Aspergillus fumigatus Cell Wall Promotes Apical Airway Epithelial Recruitment of Human Neutrophils

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ABSTRACT *Aspergillus fumigatus* is a ubiquitous fungal pathogen capable of causing multiple pulmonary diseases, including invasive aspergillosis, chronic necrotizing aspergillosis, fungal colonization, and allergic bronchopulmonary aspergillosis. Intact mucociliary barrier function and early airway neutrophil responses are critical for clearing fungal conidia from the host airways prior to establishing disease. Following inhalation, *Aspergillus* conidia deposit in the small airways, where they are likely to make their initial host encounter with epithelial cells. Challenges in airway infection models have limited the ability to explore early steps in the interactions between *A. fumigatus* and the human airway epithelium. Here, we use inverted air-liquid interface cultures to demonstrate that the human airway epithelium responds to apical stimulation by *A. fumigatus* to promote the transepithelial migration of neutrophils from the basolateral membrane surface to the apical airway surface. Promoting epithelial transmigration with *Aspergillus* required prolonged exposure with live resting conidia. Swollen conidia did not expedite epithelial transmigration. Using *A. fumigatus* strains containing deletions of genes for cell wall components, we identified that deletion of the hydrophobic rodlet layer or dihydroxynaphthalene-melanin in the conidial cell wall amplified the epithelial transmigration of neutrophils, using primary human airway epithelium. Ultimately, we show that an as-yet-unidentified nonsecreted cell wall protein is required to promote the early epithelial transmigration of human neutrophils into the airspace in response to *A. fumigatus*. Together, these data provide critical insight into the initial epithelial host response to *Aspergillus*.

KEYWORDS *Aspergillus fumigatus*, epithelial cells, host-pathogen interactions, neutrophils, respiratory pathogens

Aspergillus fumigatus is a ubiquitous environmental fungus that causes a wide range of diseases. Humans inhale hundreds of *Aspergillus* conidia daily, yet they do not develop an infection due to the airway epithelial and innate immune defenses acting to clear the conidia. In patients with recent influenza virus infection or immunosuppressed patients, *A. fumigatus* can cause invasive aspergillosis (IA), which has a mortality rate exceeding 50% (1–4). Following inhalation, the majority of *Aspergillus* conidia deposit in small airways, based on their aerodynamic profile (5). If the conidia survive,

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they can germinate and form hyphae in the small airways of the human host, leading to disease (6). The small-airway epithelium is an immunologically active tissue that contributes to host defense through the production of antimicrobial compounds, barrier defense, the mucociliary clearance of small particles, and the recruitment of innate immune cells (e.g., neutrophils). Unfortunately, the *A. fumigatus*-human airway epithelium interactions leading to airway neutrophil recruitment are not well characterized.

The cell wall of *A. fumigatus* is primarily composed of polysaccharides, including α -1,3-glucan, galactosaminogalactan, β -1,3-glucan, β -1,4-glucan, and chitin, which can stimulate or inhibit the host immune response (7–9). While the core structural components of the *Aspergillus* cell wall are similar between conidia and hyphae, the outer layer is distinct (10). *A. fumigatus* conidia contain an outer hydrophobic rodlet layer composed of hydrophobins, encoded in part by the *rodA* gene (11). *A. fumigatus* contains multiple redundant proteases capable of degrading the rodlet layer (12). The rodlet layer restricts the host immune response by decreasing conidial phagocytosis (13). Pulmonary infection with *rodA*-deficient *A. fumigatus* increased neutrophil recruitment and chemokine induction (i.e., CXCL1, CXCL2, interleukin-6 [IL-6], and IL-10) (14).

Conidial melanin underlying the rodlet layer has been shown to play a role in regulating the host immune response and conidial recognition. Shedding of the rodlet layer due to conidial swelling exposes underlying immunogenic cell wall components and dihydroxynaphthalene (DHN)-melanin (15). Synthesis of DHN-melanin, the dominant form in *A. fumigatus*, is dependent on a cluster of biosynthetic genes, including *ayg1*, *abr1*, *abr2*, *arp1*, *arp2*, and *pkpP*. DHN-melanin modulates the host response both by reducing phagolysosomal acidification and by inhibiting the apoptosis of phagocytic cells (16, 17). Specifically, *A. fumigatus* cell wall melanin was shown to inhibit LC3-associated phagocytosis by excluding the p22phox subunit from the phagosome (18). *pkpP*-deficient *Aspergillus* strains produce white conidia lacking melanin and have decreased virulence and increased susceptibility to reactive oxygen species (ROS)-mediated killing (19–22). It is unlikely that this virulence change is due to an altered ROS response since other mutations modulating ROS-mediated killing do not alter virulence (23, 24). The detection, signaling, and recruitment of innate immune cells triggered by *rodA* and DHN-melanin in airway epithelial cells remain poorly understood.

The innate immune system is critical to the identification and clearance of fungal organisms. Early neutrophil recruitment to the airways is critical in protecting against IA, as demonstrated by the increased risk of IA in neutropenic patients (25). In a murine model of IA, eliminating pulmonary neutrophils before or up to 3 h after intratracheal infection with *A. fumigatus* led to a significant reduction in host survival, whereas no difference was observed when neutrophil depletion occurred at 6 h postinfection (26). Additionally, depletion of macrophages did not diminish the neutrophil quantity or neutrophil survival in response to pulmonary *A. fumigatus* infection (26). Taken together, these data suggest that the airway epithelium plays a crucial role in promoting the recruitment of neutrophils to the host airway, leading to fungal clearance prior to hyphal generation. In bacterial models of epithelial inflammation, apical infection with *Pseudomonas aeruginosa* leads to the bidirectional secretion of cytokines, including IL-8, leading to neutrophil recruitment to the subepithelial space (27, 28). Epithelial production of heparin A3 stimulated neutrophil migration from the basolateral epithelial surface into the airway lumen at the apical surface (28–33). While the underlying mechanism has been studied in bacterial systems, little is known about the airway response and subsequent neutrophil recruitment to the site of *A. fumigatus* infection. It remains unclear which *Aspergillus* epitope leads to the epithelial response promoting neutrophil transepithelial migration. While numerous studies have dissected fungal receptors in immune cells, differentiated airway epithelial cells lack many of these receptors at the baseline (e.g., Dectin-1 and Dectin-2) (34).

Here, we used an inverted air-liquid interface (ALI) culture model to investigate the role of the airway epithelium in coordinating early neutrophil transepithelial migration into the airways. Using NCI-H292 (H292) epithelial cells or primary human epithelial

FIG 1 Prolonged epithelial exposure to live *A. fumigatus* promotes neutrophil migration. (A) Cartoon representation of an inverted ALI neutrophil transmigration experiment. It should be noted that while this schematic depicts primary human epithelium, a majority of the studies were carried out in an inverted uniform H292 cell monolayer culture system. Critical results in H292 cells were confirmed by use of an inverted primary ALI culture model. (B and C) Light microscopy (B) and MPO assay quantification (C) of migrated neutrophils following epithelial stimulation with HBSS⁺, 100 nM fMLP, *P. aeruginosa* (Pa01), Af293 live and heat-killed (HK) resting conidia, and CEA10 live and HK resting conidia. All infections were performed with 1×10^7 conidia or bacteria/cm² of epithelial surface area for 1 h or 4 h, as noted. Data are presented as the mean $\pm$ SD. *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 the HBSS⁺ control; #, *P* < 0.0001 versus the results at 1 h; ^, *P* < 0.0001 versus the results for live conidia.

cells derived from the airway basal stem cells of a healthy donor, we demonstrate that wild-type (WT) *A. fumigatus*-stimulated epithelium is relatively poor at recruiting neutrophils to the airway surface early compared to the recruitment ability of epithelium stimulated by *P. aeruginosa*, as determined by the enzymatic quantification of neutrophils and micro-optical coherence tomography (μ OCT). Efficient transepithelial migration of neutrophils required prolonged infection with *Aspergillus*, and heat-killed *Aspergillus* was unable to promote neutrophil transmigration. Furthermore, we show that deletion of the rodlet protein or DHN-melanin in *Aspergillus* conidia amplifies the epithelial response to *A. fumigatus*. Using *Aspergillus* cell wall fractions, we identify that a cell wall-associated protein epitope is responsible for stimulating the epithelial cell-driven neutrophil transmigration.

RESULTS

Neutrophil recruitment to the apical surface of airway epithelium requires prolonged exposure with live wild-type *Aspergillus fumigatus* conidia. Based on prior observations with *P. aeruginosa* (35, 36), we hypothesized that WT *A. fumigatus* conidia would also promote epithelial cell-driven recruitment of neutrophils. Thus, we used the human mucoepidermoid pulmonary carcinoma immortalized cell line H292 to monitor neutrophil recruitment from the basolateral surface to the apical surface. To assess epithelial cell-driven neutrophil recruitment to the apical surface, an epithelial cell monolayer was infected on the apical side and neutrophils were applied to the basolateral side to mimic pathogen inhalation and immune cell recruitment (Fig. 1A). Using this experimental system, we examined the ability of two different WT *A.*

fumigatus strains, Af293 and CEA10, to stimulate neutrophil recruitment to the apical surface of the airway H292 epithelial cell monolayer in simple Hanks' balanced salt solution (HBSS⁺). The neutrophils in the apical compartment were observed by light microscopy (Fig. 1B) and quantified by determination of enzymatic myeloperoxidase (MPO) activity (Fig. 1C). Although it provides an indirect measurement of neutrophil migration, the MPO assay has been used extensively to measure neutrophil migration (37–39), and in our studies, the results were confirmed by light microscopy (Fig. 1B). Consistent with our previously published results (40), H292 epithelium stimulated with HBSS⁺ alone showed no measurable recruitment of neutrophils to the apical compartment. Addition of the direct neutrophil chemoattractant *N*-formyl-methionyl-leucyl-phenylalanine (fMLP; 100 μ M, which was added on the apical side only) during the neutrophil migration step led to significant neutrophil migration to the apical compartment ($P < 0.0001$). Additionally, infection with *P. aeruginosa* (Pa01; 10^7 bacteria/cm² of membrane surface area) for 1 h promoted the significant migration of neutrophils to the apical surface compared to that for the HBSS⁺ control ($P < 0.001$). Interestingly, a 1-h apical infection with either live Af293 or CEA10 resting conidia (10^7 conidia/cm²) failed to stimulate apical neutrophil recruitment. Additionally, live Af293 and CEA10 conidia failed to stimulate neutrophil recruitment following a 1-h membrane infection over a wide range of infection concentrations ranging from 10^5 conidia/cm² to 10^8 conidia/cm² (see Fig. S1 in the supplemental material). To test whether live *Aspergillus* conidia were actively suppressing epithelial neutrophil recruitment, epithelium was stimulated for 1 h with heat-killed (HK) Af293 and CEA10. Neither HK Af293 nor HK CEA10 stimulated neutrophil recruitment following a 1-h infection (Fig. 1C).

Many patients who acquire *A. fumigatus* pulmonary infections have underlying airway disorders that impair mucociliary clearance (41). To model the impaired mucociliary clearance of *Aspergillus* conidia, we performed a neutrophil migration assay through H292 cells with a 4-h infection time prior to washing. In sharp contrast to the findings with the 1-h exposure, both live Af293 and CEA10 conidia stimulated neutrophil migration to the apical surface, although to a lesser extent than Pa01 ($P < 0.0001$). HK Af293 and CEA10 were unable to stimulate epithelial cell-driven neutrophil migration following a 4-h infection (Fig. 1C), indicating that viable fungal organisms were required for this recruitment. To confirm that neutrophil migration was not due to direct damage to the epithelium, cellular damage was assessed by measuring epithelial lactate dehydrogenase (LDH) production and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) conversions. The integrity of the epithelial barrier was assessed by measuring the movement of horseradish peroxidase (HRP) from the apical to the basolateral side postinfection. Infection with both WT *A. fumigatus* strains following a 1-h infection demonstrated no increase in LDH production or HRP flux, nor was there a reduction in MTT conversion, indicating that the H292 cell membranes were not damaged by this exposure (Fig. S2).

Rearrangement of the *A. fumigatus* cell wall during conidial swelling and germination does not accelerate epithelial cell-driven apical neutrophil recruitment. Resting *Aspergillus* conidia contain layers of hydrophobic rodlet protein and DHN-melanin, which cover the polysaccharide-rich cell wall (7). In the first few hours after exposure to moisture and heat, live resting conidia undergo an enzymatically mediated swelling process which involves shedding of the rodlet layer, rearrangement, and exposure of the *Aspergillus* cell wall (7) (Fig. 2A). Based on our observation that 4 h but not 1 h of infection with live WT *A. fumigatus* stimulates epithelial neutrophil transmigration, we hypothesized that conidial swelling may reveal an *Aspergillus* epitope that drives neutrophil migration. Therefore, we quantified neutrophil transepithelial migration across the H292 epithelium stimulated with previously swollen WT *A. fumigatus* conidia for 1 h (Fig. 2B). As previously noted, treatment with HBSS⁺ alone did not stimulate neutrophil transepithelial migration to the apical surface, while treatment with fMLP or Pa01 caused a significant movement of neutrophils to the apical side. Af293 or CEA10 in the resting or swollen conidial form did not promote significant

FIG 2 Preswelling of the conidia does not alter neutrophil transmigration. (A) Cartoon depiction of the structural changes that occur in *A. fumigatus* conidia when transitioning from the resting to the swollen state. Live *A. fumigatus* produces secreted proteases which degrade the outer hydrophobic rodlet layer, encoded by the fungal *rodA* gene. The DHN-melanin layer is disrupted but remains attached to the conidia. (B) Neutrophil migration to the apical epithelial surface of H292 cells was measured following a 1-h infection with HBSS⁺ medium, 100 nM fMLP, Pa01, Af293 resting and swollen conidia, and CEA10 resting and swollen conidia. All infections were performed with 1×10^7 conidia or bacteria/cm² of epithelial surface area for 1 h. Data are presented as the mean $\pm$ SD. *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 the HBSS⁺ control; #, $P < 0.0001$ versus the results for fMLP treatment.

transepithelial migration following a 1-h infection (Fig. 2B). Additionally, a 1-h infection with swollen conidia did not alter the LDH production, MTT conversion, or barrier integrity of the H292 cell monolayers (Fig. S3). The adhesion of resting conidia to the epithelial membrane did not significantly differ from the adhesion of swollen conidia (Fig. S4).

Genetic modification of the fungal cell wall via deletion of *rodA* or *pksP* enhances epithelial neutrophil transmigration. Based on our findings that swollen *A. fumigatus* conidia did not increase the transepithelial migration of neutrophils at 1 h compared to that for resting conidia, we hypothesized that exterior components of the cell wall (i.e., the rodlet and melanin layers) may actively inhibit the H292 epithelial response to *A. fumigatus*. To test this hypothesis, transepithelial neutrophil migration in response to the *A. fumigatus* $\Delta rodA$ and $\Delta pksP$ strains was assessed. The $\Delta rodA$ strain failed to produce the conidial hydrophobic rodlet layer, while the $\Delta pksP$ strain lacked a key polyketide synthesis enzyme in melanin biosynthesis and thus failed to produce cell wall DHN-melanin. Following a 1-h infection, neither Af293 conidia nor B5233 conidia (where strain B5233 is the WT parent strain of both the $\Delta rodA$ and $\Delta pksP$ strains) stimulated neutrophil epithelial transmigration, as expected. In contrast, resting conidia of both the $\Delta rodA$ and $\Delta pksP$ strains induced significant migration following 1 h of stimulation, with the melanin-deficient strain providing the largest increase in apical neutrophils ($P < 0.0001$) (Fig. 3A). The complemented $\Delta pksP$ strain, which consists of the $\Delta pksP$ strain into which a plasmid-encoded *pksP* gene has been introduced, did not induce neutrophil recruitment across the epithelium, recapitulating the WT B5233 strain phenotype. The conidia of the complemented $\Delta rodA$, $\Delta pksP$, and $\Delta pksP$ strains did not alter H292 cell monolayer LDH production, MTT conversion, or HRP flux, indicating that the increased neutrophil migration observed with these strains was not

FIG 3 Conidia of *A. fumigatus* $\Delta rodA$ and $\Delta pksP$ stimulate rapid neutrophil migration across epithelial borders. Neutrophil migration to the apical surface of H292 epithelium (A) and primary human airway epithelium (B) following epithelial stimulation with the conidia of *A. fumigatus* WT (Af293, B5233), $\Delta rodA$, $\Delta pksP$, and *pksP*-complemented $\Delta pksP$ ($\Delta pksP$ Compl.) strains is shown. H292 epithelium was stimulated with 1×10^7 conidia or bacteria/cm² for 1 h. Primary human epithelium samples were stimulated with 1×10^7 bacteria/cm² of Pa01 and either 1×10^7 or 1×10^8 conidia/cm² of *A. fumigatus* B5233 or $\Delta pksP$ mutant conidia for 2 h prior to neutrophil migration. Data are presented as the mean $\pm$ SD. *P* values were determined by one-way ANOVA with Tukey's *post hoc* test for multiple comparisons ($n = 4$ to 6). ***, $P < 0.0001$ versus the results for the HBSS⁺ control; **, $P < 0.01$ versus the results for the HBSS⁺ control; #, $P < 0.0001$ versus the results for the WT; Δ, $P < 0.0001$ versus the results for $\Delta pksP$ mutant conidia.

due to membrane toxicity (Fig. S5). Additionally, neither the $\Delta rodA$ strain nor the $\Delta pksP$ strain demonstrated increased epithelial adhesion compared to that for the WT strains (Fig. S6).

The H292 epithelium is a model epithelial tissue; however, it lacks the functionally and structurally diverse cell types as well as the pseudostratified layering seen in primary human airway epithelial tissue (42). Since neutrophil recruitment was more robust in response to the conidia of the $\Delta pksP$ strain than in response to the conidia of the other strains (i.e., WT and *rodA* strains) and melanin has immunomodulatory properties, we focused on this strain. To confirm that the $\Delta pksP$ strain was better able than the other strains to stimulate neutrophil epithelial transmigration, neutrophil migration was assessed in fully differentiated primary airway epithelial tissue in an inverted ALI system (35, 36). Airway epithelium was cultured from primary human airway stem cells by a previously established method (43, 44). Using this approach, we were able to generate epithelium which contained both common cell types (basal, club, goblet, and ciliated cells) and rare cell types (tuft, neuroendocrine, and ionocyte cells) (43–45). Membrane integrity was assessed immediately prior to infection by measurement of transepithelial electrical resistance (TEER), with all TEER measurements being $>2,000$ m Ω . Membrane differentiation was confirmed by immunohistochemistry staining of a subset of membranes for markers of differentiated epithelial cells, including acetylated tubulin (ACT) and mucin 5B (MUC5B) (Fig. S7). As observed in H292 cell monolayers, both fMLP and Pa01 stimulated the robust migration of neutrophils across the fully differentiated primary human airway epithelium, while treatment with HBSS⁺

FIG 4 μ OCT reveals differences in the pattern of neutrophil migration through primary human epithelium in response to melanin-deficient *A. fumigatus* compared to that in response to WT *A. fumigatus*. (A) Schematic of the μ OCT imaging setup. (B) The μ OCT imaging beam was scanned across a 1-mm² area to generate volumetric images of the neutrophil migration every 10 min over 4 h. Images show neutrophils (top layer), the Transwell membrane (middle layer; white lines), and the apical side with human airway epithelium, *A. fumigatus* conidia, and neutrophils at 4 h. A comparison of the volumetric images at the beginning (time [t] zero) and end of the 4-h experiments shows that neutrophil migration through the epithelium in response to $\Delta pksP$ mutant conidia was more abundant over the 4 h than the migration in response to WT conidia. Arrows indicate neutrophil projections on the apical side. (C) In addition to elevated neutrophil migration in $\Delta pksP$ mutant-stimulated epithelium, μ OCT also revealed a transepithelial migration pattern that favored many sparsely separated breaching points, which is depicted in the representative binarized en face images from one planar view ($\sim 4 \mu\text{m}$ away from the center of the apical side). (D) Rates of neutrophil migration were analyzed by measuring from μ OCT recordings the change in the number of voxels at the apical side of the cells from the number at the basolateral side.

alone resulted in the minimal movement of neutrophils (Fig. 3B). WT strain B5233 conidia did not stimulate neutrophil transepithelial migration at infection rates of either 10^7 or 10^8 conidia/cm². In contrast, conidia of the $\Delta pksP$ strain (10^8 conidia/cm²) stimulated significant neutrophil migration across the epithelium ($P < 0.01$) (Fig. 3B). In contrast to what has been observed with A549 respiratory epithelial cells (46, 47), we did not observe any significant phagocytosis of *A. fumigatus* by primary human airway epithelial cells (data not shown). Collectively, these data indicate that DHN-melanin suppresses the primary human airway epithelium to trigger neutrophil transcellular migration toward the fungus (apical surface).

μ OCT of epithelial transmigration of neutrophils in primary human airway epithelium in response to *A. fumigatus*. Our data establish that live *A. fumigatus* conidia are capable of stimulating the airway epithelium to drive neutrophil transmigration into the airspaces. μ OCT is a label-free quantitative and qualitative imaging technique which allows for up to 1- μm -axial-resolution cross-sectional imaging of immune cell migration at the ALI (35) (Fig. 4A). Quantitatively, measurements of neutrophil migration using μ OCT have been shown to closely approximate the MPO assay measurements described above (35, 36). Consistent with prior published observations, uninfected membranes display some nonuniformity in thickness across the Transwell surface. Infection with *Aspergillus* conidia led to the appearance of heterogeneously distributed round foci (approximately $10 \pm 2.5 \mu\text{m}$ in diameter) on the apical surface, believed to represent *Aspergillus* conidia interacting with epithelial structures. The quantities of round foci observed were consistent with those determined from our

previously measured residual adhesion for the *A. fumigatus* conidia (Movies S1 and S2). We investigated the change in movement of neutrophils across the primary airway epithelium in response to WT and $\Delta pksP$ mutant conidia over a 4-h infection. Human epithelium stimulated with WT *A. fumigatus* for 4 h demonstrated little observable clustering of neutrophils, no apparent decrease in neutrophil volume on the basolateral surface, and a minimal apparent increase in apical epithelial cell/neutrophil volume, suggesting that minimal meaningful neutrophil migration occurred compared to that seen at time zero. The presence of some peaks for the WT supports MPO data showing some increase in neutrophil migration over 4 h (Fig. 1C). In contrast, following infection with $\Delta pksP$ mutant conidia, the progressive development of diffuse heterogeneous spike-like areas (white arrows) of increased apical volume compared to that at time zero, along with a corresponding decrease in the basolateral volume of neutrophils, was observed (Fig. 4B).

To quantitate the differences in neutrophil migration across the airway epithelium in response to WT B5233 and $\Delta pksP$ strain stimulation captured by μ OCT, a thresholding approach was used to quantify the increase in apical volume following neutrophil migration (Fig. 4C and D). Images of the entire 1-mm² imaging area were collected at the initial and final time points for the neutrophil migration experiments described above. The peak and end values of the histogram of a representative image were used as the minimum and maximum values, respectively, for the binary image. Quantification confirmed our visual observation that WT conidia stimulated little neutrophil migration compared to that seen with unstimulated epithelium. In contrast, $\Delta pksP$ mutant conidia stimulated a substantial increase in the area above the threshold, which indicated neutrophil recruitment. No difference in neutrophil migration between stimulation with WT conidia and that with $\Delta pksP$ mutant conidia was observed at time zero (Fig. 4D).

Epithelial cell-driven neutrophil transmigration requires direct contact between *A. fumigatus* conidia and epithelial tissue. Our observations confirmed that $\Delta pksP$ mutant conidia efficiently stimulate epithelial transmigration in both the H292 epithelium and fully differentiated primary human airway epithelium. It remained unclear whether a conidium-secreted product or direct conidium-epithelium contact is required to drive neutrophil transepithelial migration. *Aspergillus* secretes proteases and toxins which could potentially affect the epithelial membrane (48). Transepithelial neutrophil migration was assessed following stimulation with $\Delta pksP$ mutant conidia or $\Delta pksP$ mutant conditioned medium (CM) to determine if direct contact is necessary. H292 cells exposed to $\Delta pksP$ mutant conidia stimulated the significant recruitment of neutrophils to the apical surface ($P < 0.0001$) (Fig. 5). Freshly prepared CM failed to promote any significant transepithelial migration of neutrophils. These data suggest that direct contact between *Aspergillus* conidia and the epithelial surface is necessary to stimulate epithelial cell-mediated neutrophil recruitment to the apical surface.

A protein component of the *A. fumigatus* cell wall is responsible for stimulating epithelial transmigration of neutrophils. The fungal cell wall contains multiple major epitopes known to stimulate the host immune response (7). The major epitopes involved in stimulation of the innate immune system are fungal carbohydrates, including β -1,3-glucan and galactomannan (7). Based on our studies with CM, we hypothesized that an epitope on the fungal cell wall may drive epithelial cell-driven neutrophil migration. To test this hypothesis, fungal cell wall fractions were isolated from mechanically disrupted B5233 and $\Delta pksP$ mutant conidia. Isolated $\Delta pksP$ mutant cell wall fraction stimulation of the airway epithelium caused a significant dose-dependent recruitment of neutrophils to the apical surface of the H292 epithelium over a range of cell wall concentrations (0.1 mg/ml to 10 mg/ml) (Fig. 6A). On the contrary, WT cell wall fractions demonstrated no ability to drive neutrophil migration over the same concentration range (Fig. 6B). To confirm that the mechanically disrupted conidia had been appropriately killed by this mechanism and to ensure that the observed recruitment was not due to the presence of intact live conidia, cell wall fractions were plated at 30°C

FIG 5 Direct contact between *A. fumigatus* $\Delta pksP$ mutant conidia and the airway epithelium is required for neutrophil transmigration. H292 epithelium was stimulated with HBSS⁺, fMLP (100 nM), Pa01 (10^7 bacteria/cm²), WT B5233 resting conidia (10^7 conidia/cm²), $\Delta pksP$ mutant resting conidia (10^7 and 10^8 conidia/cm²), and $\Delta pksP$ mutant conditioned medium (CM) generated from equivalent infecting concentrations. Neutrophil transmigration was quantified by the MPO assay. Data are presented as the mean $\pm$ SD. *P* values were determined by one-way ANOVA with Tukey's *post hoc* test for multiple comparisons ($n = 4$ to 5). ***, $P < 0.0001$ versus the results for the HBSS⁺ control; #, $P < 0.0001$ versus the results for strain B5233; ^, $P < 0.0001$ versus the results for conidia.

on Sabouraud dextrose agar, and no fungal growth was observed over 7 days (data not shown).

Since the $\Delta pksP$ mutant cell wall fraction was sufficient to stimulate neutrophil transepithelial migration, we hypothesized that melanin may limit the exposure of a cell wall carbohydrate or protein that may lead to epithelial stimulation. To identify the type of cell wall epitope driving transepithelial migration, neutrophil migration was performed using cell wall fractions treated with pronase (protease cocktail; 37°C for 2 h) or heat (95°C for 30 min). The pronase-treated $\Delta pksP$ mutant cell wall failed to stimulate transepithelial migration ($P < 0.0001$ versus the results obtained with the $\Delta pksP$ mutant cell wall), while heat-treated cell wall fragments retained their ability to stimulate the transepithelial migration of neutrophils (Fig. 6C). These findings suggest that a heat-stable fungal cell wall protein is responsible for driving the transepithelial migration of neutrophils in response to *A. fumigatus*.

DISCUSSION

Aspergillus infections are detrimental to immunosuppressed patients and those with recent influenza virus infections (1–4). The early recruitment of neutrophils to the apical surface of the airway epithelium is a critical step in preventing the development of IA after the inhalation of *A. fumigatus* conidia. In murine models, neutrophil clearance of conidia within hours of infection is needed to prevent the development of IA (26). The airway epithelium plays a critical role in this early neutrophil recruitment, since epithelial MyD88 signaling facilitates CXCL1 and CXCL5 production and neutrophil recruitment to the lungs in mice following pulmonary *A. fumigatus* exposure (49). Unfortunately, little is currently known about which *A. fumigatus* epitopes promote or suppress epithelial inflammation, as signaled by neutrophil recruitment and transmigration to the airspaces where neutrophils can interact with conidia in the first hours following infection. In the present study, we demonstrate that direct epithelial cell-conidium contact via a non-heat-labile protein stimulates neutrophil recruitment to the airways following *A. fumigatus* infection. Furthermore, melanin masks this protein, resulting in decreased neutrophil recruitment (Fig. 7).

There have been numerous studies which have explored the mechanisms by which bacteria stimulate epithelial neutrophil recruitment and transmigration (28, 30, 35, 40, 50–52). When stimulated with *P. aeruginosa*, the airway epithelium secretes cytokines (e.g., IL-6, IL-8) that promote neutrophil localization to the lung. Epithelial cell-produced heparin A3 is then released to stimulate neutrophil migration to the apical surface (28, 30). Neutrophils reinforce epithelial transmigration to the apical surface by producing

FIG 6 Cell wall fragments from $\Delta pksP$ mutant resting conidia are sufficient to drive epithelial neutrophil transmigration. (A and B) Neutrophil epithelial transmigration to the apical surface of H292 epithelium was measured following stimulation with HBSS⁺, fMLP (100 nM), Pa01 (10^7 bacteria/cm²), WT B5233 resting conidia (10^7 conidia/cm²), $\Delta pksP$ mutant resting conidia (10^7 conidia/cm²), and various concentrations of the $\Delta pksP$ mutant resting conidium cell wall fraction (A) or B5233 cell wall fraction (B). (C) Additionally, neutrophil migration was assessed following epithelial stimulation with 1-mg/ml $\Delta pksP$ mutant resting conidium cell wall fraction treated with a protease cocktail at 37°C for 2 h or heat treated at 95°C for 30 min. Data are presented as the mean $\pm$ SD. *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 the HBSS⁺ control; #, *P* < 0.0001 versus the results for strain B5233; ^, *P* < 0.0001 versus the results for the $\Delta pksP$ mutant.

leukotriene B4 (52). Dynamic structural observations using μ OCT show that this process involves the clustering of neutrophils on the basolateral surface, followed by clustered migration across the epithelium (35, 36, 51). While a role of host leukotriene B4 in bacterial infections has been found (50), it is unknown whether a similar mechanism is responsible in *Aspergillus* infections.

Here, using an inverted ALI culture system, we demonstrate that live WT *A. fumigatus* conidia are capable of stimulating neutrophil epithelial transmigration; however, this response requires an exposure time substantially longer than that required by the Gram-negative bacterial airway pathogen *P. aeruginosa*. Allowing the conidia to swell

FIG 7 Schematic representation of *A. fumigatus*-driven epithelial neutrophil transmigration. (Left) WT *A. fumigatus* recruits minimal neutrophils to migrate early in infection. (Right) On the contrary, conidium lacking the melanin biosynthesis enzyme *pksP* triggers ample neutrophil recruitment. This robust response occurs due to direct contact and is by a non-heat-labile fungal cell wall protein.

prior to infection did not promote the more rapid neutrophil migration across the epithelial border. During conidial swelling, the rodlet layer is shed as an active process driven by proteases produced by *Aspergillus* (7). Deletion of *rodA* may reveal a ligand on the *A. fumigatus* cell wall that promotes neutrophil migration. It is likely that this change in cell wall structure is not replicated by conidial swelling. Based on these findings, we hypothesized that conidial DHN-melanin may modulate the epithelial response to *A. fumigatus*. While *rodA* is shed in this process, melanin remains associated with the swollen conidia. Indeed, melanin-deficient $\Delta pksP$ mutant conidia rapidly promoted neutrophil migration across the airway epithelium.

Aspergillus produces numerous secreted products which can influence the host response. Conidia produce epithelial toxins, including tryptochvaline F, fumiquinazoline C, questin, monomethylsulochrin, and trypacidin (53). Additionally, alkaline protease 1 (Alp1), the most abundant protein secreted by *Aspergillus*, promotes eosinophil recruitment via epithelial TRPV4 mechanosensory-dependent signaling (54). Despite the known production of epithelial toxins, promotion of the transmigration of neutrophils required direct contact between *Aspergillus* conidia and the epithelial border, as shown by a lack of transmigration with *A. fumigatus* CM. Additionally, we did not observe epithelial toxicity caused by *A. fumigatus* during the time frame required for neutrophil transmigration, suggesting that these toxins are unlikely to play a critical role in this process. Our data demonstrated that cell wall fractions from mechanically disrupted $\Delta pksP$ mutant conidia alone are sufficient to drive neutrophil transmigration without inducing epithelial toxicity but lose this ability when treated with pronase. These data suggest that stimulation of the airway epithelium is driven by a protein or protein-linked epitope rather than by the typical carbohydrate epitopes known to trigger an innate response (e.g., C-type lectin receptors [CLRs]). *Aspergillus* is capable of causing a wide range of host diseases. It is distinctly possible that the balance of signals driven by secreted factors (e.g., Alp1) and pathogen-associated factors may drive the difference between allergic (eosinophilic) and inflammatory (neutrophilic) responses in different hosts. Further studies are warranted to investigate this balance.

The lung epithelium contributes to both neutrophil recruitment to the airspaces and the coordination of fungal killing by innate lymphocytes. Epithelial IL-1 α -interleukin-1 receptor (IL-1R) signaling was shown to be critical for neutrophil recruitment in a murine pulmonary model of IA (49). IL-1 α -IL-1R-NF- κ B signaling by the airway epithelium in a CLR-independent fashion is observed within 1 h following fungal infection (55). Furthermore, epithelial cells can produce CCL20, which contributes to Th17-mediated epithelial immunity regulating fungal killing by innate immune cells. Here, we

demonstrate using both quantitative enzymatic assays and μ OCT visualization that *Aspergillus* conidial stimulation of the human airway epithelium is sufficient to stimulate neutrophil transmigration into the airspaces in the absence of other immune cells within the first few hours following host-pathogen contact. It remains unclear which *Aspergillus* epitopes stimulate the airway epithelium. Data regarding the expression of CLRs in airway epithelial cells have been inconsistent (34, 56–59), but neither recent single-cell RNA sequencing data (45, 60) nor data from our own immunoblotting and flow cytometry experiments (data not shown) support the presence of CLR expression in primary human airway epithelial cells. Our data suggest that a protease-degradable, heat-stable epitope in the *Aspergillus* cell wall is sufficient to promote neutrophil transepithelial migration. In addition, our data suggest that phagocytosis is not required to drive neutrophil recruitment. Further experiments are needed to fully elucidate the pathogen epitope identity and signaling cascade.

We further found that the presence of *Aspergillus* cell wall DHN-melanin decreased the ability of conidia to stimulate neutrophil movement. CLEC1a (MelLec) was recently described to be a host membrane-bound receptor for fungal DHN-melanin; however, expression of MelLec has not been identified in airway epithelial cells (61). Melanin either may serve to shield a pathogen epitope or may act through a receptor-dependent pathway to modulate epithelial signaling. Furthermore, while arachidonic acid derivatives, such as hepoxilin A3, have a necessary and well-understood role in driving neutrophil recruitment in response to bacterial pathogens, their role in coordinating the response to fungal pathogens remains unknown. The structural differences in *Aspergillus*-driven versus *Pseudomonas*-driven epithelial migration of neutrophils observed suggest that the underlying mechanisms may be distinct for different pathogens.

Early neutrophil recruitment into the airways is a critical step that must occur within hours of host exposure to inhaled fungal conidia. Here, we add to the growing body of literature that the airway epithelium is essential in coordinating the host response to fungal spores and is sufficient to promote neutrophil transmigration in the absence of additional immune cells. Furthermore, *Aspergillus* has evolved tools, such as melanin production, that allow it to evade efficiently host epithelial signaling.

MATERIALS AND METHODS

Human airway epithelium cell culture. Human NCI-H292 mucoepidermoid pulmonary carcinoma immortalized cells or primary airway epithelial cells were used. NCI-H292 cells were grown in Dulbecco modified Eagle medium (DMEM) culture medium with 10% heat-inactivated fetal bovine serum (Invitrogen) and 0.01% penicillin and streptomycin (Invitrogen). Human adult airway basal cells were isolated and expanded as previously described (44). Briefly, adult basal stem cells were isolated from the New England Organ Bank under Massachusetts General Hospital Institutional Review Board (IRB)-approved protocol 2017P001479 (Hongmei Mou). The basal cells were propagated in small-airway epithelial cell medium (growth media kit; PromoCell) with 5 μ M Y-27632 (Tocris), 1 μ M A-83-01 (Tocris), 0.2 μ M DMH-1 (Tocris), and 0.5 μ M CHIR99021 (Tocris) on laminin-enriched 804G-conditioned medium-coated plates. Mucociliary differentiation was performed by seeding basal stem cells onto 0.4- μ m Transwell membranes with 804G-conditioned medium at a density of $>6,000$ cells/mm² to achieve 100% confluence. The cells were allowed to attach for at least 12 h, and excess cells were removed through the replacement of medium with complete PneumaCult-ALI medium (StemCell Technology), filling both chambers. On the following day, PneumaCult-ALI medium was added to the lower chamber only to initiate an airway-liquid interface. The medium was changed daily for 14 to 16 days. All methods were performed in accordance with relevant guidelines and regulations by the Partners Human Research Committee (Massachusetts General Hospital).

***Aspergillus fumigatus* strains.** *A. fumigatus* WT (AF293, CEA10, and B5233), Δ rodA, Δ pksP, and complemented Δ pksP strains were used. The B5233, Δ rodA, Δ pksP, and complemented Δ pksP *A. fumigatus* strains were gifted by K. J. Kwon-Chung (National Institutes of Health [NIH]). All *A. fumigatus* strains were grown on Difco Sabouraud dextrose agar plates with 100 μ g/ml ampicillin at 30°C for 3 to 4 days. The conidia were harvested by gentle scraping and washed 4 times with ice-cold phosphate-buffered saline (PBS). The conidia were used immediately or stored at 4°C for up to 1 week. For heat-killed conidia, freshly harvested conidia were diluted in PBS and heated at 95°C for 30 min. Swollen conidia were produced by incubating WT strains in DMEM culture medium (Gibco) with 10% heat-inactivated fetal bovine serum (Invitrogen) and 0.01% penicillin and streptomycin (Invitrogen) for 4 h at 37°C with shaking. The mean particle size was measured pre- and postswelling by use of a Luna hemocytometer (Logos Biosystems) to ensure that the conidia were indeed swollen. Before swelling, Af293 and CEA10 conidia had average mean particle sizes of 3.4 μ m and 2.6 μ m, respectively. Af293 and CEA10 conidia

increased to mean particle sizes of 6.2 μm and 4.2 μm , respectively, after 4 h in culture medium. All working concentrations were calculated via a Luna hemocytometer (Logos Biosystems) specified to 60% roundness and consistent 1- to 7- μm size exclusion.

Neutrophil isolation. Neutrophils were isolated from healthy volunteers under an IRB-approved protocol (protocols 2015P000818 and 1999P007782) at Massachusetts General Hospital as previously described (39). Briefly, blood was drawn by venipuncture into a syringe containing anticoagulant and acid citrate-dextrose. Blood was then centrifuged at $850 \times g$ for 20 min at room temperature through Ficoll to allow for buffy coat layer formation. Plasma and mononuclear cells were removed by aspiration. Red blood cells (RBCs) were removed via the 2% gelatin sedimentation technique and a wash with RBC lysis buffer. The cells were washed and resuspended in HBSS without calcium or magnesium (HBSS⁻) to a concentration of 5×10^7 neutrophils/ml.

Neutrophil transepithelial migration. The neutrophil transepithelial migration assay was adapted from previous studies (35, 36, 39, 40, 51) and has been reliably used over the past 20 years (37–39). H292 cell monolayers or primary airway epithelial cells were grown on the underside of Transwell filters with 3.0- μm pore sizes (Corning Life Sciences) to allow neutrophil migration from the basolateral side to the apical side. Cells were stimulated with medium only (HBSS; negative control), *P. aeruginosa* (Pa01; infected positive control), 100 nM *N*-formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma; uninfected positive control), or *A. fumigatus* conidia. Infections were for 1 h or 4 h on H292 cell monolayers and 2 h on primary airway epithelium. The apical membrane was washed following the infection incubation to remove excess conidia. Neutrophil migration was allowed to occur for 2 h in H292 cells and 4 h in primary airway epithelium. After the migration period, the Transwells were discarded and migrated neutrophils were quantified using a myeloperoxidase (MPO) activity assay. Briefly, apically migrated neutrophils were lysed with 0.5% Triton X-100 (Sigma), and neutrophil MPO activity was assessed using citrate buffered 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Sigma) solution. The magnitude of MPO activity (the optical density [OD] at 405 nm) was measured using a colorimetric enzyme assay and directly correlated to the number of migrated neutrophils in a linear fashion ($R^2 > 0.99$). Standard curves were produced for each experiment to control for possible variability between groups. Data are displayed as the number of neutrophils to reflect the magnitude of migration. Figure 1A depicts the neutrophil transepithelial migration in cartoon form. Since MPO activity is an indirect measure of neutrophil epithelial migration, migrated neutrophils were also captured by an Olympus Q-Fire camera on a Nikon Eclipse T5100 microscope under a Nikon $\times 10$ objective.

Conditioned medium experiment. Neutrophil transepithelial migration was tested with conditioned HBSS⁺ medium to determine if *A. fumigatus* requires direct contact with airway epithelial cells. Conditioned HBSS⁺ medium was generated by incubating $\Delta pksP$ mutant conidia in HBSS⁺ at 37°C with shaking for 1 h. The conidia were pelleted through low-speed centrifugation at $100 \times g$, and the resulting supernatant was filter sterilized through a 0.22- μm -pore-size filter. H292 cells were grown on 24-well 3.0- μm -pore-size Transwell plates. Cells were stimulated with medium only (HBSS; negative control), Pa01 (infected positive control), 100 nM fMLP (uninfected positive control), $\Delta pksP$ mutant conidia, and $\Delta pksP$ mutant conditioned HBSS⁺ medium (CM). All cells were infected for 1 h and washed 3 times in HBSS⁺, followed by a 2-h neutrophil migration. Neutrophil migration was quantified via the MPO assay (OD at 405 nm).

μOCT imaging. High-resolution micro-optical coherence tomography (μOCT) is a custom-built imaging technology that provides optical resolutions of 2 μm and 1 μm in the lateral/horizontal and axial/vertical directions, respectively. μOCT imaging of neutrophils has been previously reported (35). In brief, the μOCT instrument was inverted, and the imaging laser beam was directed to the sample from below in a transparent OCT-compatible bottom holding Transwell plates containing the airway epithelium and neutrophils approximately 100 μm from the glass bottom. Immediately following placement of the neutrophils in the basolateral compartment, μOCT captured time-lapse three-dimensional (3D) images every 10 min over 4 h. During the imaging process, samples were maintained at 33°C via an incandescent heat source. The 3D Viewer plug-in in ImageJ software was used to render 3D μOCT volume sequences. The neutrophil migration volume was determined by measuring the time-dependent increase (at 4 h compared to that at time zero) in the number of voxels in the entire imaged apical region exceeding the brightness threshold set by the end value of the histogram of the representative image.

Cell wall fragmentation. Cell walls of B5233 or $\Delta pksP$ mutant conidia were dissociated by a bead beater (Bertin Precellys 24) in 2-ml CK-14 bead-beating tubes with 1.4-mm ceramic beads, followed by low-speed centrifugation (at 6,800 rpm 3 times for 20 s each time with 60-s breaks) to remove intracellular components. The cell wall fraction was pelleted by filtration through a 40- μm -mesh-size mesh and centrifugation at $2 \times g$ for 5 min. The supernatant was removed, and cell wall fragments were washed twice in PBS. Prior to the initiation of the experiment, cell wall fragments were suspended in a solution of HBSS⁺ to achieve a concentration of 10 mg/ml and sonicated (Branson 1200 tabletop sonicator) for 2 min with repeating cycles of 3 to 6 cycles.

Drug treatment. Pronase (Sigma-Aldrich) was prepared in HBSS⁺ to a 1-mg/ml solution, incubated for 2 h at 37°C, and subjected to high-speed centrifugation at $15,000 \times g$ for 5 min. Supernatants were collected and resuspended to 1 mg/ml. The cell walls of $\Delta pksP$ mutant conidia were dissociated as described above and treated with 1 mg/ml of pronase under conditions similar to those described above.

Statistical analysis. Data are reported as means $\pm$ standard deviations. Differences were compared for statistical significance by one-way analysis of variance (ANOVA). In cases of significant differences, Tukey's posttest analysis was used to assess differences between groups. A *P* value of 0.05 or less was considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 10, AVI file, 2.6 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.4 MB.

SUPPLEMENTAL FILE 4, PDF file, 0.3 MB.

SUPPLEMENTAL FILE 5, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 6, PDF file, 0.3 MB.

SUPPLEMENTAL FILE 7, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 8, PDF file, 0.6 MB.

SUPPLEMENTAL FILE 9, AVI file, 2.8 MB.

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