Cystic Fibrosis Transmembrane Conductance Regulator Regulates Epithelial Cell Response to *Aspergillus* and Resultant Pulmonary Inflammation

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Rationale: Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) alter epithelial cell (EC) interactions with multiple microbes, such that dysregulated inflammation and injury occur with airway colonization in people with cystic fibrosis (CF). *Aspergillus fumigatus* frequently colonizes CF airways, but it has been assumed to be an innocent saprophyte; its potential role as a cause of lung disease is controversial.

Objectives: To study the interactions between *Aspergillus* and EC, and the role of the fungus in evoking inflammatory responses.

Methods: *A. fumigatus* expressing green fluorescent protein was developed for *in vitro* and *in vivo* models, which used cell lines and mouse tracheal EC.

Measurements and Main Results: Fungal spores (conidia) are rapidly ingested by ECs derived from bronchial cell lines and murine tracheas, supporting a role for EC in early airway clearance. Bronchial ECs harboring CFTR mutations ($\Delta F508$) or deletion demonstrate impaired uptake and killing of conidia, and ECs with CFTR mutation undergo more conidial-induced apoptosis. Germinated (hyphal) forms of the fungus evoke secretion of inflammatory mediators, with CFTR mutation resulting in increased airway levels of macrophage inflammatory protein 2 and KC, and higher lung monocyte chemoattractant protein-1. After *A. fumigatus* inhalation, CFTR^{-/-} mice develop exaggerated lymphocytic inflammation, mucin accumulation, and lung injury.

Conclusions: Data demonstrate a critical role for CFTR in mediating EC responses to *A. fumigatus*. Results suggest that the fungus elicits aberrant pulmonary inflammation in the setting of CFTR mutation, supporting the potential role of antifungals to halt progressive CF lung disease.

Keywords: *Aspergillus*; cystic fibrosis; epithelial

Chronic pulmonary inflammation is exacerbated by airway colonization by certain microbes, such as *Pseudomonas aeruginosa*, which infects more than 80% of people with cystic fibrosis (CF)-related chronic lung disease (1). Microbial specificity in invoking pulmonary inflammation is a subject of interest; studies have shown that epithelial cells (ECs) interact with specific organisms, such as *P. aeruginosa*, by mechanisms that rely on functional CF transmembrane conductance regulator (CFTR) (2). *P. aeruginosa* stimulates the formation of lipid rafts containing CFTR and caveolin-1, which facilitates organism entry into ECs; deficiency

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Aspergillus fumigatus is frequently recovered from airway samples in people with cystic fibrosis. Historically, its recovery has been considered to be an indicator of lung dysfunction, with the organism acting as an innocent saprophyte; hence, antifungal therapy has not been favored.

What This Study Adds to the Field

Our data suggest that cystic fibrosis transmembrane conductance regulator has an important role in airway epithelial cell clearance of *Aspergillus* spores. Deficiency in cystic fibrosis transmembrane conductance regulator results in epithelial cell susceptibility to apoptosis, and aberrant inflammatory responses to mature fungal morphotypes.

of either alters bacterial uptake, burden, and inflammatory responses (2, 3). Multiple other mechanisms by which CFTR mutations impact host-pathogen interactions have been described, with defects in organism clearance associated with ceramides in ECs and macrophages (4, 5), and the more generalized effects of impaired mucous clearance. These observations support the development and application of antimicrobial strategies now widely used to decrease airway burden of specific microbes, especially *P. aeruginosa*, with the overall goal of halting the progression of lung disease.

Aspergillus fumigatus, a filamentous fungus, is isolated from respiratory secretions of patients with CF, with reported prevalence ranging from 9–57% (6, 7). Up to 15% of patients with CF mount an allergic response, known as allergic bronchopulmonary aspergillosis (ABPA), which is associated with exaggerated Th2 responses to the organism. Although the significance of *A. fumigatus* colonization in the absence of ABPA is unknown, recent studies suggest that airway exposure or persistent colonization functions to elicit dysregulated inflammation. A retrospective cohort study found that persistent *A. fumigatus* recovery from the airway is a risk factor for hospital admissions, independent of lung function (8). Results of murine studies showed that mice with abnormal ($\Delta F508$) or absent CFTR demonstrate a profound Th2 response to inhaled inactivated *A. fumigatus* crude hyphal antigens *in vivo* (9); this is at least in part associated with aberrant responses generated by T cells harboring CFTR dysfunction (9, 10).

Aspergillus conidia (spores) are inhaled frequently and usually cleared without development of inflammation or invasion in the lung. Mechanisms by which hosts clear *A. fumigatus* from the lungs are being elucidated. Recent studies have outlined that the morphologic transition of the organism from conidia into filamentous cells, or hyphae, serves to functionally expose

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Figure 1. Δ F508 epithelial cell line IB3 and cystic fibrosis transmembrane conductance regulator (CFTR)^{-/-} mouse tracheal epithelial cells (MTEC) demonstrate reduced binding and uptake of green fluorescent protein (GFP)-Af293 conidia. (A) Calcofluor white counterstaining of GFP-expressing conidia distinguishes between internalized conidia (green, arrow) and external conidia (blue). Triplicate wells were prepared for each cell type and six random fields were examined to quantify internalized and external conidia. Average numbers of conidia bound to cell lines (blue) per field are shown in B, and the number of conidia per cell (green) taken up by epithelial cell lines are shown in C. Average numbers of conidia bound and taken up by wild-type (WT) and CFTR^{-/-} MTEC are shown in D and E, respectively. Data represent means $\pm$ SEM from three independent experiments. * Statistical significance (two-tailed Student *t* test; *P* < 0.05) of conidial uptake by CFTR^{-/-} and Δ F508 IB3 cells.

different cell surface molecules, which evoke inflammatory responses by professional phagocytes (11). Hence, inhaled conidia can be cleared in an immunologically “silent” fashion. Although most studies have focused on defining the role of myeloid-derived cells in mediating inflammatory responses to *A. fumigatus*, airway ECs anatomically form a first-response to inhaled conidia, potentially mediating both airway organism clearance and local inflammatory responses (12–16). Little is known regarding the mechanisms by which ECs interact with different forms of the fungus.

These studies were performed to elucidate EC inflammatory responses to *A. fumigatus*, and to determine the importance of CFTR mutation. Results show that *A. fumigatus* conidia are

Figure 2. *Aspergillus fumigatus* (Af293) conidia remain viable inside cystic fibrosis (CF) epithelial cells. Intracellular viability of Af293 conidia inside (A) S9 and (B) IB3 cells was measured by FUN-1 metabolism from green to orange. S9 and IB3 cells grown on 18-mm coverslips were infected with FUN-1-labeled Af293 live conidia (2×10^6) for 7–8 hours at 37°C/5% CO₂. Extracellular conidia were counterstained with calcofluor white (blue), and plates were washed and fixed in 1% paraformaldehyde. Conidia inside S9 epithelial cells fluoresced green (A, arrows), whereas germinating conidia that accumulate orange vacuoles were observed more frequently in IB3 cells, indicating fungal metabolic activity (B, arrows), $\times 63$. (C) Green fluorescent protein-Af293 inside mouse tracheal epithelial cells harvested from cystic fibrosis transmembrane conductance regulator deficient mice retained viability after 8 hours, as observed by swelling and germination of intracellular (green) organisms (arrow), $\times 40$.

Figure 3. Defective clearance of *Aspergillus fumigatus* (Af293) conidia by cystic fibrosis transmembrane conductance regulator (CFTR)^{-/-} airways. Representative lung sections with Gomori methenamine silver staining (×100) showing the presence of conidia (arrowheads) in lung parenchyma and in the airways of Af293-challenged (A) wild-type (WT) and (B) CFTR^{-/-} mice at 6 hours. (C) At 24 hours, Af293-challenged WT mice demonstrated no visible conidia or hyphal elements in the airways, whereas hyphal elements were seen (arrowhead) in the large airways of (D) Af293-challenged CFTR^{-/-} mice, ×100.

rapidly ingested by ECs derived from bronchial cell lines and murine tracheas. The presence of CFTR Δ F508 mutation (or CFTR deletion) is associated with decreased conidial uptake, with an increased cellular susceptibility to apoptosis. Altered cellular conidial interaction is associated with differences in secreted

inflammatory mediators, which occur significantly only after exposure to more mature hyphal morphotypes. CFTR^{-/-} mice develop profound pulmonary inflammation, mucin accumulation, and injury after inhalational exposure to *A. fumigatus*. These results outline a novel role of CFTR in mediating EC interactions with *A. fumigatus*. Because results support a causative role for the fungus in evoking pulmonary inflammation in the setting of dysfunctional CFTR, therapeutic algorithms that minimize airway *Aspergillus* exposure should be explored. A portion of this study has been reported elsewhere in abstract form (17).

METHODS

Reagents

Aspergillus fumigatus (isolate Af293) conidia were collected from mature colonies grown on potato dextrose agar. Germ tubes (GTs) were prepared from conidia (10⁶/ml) in Sabouraud dextrose broth for 6 hours at 37°C, and then heat-killed (90°C, 60 min). Af293 that constitutively expresses green fluorescent protein (GFP-Af293) was prepared as previously described (16). IB3 cell line (Δ F508/W1282X) and CFTR-corrected wild-type (WT) cell line (S9) were purchased from ATCC (Manassas, VA). Both were grown in LHC-8 medium (Invitrogen, Grand Island, NY) with 5% fetal-bovine serum (Gemini Bio-Products, West Sacramento, CA) and antibiotics.

Murine Experiments

Studies were approved by the Johns Hopkins University Institutional Animal Care and Use Committee. CFTR-deficient (CFTR^{-/-}) mice on a mixed genetic background (18) and age-matched (6–8 wk) C57BL/6J mice were used. Mice were infected by the inhalation route, using an aerosol chamber (16). WT and CFTR^{-/-} mice were infected in the same flasks, and inoculum control animals were evaluated 1 hour after exposure (5 × 10⁶ colony forming units per lung). Lungs were harvested at 24, 72, 96, and 120 hours for histopathologic analysis after staining with hematoxylin and eosin, Gomori methenamine silver, and

Figure 4. Inflammatory mediators in well-differentiated wild-type (WT) and cystic fibrosis transmembrane conductance regulator (CFTR)^{-/-} mouse tracheal epithelial cells after exposure to *Aspergillus fumigatus* conidia or germ tubes (GTs). Levels of cytokines and chemokines were measured using Luminex multiplex technology, after exposure to Af293 heat-inactivated conidia/GT (1 × 10⁷; multiplicity of infection of 20:1) or LPS (1 μg/ml) for 24 hours. Only cytokines and chemokines that were differentially measured are shown. Data are representative of two independent experiments. Significance of difference between independent groups of data (mean ± SEM) was analyzed by Student *t* test (two-tailed); one sample *t* test was performed using the lower limit of detection for the groups in which the cytokine values were below the assay detection limits (nd, not detected). * *P* < 0.05 for all comparisons between WT and CFTR^{-/-}. # *P* < 0.05 for all comparisons of conidia- or GT-treated versus untreated cells in WT and CFTR^{-/-}. IP = IFN-γ induced protein; MCP = monocyte chemoattractant protein; MIP = macrophage inflammatory protein.

Figure 5. Epithelial cells harboring $\Delta F508$ mutation or cystic fibrosis transmembrane conductance regulator (CFTR) deletion exhibit increased susceptibility to apoptosis after exposure to Af293 conidia. The percentage of apoptotic S9 (wild-type [WT]) and IB3 ($\Delta F508$) epithelial cell lines treated with Af293 conidia (4×10^6) and staurosporine (STS) (1 μ M) (positive control) for 4 hours was measured by flow-cytometry using the (A) fluorescein isothiocyanate anti-poly-ADP ribose polymerase (PARP)-1 antibody and fluorescein isothiocyanate M-30 cytodeath antibody (see METHODS). Data (mean $\pm$ SEM) represent two independent experiments. * $P < 0.05$ for comparisons between IB3 and S9 cells, calculated by the Student two-tailed t test. (B) The percentage of apoptotic mouse tracheal epithelial cells (appear green) from WT and CFTR^{-/-} mice was assessed after exposure to Af293 conidia and tumor necrosis factor (TNF)- α (100 ng/ml) (positive control) by fluorescein isothiocyanate M-30 staining ($\times 40$).

alcian blue. In some studies, mouse tracheal ECs (MTEC) were isolated and differentiated on an air-liquid interface, as described (19).

Binding and Viability Studies

Cell lines (5×10^5 per well, 18-mm coverslips) grown for 18 hours and MTEC (1×10^5 per well) proliferated in MTEC-plus media (19) for 3–4 days at 37°C/5% CO₂ were infected with GFP-expressing Af293 conidia (2×10^6) or GF-latex beads (2 μ m; Sigma, St. Louis, MO). Plates were centrifuged at 1,200 rpm for 10 minutes; incubated for 2–3 hours at 37°C/5% CO₂; washed with warm phosphate-buffered saline and incubated with 25 μ M calcofluor white in ice-cold phosphate-buffered saline for 10 minutes; washed twice (phosphate-buffered saline); and fixed in 1% paraformaldehyde. Coverslips were then mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and examined by LSM-510 (Carl Zeiss Micro-imaging, Thornwood, NY) MetaView Image Analyzer (MetaView, West Chester, PA). In viability studies, Af293 conidia were labeled with FUN-1 dye (Molecular Probes, Invitrogen) (20) before cell line or MTEC exposure.

Cytokines and Flow Cytometry

Milliplex MAP mouse immunoassay kits (Millipore, Billerica, MA) were used to measure IL-1 α , IL-1 β , IL-6, KC, IL-4, IL-5, IFN- γ , macrophage inflammatory protein (MIP)-2, monocyte chemoattractant protein (MCP)-1, IFN- γ -induced protein (IP)-10, MIP-1 α , MIP-1 β , regulated

upon activation normal T-cell expressed and secreted, and tumor necrosis factor (TNF)- α from supernatants of MTEC and in bronchoalveolar lavage (BAL) fluid.

Cellular population in BAL after 24 hours and in lung suspensions 96 and 120 hours after Af293 exposure (21) were determined as described (16, 22). Immunostaining for cell surface molecules was performed for 30 minutes at 4°C, using antibodies against CD3 (CD3/17A2-PerCpCy5.5); CD4 (CD4-L3T4/GK1.5-phycoerythrin); CD8b (CD8b-Ly-3/H35/17.2-phycoerythrin); CCR4 (CD194/2G12-allophycocyanin); CCR5 (CD195/HM-Alexafluor 488); Ly6-G/Ly6-C-PE; CD11c (CD11c/HL3-fluorescein isothiocyanate [FITC]); and F4/80 (F4/80-phycoerythrin). All antibodies except for F4/80-phycoerythrin (eBioscience Inc., San Diego, CA) were purchased from BD Pharmingen (San Diego, CA). Samples were analyzed using a FACSCalibur with CellQuest software (both from BD Immunocytometry Systems, San Jose, CA).

Apoptosis

Apoptosis was measured by M-30 cytodeath-fluorescein antibody (Roche Applied Science, Indianapolis, IN), FITC-labeled poly-ADP ribose polymerase (PARP)-1 antibody (Invitrogen), and Annexin V/PI staining (BD Pharmingen). Staurosporine (STS) induced a greater number of F4/80-PARP-1-positive cells than TNF- α and was used as a positive control for cell lines. TNF- α was used as a positive control in MTEC experiments because STS led to nonspecific M-30 staining.

Figure 6. Increased cellular infiltration and elevated levels of inflammatory mediators in bronchoalveolar lavage (BAL) of Af293-challenged cystic fibrosis transmembrane conductance regulator (CFTR)^{-/-} mice. BAL fluid collected after 24 hours from Af293-challenged wild-type (WT) and CFTR^{-/-} mice were evaluated for cellular analysis using flow cytometry (see METHODS) and were also analyzed for cytokines and chemokines using the Luminex assay. (A) Higher numbers of macrophages (MΦ) and polymorphonuclear leukocytes (PMN) were observed in BAL of Af293-challenged CFTR^{-/-} mice after 24 hours of conidial exposure than WT mice. (B) Increased levels of macrophage inflammatory protein (MIP)-2 and KC chemokines were also observed in BAL of Af293-challenged CFTR^{-/-} at this time point. Cellular populations were also measured in lung homogenates by flow cytometry at basal levels (untreated) and after 96 hours of conidial exposure in CFTR^{-/-} and WT mice. (C) Higher percentage of T cells (CD3⁺/CD4⁺, CD3⁺/CD8⁺) were present in the lungs of CFTR^{-/-} mice recovered 96 hours after exposure to Af293 conidia than WT mice. The data are representative of two independent experiments, with BAL and lung cells (pooled) from three mice per condition. Total of 10,000 events were analyzed for flow cytometry data. Significance between independent groups of data was analyzed by Student *t* test (two-tailed). * *P* < 0.05, comparison of Af293-challenged WT and CFTR^{-/-}. # *P* < 0.05, comparison of Af293-challenged WT and CFTR^{-/-} versus basal levels. IP = IFN-γ-induced protein; MCP = monocyte chemotactic protein.

Statistical Analysis

Two-tailed Student *t* test was used to evaluate significance of difference between independent groups (GraphPad Prism v4, GraphPad Software, La Jolla, CA), unless indicated otherwise. *P* less than or equal to 0.05 was considered significant.

RESULTS

CF ECs Show Defective Interaction with *A. fumigatus* Conidia *In Vitro*

EC lines and MTEC were challenged with GFP-Af293. The cell-impermeant calcofluor white, which stains extracellular fungal cells

blue, was used to differentiate between intracellular (green) and extracellular (blue) conidia after exposure to ECs (Figure 1A). The EC line IB3, harboring the ΔF508 mutation, demonstrated reduced binding and uptake of GFP-Af293 compared with the corrected WT cell line S9 (Figures 1B and 1C). The defect in binding and uptake was specific to conidia, because both cell types demonstrated similar interactions with green fluorescent latex beads of similar size (2 μm) (data not shown). To validate the data obtained with EC lines, studies were performed with MTEC isolated from CFTR^{-/-} and age-matched WT C57BL/6J control animals. Similar to ΔF508 mutant IB3 cells, CFTR^{-/-} MTEC bound to and internalized

TABLE 1. DISTRIBUTION OF CD3⁺ GATED CCR4⁺ AND CCR5⁺ T CELLS (CD4⁺ AND CD8⁺) IN THE LUNGS OF WT AND CFTR^{-/-} MICE AT BASELINE AND 96 HOURS AFTER *ASPERGILLUS FUMIGATUS* AF293 CHALLENGE

Condition	T Cell (CD3 ⁺)	CCR Marker	Strain*	
			WT	CFTR ^{-/-}
None (basal)	CD4 ⁺	CCR4 ⁺	3.8	6.9
		CCR5 ⁺	3.2	6.2
	CD8 ⁺	CCR4 ⁺	1.7	4.7
		CCR5 ⁺	1.5	4.0
Post-challenge	CD4 ⁺	CCR4 ⁺	3.5	14.0
		CCR5 ⁺	2.9	11.4
	CD8 ⁺	CCR4 ⁺	5.4	9.9
		CCR5 ⁺	4.4	8.4

Definition of abbreviations: CFTR = cystic fibrosis transmembrane conductance regulator; WT = wild-type.

*Percentage of total cells, calculated from experiments using pooled lung cells from three mice per condition.

GFP-Af293 less well compared with WT MTEC (Figures 1D and 1E).

Intracellular viability of *Aspergillus* conidia was measured by conidial metabolism of the viability dye, FUN-1, and by visual observation of intracellular conidial germination into hyphal cells. IB3 cells, but not S9 cells, contained viable conidia, as assessed by the development of orange cylindrical intravacuolar structures and by the observation of intracellular GTs (Figures 2A and 2B) (20). Tracheal ECs harvested from CFTR^{-/-} mice also demonstrated a defect in killing of internalized conidia, observed by swelling and development of GTs from cells that retained GFP-fluorescence after calcofluor white costaining (Figure 2C). No swollen or germinated conidia were recognized in MTEC harvested from WT mice. Collectively, these data suggest defects in early and late interactions of ECs with conidia in the presence of ΔF508 CFTR mutation or deletion *in vitro*; specifically, cell lines and murine tracheal cells that harbor defects in CFTR bind and internalize and kill conidia less well compared with control cells.

Defective Clearance of *A. fumigatus* Conidia by CFTR^{-/-} Mice

Conidia were visible in the lung parenchyma and in small and large airways of WT (Figure 3A) and CFTR^{-/-} mice (Figure 3B) at 6 hours post-Af293 challenge. In contrast to Af293-challenged WT mice (Figure 3C), airways of CFTR^{-/-} mice demonstrated the presence of fungal hyphae at 24 hours (Figure 3D) suggesting poor clearance of the fungus in the absence of the CFTR.

Aberrant Secretion of Inflammatory Mediators in Response to *A. fumigatus* Exposure by CFTR^{-/-} MTEC

ECs function in early innate immune defense by secretion of multiple inflammatory mediators. Proinflammatory mediators were measured after MTEC exposure to inactivated conidia and hyphal cells. Compared with WT MTEC, unstimulated CFTR^{-/-} cells demonstrated an overall decreased production of MIP-2, IL-6, and IP-10, and an increased production of MCP-1 (Figure 4). LPS stimulated release of all mediators, with a disproportionate increase of MCP-1 by CFTR^{-/-} cells, as previously reported (23–26). WT ECs exposed to *A. fumigatus* demonstrated morphotype specificity. Conidia induced secretion of very little or no cytokines (TNF-α, IL-6, IP-10, MCP-1, MIP-2, prostaglandin E₂, KC, and regulated upon activation normal T-cell expressed and secreted) from WT cells (Figure 4, and data not shown). GTs stimulated increased secretion of MIP-2 compared with unstimulated WT cells (Figure 4). Exposure to fungal products

also decreased WT cell secretion of several mediators compared with amounts released from nonstimulated cells. Specifically, WT cells released less MCP-1 after exposure to conidia, and WT cells released less IL-6 after exposure to conidia and GT (Figure 4).

Compared with WT, MTEC with CFTR deletion exposed to fungal cells secreted increased amounts of MCP-1 after GT exposure (Figure 4).

Conidia Induce Excessive Apoptosis of CF ECs

CFTR mutations alter EC apoptotic responses (27). We postulated that the baseline decrease in mediator production after exposure to conidia was at least partially secondary to microbial-induced apoptosis or cell death. Apoptosis and cellular viability were measured in bronchial EC lines and MTEC, using three different methods. As expected, the CFTR mutation was associated with higher basal and STS-induced apoptosis in IB3 cells compared with S9 cells, as measured using PARP-1 cleavage and M-30-FITC staining (Figure 5A). IB3 cells were more susceptible to apoptosis when stimulated with Af293 conidia (Figure 5A). Similar results were obtained by measurement of annexin V-PI staining (data not shown).

Similarly, apoptosis of MTEC harvested from WT and CFTR^{-/-} mice was measured after exposure to Af293 conidia and TNF-α, the latter as a positive control (Figure 5B), by M-30 staining. More MTEC harvested from CFTR^{-/-} mice were apoptotic at baseline, and after exposure to conidia and TNF-α.

Increased Cellular Infiltration and Elevated Levels of Inflammatory Mediators in BAL of Af293-challenged CFTR^{-/-} Mice

To examine whether CFTR deficiency also impacts pulmonary inflammatory responses to Af293 *in vivo*, CFTR^{-/-} and WT mice were challenged by inhaled delivery of conidia. Cellular populations in BAL were examined by flow cytometry 24 hours after conidial challenge because this time point captures the early infiltration of effector cells into the lungs. WT and CFTR^{-/-} mice had higher total BAL cell counts after Af293 challenge, compared with basal levels (Figure 6A). CFTR^{-/-} mice had more cells compared with WT (Figure 6A). This difference seemed to be associated with more macrophages and polymorphonuclear leukocytes in Af293-challenged CFTR^{-/-} mice (Figure 6A). There were also higher levels of MIP-2 and KC in BAL of Af293-challenged CFTR^{-/-} mice compared with WT (Figure 6B). No significant differences were observed in basal levels of these or other cytokines (data not shown).

Cellular populations were measured in lung homogenates, later (96 h) after Af293 exposure (Figure 6C and Table 1). More lymphocytes (CD3⁺/CD4⁺, CD3⁺/CD8⁺, CD4⁺/CCR4⁺, and CD4⁺/CCR5⁺ T cells) were present in the lungs of Af293-challenged CFTR^{-/-} mice compared with WT mice. Both WT and CFTR^{-/-} mice showed higher numbers of CD8⁺/CCR4⁺ and CD8⁺/CCR5⁺ CD3⁺ T cells after Af293 challenge (Table 1).

Cellular inflammation was also examined by histopathology. Nonchallenged lungs of CFTR^{-/-} and WT mice were indistinguishable (Figures 7A and 7D). WT mice challenged with Af293 demonstrated acute inflammation in the area of luminal, respiratory bronchioles and in the adjacent alveoli (Figures 7B and 7C). Acute inflammation was also observed intraalveolar in the lungs of the CFTR^{-/-} mice. However, lungs of these mice were further characterized by epithelial necrosis in small airways with loss of respiratory epithelium and fibrin (F) deposition in airways (Figures 7E and 7F). In addition, CFTR^{-/-} mice had prominent mucin (M) accumulation with acute inflammation (AI) within airways (Figures 7G and 7H).

Figure 7. Lung sections stained with hematoxylin and eosin (A–H) and alcian blue (J and K) from wild-type (WT) and cystic fibrosis transmembrane conductance regulator (CFTR)^{−/−} mice challenged with Af293 conidia, observed after 72 hours at indicated magnification. (A) Lung sections from unchallenged WT and (D) CFTR^{−/−} mice showed no basal differences in cellular infiltration and inflammation. (B) Lung sections (×20 magnification) from WT mice challenged with Af293 demonstrated acute inflammation in small airways at adjacent alveoli, bronchioles, and alveolar spaces (AS). (C) Enhanced (×100) magnification of boxed area in B showed intraluminal acute inflammation in small airways without epithelial necrosis or fibrin deposition. (E) Lung sections (×20 magnification) from Af293-infected CFTR^{−/−} mice showed prominent fibrin (F) deposition in the areas of acute inflammation. (F) Enhanced (×100) magnification of boxed region in (E) showed fibrin (F) deposition in the bronchioles with loss of respiratory epithelium (LRE). (G) Prominent intraluminal mucin (M) was present in the bronchiole shown (×20) with (H) magnified view at (×100) of boxed region showing acute inflammation (AI), with accumulated mucin, without epithelial necrosis. (I) Lung

inflammatory score was higher in Af293-challenged CFTR^{−/−}, performed using blinded scoring in lung sections (n = 2–3) at 24 and 72 hours. The scoring for the lung inflammatory changes was ranked from 0–4. This scoring parameters included the following: 0, no inflammation around the bronchioles and in alveolar spaces; 1, chronic inflammation around bronchioles; 2, acute and chronic inflammation around bronchioles, and acute inflammation in bronchiolar lumens; 3, acute and chronic inflammation around bronchioles, and acute inflammation in bronchiolar lumens and scattered airspaces; and 4, acute and chronic inflammation around bronchioles, acute inflammation in bronchiolar lumens, epithelial necrosis, and extensive fibrin deposition. (J and K) Representative lung sections (×40) stained with alcian blue demonstrating increased abundance of mucin in airways of (K) Af293-challenged CFTR^{−/−} mice than WT (J).

Lung inflammation was quantified 24 and 72 hours after exposure to WT and CFTR^{−/−} mice (Figure 7I). Using the point-counter technique with a 10 × 10 grid, lung sections of WT (Figure 7J) and CFTR^{−/−} (Figure 7K) mice demonstrated 7.7% and 11.3% of airway/airspace units with acute inflammation positive for mucin deposition (alcian blue stain), respectively, at 72 hours. These data confirm exuberant cellular inflammation and mucin production (by alcian-blue staining) at multiple time points after *Aspergillus* challenge in CFTR^{−/−} mice.

DISCUSSION

These results demonstrate that filamentous fungi, specifically *A. fumigatus*, contribute to altered pulmonary inflammation in the setting of CF, at least in part associated with ineffective EC clearance of conidia, and subsequent aberrant inflammatory responses to germinated *A. fumigatus* morphotypes. This

observation has critical importance with consideration of current treatment strategies, suggesting a potential benefit of treating airway colonization by *Aspergillus* species, even in the absence of overt ABPA.

The CFTR protein not only functions as a pathogen recognition molecule, but it also has a critical role in regulating the proinflammatory response and phenotype that is common to other, nonmicrobial lung insults. CFTR, clustered in membrane lipid rafts, is important for direct EC interactions with *P. aeruginosa* (2, 3, 28, 29). Recent studies have shown that membrane CFTR is involved in nuclear factor-κB-mediated inflammatory signaling generated by insults, such as cigarette smoke, at least in part by regulating ceramide-enriched lipid raft signaling platforms that signal cellular apoptosis (30, 31). Our data demonstrate, using bronchial EC lines, a defective uptake of *A. fumigatus* conidia, and aberrant inflammatory and apoptotic

responses to the different forms of the organism. These findings were confirmed using MTEC because transformation of cell lines with adeno12-SV40-virus may affect cellular functions. Our data suggest that interactions are altered at the level of both adherence and internalization, with more internalized cells remaining viable in ECs harboring CFTR mutations or deletions. This was also evident as *in vivo* observations showing hyphal elements in airways of CFTR^{-/-} mice after *A. fumigatus* exposure. Whether these outcomes are caused by alterations in direct microbial binding or a secondary effect is not clear; EC binding motifs and receptors involved in mediating responses to the different *Aspergillus* morphotypes have not yet been described.

Although the mechanisms by which *A. fumigatus* triggers pulmonary EC release of inflammatory mediators have not been defined, prior studies using cell lines demonstrate secretion of prostaglandin E₂, IL-6, and IL-8 (32) after exposure to live *A. fumigatus*. Results shown here confirm the immunologic silence of tracheal ECs on exposure to conidia, or “morphotype specificity,” similar to that demonstrated by cells of myeloid lineage (14, 32–34). In myeloid cells, dectin-1 recognizes β -glucan only after conidia shed a hydrophobin layer (13, 14, 33, 34). One can postulate that similar mechanisms may be involved in EC distinction between *Aspergillus* morphotypes, which seems critical to mounting effective inflammation toward potentially invasive hyphal forms of the organism while limiting proinflammatory responses to frequently inhaled, dormant conidia. The recent finding that dectin-1 is expressed on the surface of airway EC lines after activation of specific antigens, in a TLR2-dependent fashion, may be particularly relevant (35).

Our findings illustrate that ECs that harbor mutation in CFTR demonstrate exaggerated apoptotic responses to conidia. A role for CFTR in the apoptosis of ECs has been reported by several groups, and has been a matter of debate (36). Recently, studies have shown that membrane-localized CFTR has a critical role in regulating apoptotic responses to other insults, such as cigarette smoke, through increased ceramide accumulation (30). CFTR-deficient human alveolar macrophages exhibit increased apoptosis and inflammatory responses (37). Whether conidia-induced apoptosis is elicited by a specific microbial factor or a more general function, such as increased oxidative or endoplasmic reticulum stress, is unclear (36, 38).

ECs that harbor mutated CFTR demonstrated decreased clearance of, and exaggerated apoptotic responses to, conidia and increased production of MCP-1 in response to germinated cells. MCP-1 is a chemokine involved in attracting monocytes, memory T cells, and dendritic cells to the lung (39, 40), and seems to stimulate production of IL-4, with overexpression associated with biased Th2 polarization (41). Different genetic backgrounds from which WT and CFTR^{-/-} MTEC were harvested may have contributed to variable responses.

We observed an increased percentage of macrophages and polymorphonuclear leukocytes in BAL fluid of CFTR^{-/-} mice as early as 24 hours after conidial exposure, with increased levels of CXC neutrophilic chemokines, MIP-2, and KC compared with WT. In this model, acute (neutrophilic) lung inflammation was followed by the more chronic manifestations of excessive lymphocytic populations, fibrin deposition, mucin accumulation, and injury. It is important to note that there were some discrepancies observed in the cytokines measured from the MTEC model and from BAL fluid, especially MIP-2 and MCP-1. We believe that direct comparisons of results from these models should be made with caution, because they used different fungal inocula and measured mediators at different time points after exposure. Moreover, the cytokine milieu and other resident and structural cells in the lung could also participate in the differences observed in *in vivo* versus *in vitro* models.

Excessive inflammation is likely multifactorial in origin, in part mediated by early, aberrant responses by airway ECs and excessive inflammatory responses generated by myeloid cells that are stimulated by the disproportionate amount of germinated conidia and hyphae that result from ineffectual conidial killing. A schematic of these early differences in fungal–epithelial interactions is presented in Figure 8. Finally, we cannot discount the potential impact of primary differences in lymphocyte responses to the fungal antigens. The latter is supported by recent studies that show excessive Th2-type CD4⁺ stimulation from CF lymphocytes exposed to *A. fumigatus*, which has now been demonstrated in both murine and human samples (9, 10, 42). Thus, primary EC–fungus interactions contribute to exuberant and aberrant cellular inflammation, which couple with inherently Th2-biased lymphocyte responses to generate chronic inflammation and lung injury.

These mechanistic insights add to the results of recent clinical studies that suggest that fungal airway colonization may

Figure 8. Schematic representation of early events occurring after exposure of wild-type and cystic fibrosis transmembrane conductance regulator (CFTR)^{-/-} epithelial cells (EC) to conidia. Conidia are normally internalized and inactivated by ECs, without evoking secretion of inflammatory mediators (upper panel). In the presence of CFTR mutations, conidia induce increased EC apoptosis. Inefficient conidial uptake and killing results in more luminal conidial germination to hyphal cells, which subsequently trigger increased production of specific inflammatory mediators (e.g., monocyte chemoattractant protein-1), resultant dysregulated cellular inflammation.

perpetuate chronic lung disease rather than simply reflect degree of pulmonary dysfunction (6, 8, 43). Some controversy has been generated, with some studies suggesting that colonization can function as an independent risk for functional decline or related complications (hospitalizations), whereas others showed no independent contribution of *Aspergillus* colonization (44). Perhaps some insight may be garnered from genetic association studies, in which people with ABPA have been shown to carry CFTR mutations more frequently than the general population, suggesting that dysfunctional CFTR generates an environment conducive to a Th2 bias, even in the absence of CF or severe pulmonary dysfunction (45). Our data suggest that *Aspergillus* colonization in the airway may contribute to progressive lung dysfunction. Further defining the mechanisms by which CFTR mutation alters host–*Aspergillus* interactions, and the clinical use of antifungal therapy for decreasing CF lung disease, is high priority.

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References

- Lyczak JB, Cannon CL, Pier GB. Lung infections associated with cystic fibrosis. *Clin Microbiol Rev* 2002;15:194–222.
- Pier GB, Grout M, Zaidi TS. Cystic fibrosis transmembrane conductance regulator is an epithelial cell receptor for clearance of *Pseudomonas aeruginosa* from the lung. *Proc Natl Acad Sci USA* 1997;94:12088–12093.
- Gadjeva M, Paradis-Bleau C, Priebe GP, Fichorova R, Pier GB. Caveolin-1 modifies the immunity to *Pseudomonas aeruginosa*. *J Immunol* 2010;184:296–302.
- Teichgraber V, Ulrich M, Endlich N, Riethmüller J, Wilker B, De Oliveira-Munding CC, van Heeckeren AM, Barr ML, von Kurthy G, Schmid KW, *et al.* Ceramide accumulation mediates inflammation, cell death and infection susceptibility in cystic fibrosis. *Nat Med* 2008;14:382–391.
- Zhang Y, Li X, Grassme H, Döring G, Gulbins E. Alterations in ceramide concentration and pH determine the release of reactive oxygen species by CFTR-deficient macrophages on infection. *J Immunol* 2010;184:5104–5111.
- Sudfeldt CR, Dasenbrook EC, Merz WG, Carroll KC, Boyle MP. Prevalence and risk factors for recovery of filamentous fungi in individuals with cystic fibrosis. *J Cyst Fibros* 2010;9:110–116.
- Milla CE, Wielinski CL, Regelman WE. Clinical significance of the recovery of *Aspergillus* species from the respiratory secretions of cystic fibrosis patients. *Pediatr Pulmonol* 1996;21:6–10.
- Amin R, Dupuis A, Aaron SD, Ratjen F. The effect of chronic infection with *Aspergillus fumigatus* on lung function and hospitalization in patients with cystic fibrosis. *Chest* 2010;137:171–176.
- Allard JB, Poynter ME, Marr KA, Cohn L, Rincon M, Whittaker LA. *Aspergillus fumigatus* generates an enhanced Th2-biased immune response in mice with defective cystic fibrosis transmembrane conductance regulator. *J Immunol* 2006;177:5186–5194.
- Mueller C, Braag SA, Keeler A, Hodges C, Drumm M, Flotte TR. Lack of cystic fibrosis transmembrane conductance regulator in CD3+ lymphocytes leads to aberrant cytokine secretion and hyper-inflammatory adaptive immune responses. *Am J Respir Cell Mol Biol* 2011;44:922–929.
- Goodridge HS, Wolf AJ, Underhill DM. Beta-glucan recognition by the innate immune system. *Immunol Rev* 2009;230:38–50.
- Brakhage AA, Bruns S, Thywissen A, Zipfel PF, Behnken J. Interaction of phagocytes with filamentous fungi. *Curr Opin Microbiol* 2010;13:409–415.
- Gersuk GM, Underhill DM, Zhu L, Marr KA. Dectin-1 and TLRs permit macrophages to distinguish between different *Aspergillus fumigatus* cellular states. *J Immunol* 2006;176:3717–3724.
- Steele C, Rapaka RR, Metz A, Pop SM, Williams DL, Gordon S, Kolls JK, Brown GD. The beta-glucan receptor dectin-1 recognizes specific morphologies of *Aspergillus fumigatus*. *PLoS Pathog* 2005;1:e42.
- Mircescu MM, Lipuma L, van Rooijen N, Pamer EG, Hohl TM. Essential role for neutrophils but not alveolar macrophages at early time points following *Aspergillus fumigatus* infection. *J Infect Dis* 2009;200:647–656.
- Bretz C, Gersuk G, Knoblauch S, Chaudhary N, Randolph-Habecker J, Hackman RC, Staab J, Marr KA. MyD88 signaling contributes to early pulmonary responses to *Aspergillus fumigatus*. *Infect Immun* 2008;76:952–958.
- Chaudhary N, Staab J, Marr KA. In vitro studies on the role of cystic fibrosis transmembrane conductance regulator (CFTR) in pulmonary epithelial cell interactions to *A. fumigatus* [abstract]. *J Allergy Clin Immunol* 2010;125:AB118.
- Zhou L, Dey CR, Wert SE, DuVall MD, Frizzell RA, Whittsett JA. Correction of lethal intestinal defect in a mouse model of cystic fibrosis by human CFTR. *Science* 1994;266:1705–1708.
- You Y, Richer EJ, Huang T, Brody SL. Growth and differentiation of mouse tracheal epithelial cells: selection of a proliferative population. *Am J Physiol* 2002;283:L1315–L1321.
- Marr KA, Koudadoust M, Black M, Balajee SA. Early events in macrophage killing of *Aspergillus fumigatus* conidia: new flow cytometric viability assay. *Clin Diagn Lab Immunol* 2001;8:1240–1247.
- Rivera A, Van Epps HL, Hohl TM, Rizzuto G, Pamer EG. Distinct CD4+ T-cell responses to live and heat-inactivated *Aspergillus fumigatus* conidia. *Infect Immun* 2005;73:7170–7179.
- D'Hulst AI, Vermaelen KY, Brusselle GG, Joos GF, Pauwels RA. Time course of cigarette smoke-induced pulmonary inflammation in mice. *Eur Respir J* 2005;26:204–213.
- Greene CM, Carroll TP, Smith SG, Taggart CC, Devaney J, Griffin S, O'Neill SJ, McElvaney NG. TLR-induced inflammation in cystic fibrosis and non-cystic fibrosis airway epithelial cells. *J Immunol* 2005;174:1638–1646.
- Kube D, Sontich U, Fletcher D, Davis PB. Proinflammatory cytokine responses to *P. aeruginosa* infection in human airway epithelial cell lines. *Am J Physiol* 2001;280:L493–L502.
- John G, Yildirim AO, Rubin BK, Gruenert DC, Henke MO. TLR-4-mediated innate immunity is reduced in cystic fibrosis airway cells. *Am J Respir Cell Mol Biol* 2010;42:424–431.
- McGreal EP, Davies PL, Powell W, Rose-John S, Spiller OB, Doull I, Jones SA, Kotecha S. Inactivation of IL-6 and soluble IL-6 receptor by neutrophil derived serine proteases in cystic fibrosis. *Biochim Biophys Acta* 2010;1802:649–658.
- Rottner M, Kunzelmann C, Mergey M, Freyssinet JM, Martinez MC. Exaggerated apoptosis and NF-κB activation in pancreatic and tracheal cystic fibrosis cells. *FASEB J* 2007;21:2939–2948.
- Bajmoczy M, Gadjeva M, Alper SL, Pier GB, Golan DE. Cystic fibrosis transmembrane conductance regulator and caveolin-1 regulate epithelial cell internalization of *Pseudomonas aeruginosa*. *Am J Physiol Cell Physiol* 2009;297:C263–C277.
- Pier GB, Grout M, Zaidi TS, Olsen JC, Johnson LG, Yankaskas JR, Goldberg JB. Role of mutant CFTR in hypersusceptibility of cystic fibrosis patients to lung infections. *Science* 1996;271:64–67.
- Bodas M, Min T, Vij N. Critical role of CFTR dependent lipid-rafts in cigarette smoke induced lung epithelial injury. *Am J Physiol* 2011;300:L811–L820.
- Vij N, Mazur S, Zeitlin PL. CFTR is a negative regulator of NFκB mediated innate immune response. *PLoS ONE* 2009;4:e4664.
- Bellanger AP, Millon L, Khoufache K, Rivollet D, Bieche I, Laurendeau I, Vidaud M, Botterel F, Bretagne S. *Aspergillus fumigatus* germ tube growth and not conidia ingestion induces expression of inflammatory mediator genes in the human lung epithelial cell line A549. *J Med Microbiol* 2009;58:174–179.
- Aimanianda V, Bayry J, Bozza S, Kniemeyer O, Perruccio K, Elluru SR, Clavaud C, Paris S, Brakhage AA, Kaveri SV, *et al.* Surface hydrophobin prevents immune recognition of airborne fungal spores. *Nature* 2009;460:1117–1121.
- Hohl TM, Van Epps HL, Rivera A, Morgan LA, Chen PL, Feldmesser M, Pamer EG. *Aspergillus fumigatus* triggers inflammatory responses by stage-specific beta-glucan display. *PLoS Pathog* 2005;1:e30.

35. Lee HM, Yuk JM, Shin DM, Jo EK. Dectin-1 is inducible and plays an essential role for mycobacteria-induced innate immune responses in airway epithelial cells. *J Clin Immunol* 2009;29:795–805.
36. Rottner M, Freyssinet JM, Martinez MC. Mechanisms of the noxious inflammatory cycle in cystic fibrosis. *Respir Res* 2009;10:23.
37. Xu Y, Krause A, Hamai H, Harvey BG, Worgall TS, Worgall S. Proinflammatory phenotype and increased caveolin-1 in alveolar macrophages with silenced CFTR mRNA. *PLoS ONE* 2010;5:e11004.
38. Amitani R, Taylor G, Elezis EN, Llewellyn-Jones C, Mitchell J, Kuze F, Cole PJ, Wilson R. Purification and characterization of factors produced by *Aspergillus fumigatus* which affect human ciliated respiratory epithelium. *Infect Immun* 1995;63:3266–3271.
39. Carr MW, Roth SJ, Luther E, Rose SS, Springer TA. Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. *Proc Natl Acad Sci USA* 1994;91:3652–3656.
40. Xu LL, Warren MK, Rose WL, Gong W, Wang JM. Human recombinant monocyte chemotactic protein and other C-C chemokines bind and induce directional migration of dendritic cells in vitro. *J Leukoc Biol* 1996;60:365–371.
41. Gu L, Tseng S, Horner RM, Tam C, Loda M, Rollins BJ. Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1. *Nature* 2000;404:407–411.
42. Kreindler JL, Steele C, Nguyen N, Chan YR, Pilewski JM, Alcorn JF, Vyas YM, Aujla SJ, Finelli P, Blanchard M, et al. Vitamin D3 attenuates Th2 responses to *Aspergillus fumigatus* mounted by CD4+ T cells from cystic fibrosis patients with allergic bronchopulmonary aspergillosis. *J Clin Invest* 2010;120:3242–3254.
43. Chotirmall SH, O'Donoghue E, Bennett K, Gunaratnam C, O'Neill SJ, McElvaney NG. Sputum *Candida albicans* presages FEV decline and hospital-treated exacerbations in cystic fibrosis. *Chest* 2010;138:1186–1195.
44. de Vrankrijker AM, van der Ent CK, van Berkhout FT, Stellato RK, Willems RJ, Bonten MJ, Wolfs TF. *Aspergillus fumigatus* colonization in cystic fibrosis: implications for lung function? *Clin Microbiol Infect* 2011;17:1381–1386.
45. Marchand E, Verellen-Dumoulin C, Mairesse M, Delaunois L, Brancalione P, Rahier JF, Vandenplas O. Frequency of cystic fibrosis transmembrane conductance regulator gene mutations and 5T allele in patients with allergic bronchopulmonary aspergillosis. *Chest* 2001;119:762–767.