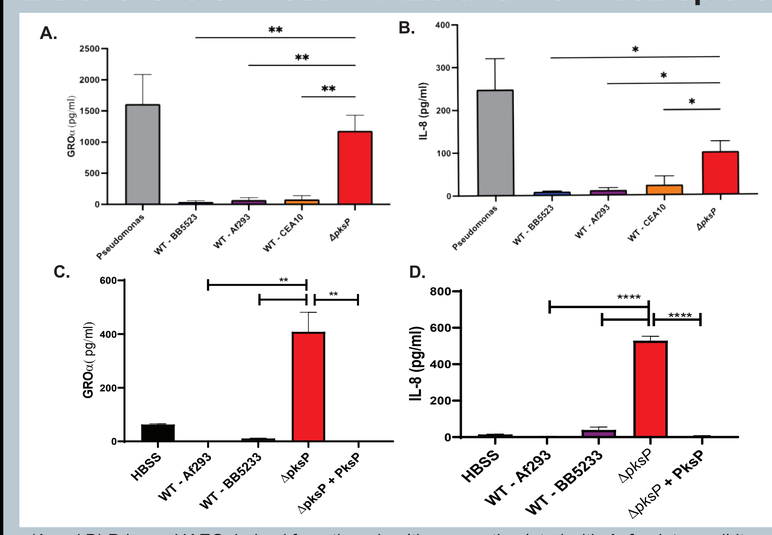
Primary HAEC derived from healthy donor basal cells was plated on 24-well transwells and allowed to differentiate for 16 days into a fully pseudostratified epithelium at air-liquid interface (ALI) Melanin ghosts were created from wildtype strain B5233. Epithelium were stimulated with HBSS, 1 x 10^7 conidia/cm^2 from ΔpksP strain or 1 x 10^7 ΔpksP conidia/cm^2 with 5 x 10^7 melanin ghosts/cm^2 or 5 x 10^7 melanin ghosts/cm^2 alone for 1 hour. PMN transmigration was measured using a myeloperoxidase assay. P values were determined by one-way ANOVA with Tukey's post hoc test for multiple comparisons; ***, $p < 0.0001$, **, $p < 0.001$.

Melanin Suppresses PMN Transepithelial Migration in Response to both Fungal and Bacterial Pathogens



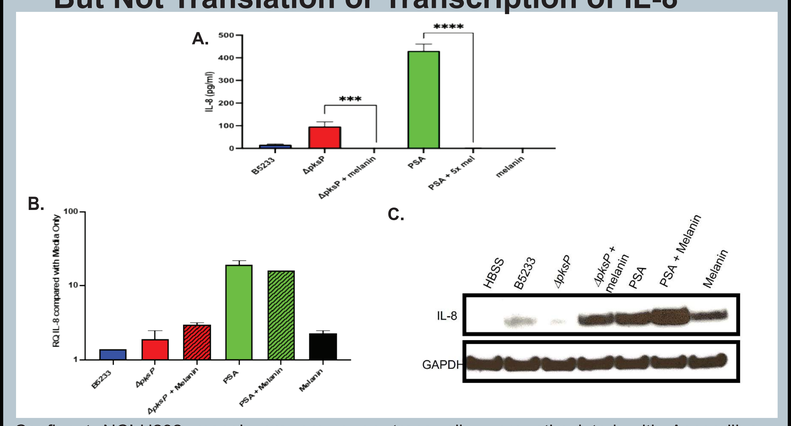
HAEC were stimulated with Aspergillus wildtype (B5233), ΔpksP conidia at 1 x 10^7 conidia/cm^2 or P. aeruginosa (PaO1) alone of in combination with 5 x 10^7 melanin ghosts/cm^2 in HBSS media. fMLP was used as a positive control for PMN transmigration. Transmigration was quantified using a myeloperoxidase assay and compared with a standard curve. Error bars represent standard error of three biologic replicates, statistics were calculated with two-way ANOVA using PRISM 9 software (** $p \le 0.01$; **** $p < 0.0001$).

A. fumigatus ΔpksP induces PMN Chemoattractants IL-8 and Gro-α in both HAEC and NCI-H292 epithelium



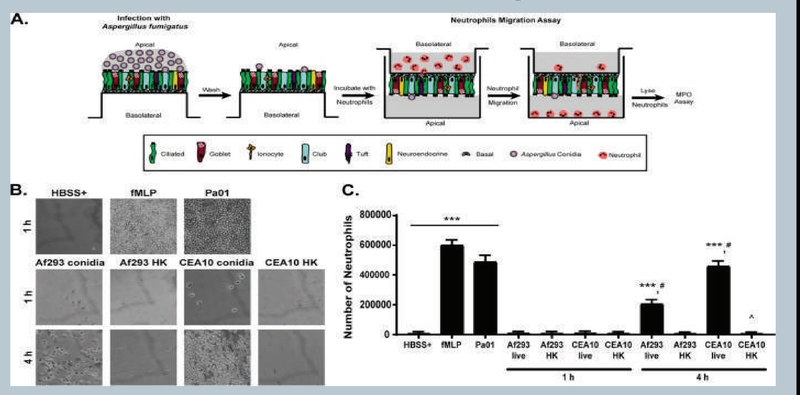
(A and B) Primary HAEC derived from three healthy were stimulated with *A. fumigtaus* wildtype strains (BB5233, Af293, CEA10), ΔpksP conidia or *P. aeroginosa* strain PaO1. Fungal stimulation were performed at 1 x 10^7 conidia/cm^2 for 4 hours and cytokines were measured using Luminex. (C and D) Confluent epithelia composed of NCI-H292 cells were stimulated using the same conditions as the HAEC and cytokine production was measured using ELISA. Error bars represent the standard deviation of the mean. P values were determined by one-way ANOVA with Tukey's post hoc test for multiple comparisons; ****, $p < 0.00001$; ***, $p < 0.0001$, **, $p < 0.001$, *. $p < 0.01$.

Aspergillus Melanin Blocks Secretion, But Not Translation or Transcription of IL-8



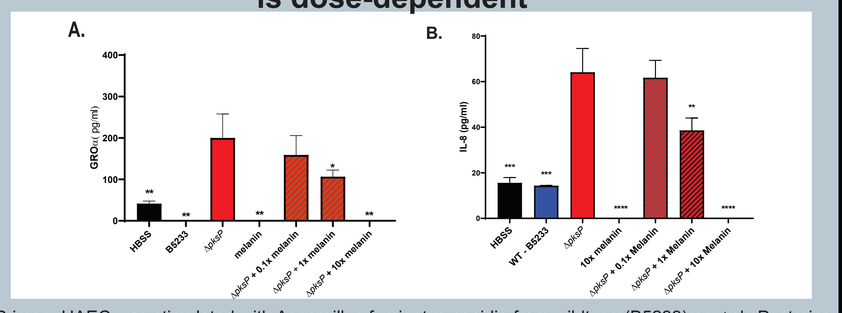
Confluent NCI-H292 monolayers grown on transwells were stimulated with Aspergillus wildtype, ΔpksP, P. aeruginosa (PSA) at 1 x 10^7 conidia/cm^2 or in combination with melanin ghosts at 5 x 10^7 ghosts/cm^2. Supernatants from the apical compartment were analyzed for IL-8 using ELISA (R&D, Duoset). Five replicates were performed for each condition, three replicates were processed to purify total RNA and two replicates were lysed using 1% NP40 lysis buffer and processed for immunoblotting. (A) Quantification of IL-8 in the apical supernatant (B) Total RNA samples were converted to cDNA using Superscript IV Vilo MasterMix (ThermoFisher) and qPCR was performed using TaqMan probes for IL-8 and GAPDH (control) and the TaqMan Advanced Master Mix (ThermoFisher) and quantified using an Applied Biosystems 7500 Fast Real-Time PCR System. RQ was computed using manufacturers software and error bars represent the standard deviation from 3 biological replicates. (C) Immunoblotting of lysates for IL-8 and GAPDH.

Prolonged Infection with live WT Aspergillus fumigatus is required for transepithelial migration of PMNs



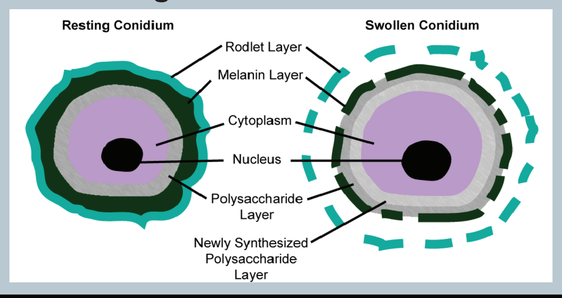
Prolonged epithelial exposure to live A. fumigatus promotes neutrophil migration. (A) Cartoon representation of an inverted ALI neutrophil transmigration experiment. (B and C) Light microscopy *B) and MPO assay quantification (C) of migrated neutrophils following epithelial stimulation with HBSS, 100nM fMLP, P. aeroginosa (PaO1), Af293 live and heat-killed (HK) resting conidia, and CEA10 live and HK resting conidia. All infections were performed with a 1 x 10^7conidia/cm^2 of epithelial surface area for 1 hour or 4 hours, as indicted. Error bars represent the SD of the mean. P values were determined by one-way ANOVA with Tukey's post hoc test for multiple comparisons (n=6). ***, $p < 0.0001$ versus the results for HBSS control; #, $p < 0.0001$ versus the results at 1 hour; ^, $p < 0.0001$ versus the results for live conidia. (Feldman, et al. Infect Immun. 2020 Feb; 88(2): e00813-19).

Aspergillus Melanin Suppression of IL-8 and Gro-α is dose-dependent

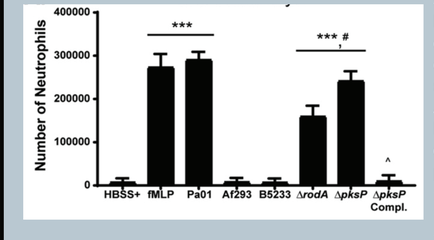


Primary HAEC was stimulated with Aspergillus fumigatus conidia from wildtype (B5233) or ΔpksP strains at 1 x 10^7 conidia/cm^2 and were mixed with melanin ghosts at the indicated concentration (relative to the ΔpksP strain). Supernatants were analyzed for Gro-α and IL-8 by ELISA (R&D, Duoset). Error bars represent SEM of three biological replicates, significance was determine by two-way ANOVA using PRISM 9 software. Signficance indicated is relative to the ΔpksP strain (not signficant, NS; *** $p \le 0.001$; **** $p < 0.0001$).

Active Remodeling of the Aspergillus Cell Wall During Conidial Germination

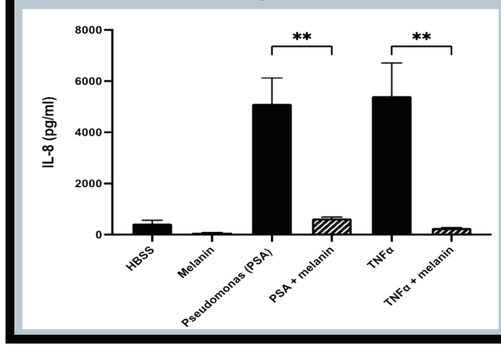


Melanin Deficient Aspergillus Promotes Early Robust PMN Transmigration



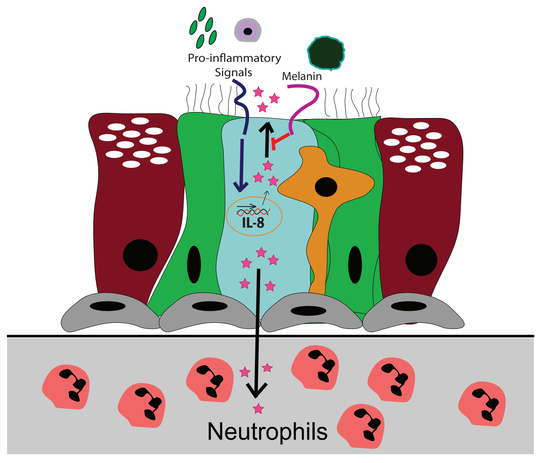
Conidia of A. fumigatus ΔrodA and ΔpksP stimulate rapid PMN migration across epithelium. PMN migration to the apical surface of H292 epithelium following stimulation with 1x10^7 conida/cm^2 of A. fumigatus conidia from WT (B5233, Af293), ΔrodA, ΔpksP, and ΔpksP-complemented strains for 1 hour. Error bars represent the SD of the mean. P values were determined using one-way ANOVA with Tukey's post hoc test for multiple comparisons. ***, $p < 0.0001$ versus the results for HBSS control; #, $p < 0.0001$ versus the results for the WT; ^, $p < 0.0001$ versus the results for theΔpksP mutant conidia (Feldman, et al. Infect Immun. 2020 Feb; 88(2): e00813-19).

Aspergillus Melanin Suppresses Fungal, Bacterial and Cytokine Induced IL-8 Production



Primary HAEC was stimulated with wildtype Aspergillus melanin ghosts at 5 x 10^7 ghosts/cm^2, P. aeruginosa (PaO1), or 100 nM TNFα alone or in combination as indicated for 4 hours. Supernatants were harvested and analyzed for IL-8 using ELISA (R&D, Duoset). Error bars represent SEM of three biological replicates, significance was determine by two-way ANOVA using PRISM 9 software. (** $p \le 0.01$).

Conclusions



A. fumigatus wildtype strains induce delayed transepithelial migration of neutrophils, however strains that lack melanin (*ΔpksP*) promote early robust neutrophil transmigration.

Aspergillus melanin suppresses pro-inflammatory cytokine production by airway epithelial cells in a dose-dependent manner.

Melanin blocks both fungal, bacterial, and cytokine induced IL-8 production, and blocks both fungal and bacterial induced transepithelial migration of neutrophils.

Melanin does not block either transcription or translation of IL-8, but inhibits secretion of IL-8, revealing a novel role for melanin for dampening HAEC-derived inflammation in response to *Aspergillus.*