Loss of Cystic Fibrosis Transmembrane Conductance Regulator Function Enhances Activation of p38 and ERK MAPKs, Increasing Interleukin-6 Synthesis in Airway Epithelial Cells Exposed to Pseudomonas aeruginosa

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In cystic fibrosis (CF), the absence of functional cystic fibrosis transmembrane conductance regulator (CFTR) translates into chronic bacterial infection, excessive inflammation, tissue damage, impaired lung function and eventual death. Understanding the mechanisms underlying this vicious circle of inflammation is important to design better therapies for CF. We found in CF lung biopsies increased immunoreactivity for p38 MAPK activity markers. Moreover, when compared with their non-CF counterpart, airway epithelial cells expressing the most common mutation in CF (CFTRΔF508) were more potent at inducing neutrophil chemotaxis through increased interleukin (IL)-6 synthesis when challenged with Pseudomonas aeruginosa diffusible material. We then discovered that in CFTRΔF508 cells, the p38 and ERK MAPKs are hyperactivated in response to P. aeruginosa diffusible material, leading to increased IL-6 mRNA expression and stability. Moreover, although TLR5 contributes to p38 MAPK activation upon P. aeruginosa challenge, it only played a weak role in IL-6 synthesis. Instead, we found that the production of reactive oxygen species is essential for IL-6 synthesis in response to P. aeruginosa diffusible material. Finally, we uncovered that in CFTRΔF508 cells, the extracellular glutathione levels are decreased, leading to a greater sensitivity to reactive oxygen species, providing an explanation for the hyperactivation of the p38 and ERK MAPKs and increased IL-6 synthesis. Taken together, our study has characterized a mechanism whereby the CFTRΔF508 mutation in airway epithelial cells contributes to increase inflammation of the airways.

Cystic fibrosis is one of the most common fatal genetic diseases affecting Caucasians of European descent. It is an autosomal recessive disorder caused by mutations of the CF transmembrane conductance regulator (CFTR). Defective CFTR function in the airway epithelium is responsible for CF lung disease, the most life-threatening complication of CF, characterized by mucus hypersecretion and neutrophil-dominated inflammation. The proinflammatory cytokines, TNFs, IL-1β, IL-6, and CXCL8 (Cys-Xaa-Cys chemokine ligand 8), are all found elevated in the airways of CF patients, with decreased levels of the anti-inflammatory cytokine IL-10 (1, 2). Pseudomonas aeruginosa infections occur in 70% of the individuals at an early age and contribute to lung destruction and mortality. Moreover, CF patients suffer from exacerbation episodes, which have a profound effect on the patient’s quality of life, where P. aeruginosa is the predominant pathogen found (3, 4). Therefore, in CF, the absence of functional CFTR translates somehow into chronic bacterial infection, excessive inflammation, tissue damage, impaired lung function, and eventual death.

CF pathogens activate common signaling pathways in airway epithelial cells, leading to the production of proinflammatory cytokines (5). Human cells have evolved to recognize pathogens through receptors that bind different molecular patterns like lipids, carbohydrates, peptides, and nucleic acids expressed by various microorganisms. Once activated, these pattern recognition receptors trigger a network of intracellular signaling events leading to the production of inflammatory mediators. The two most studied pattern recognition receptor families are the TLR and nucleotide-binding oligomerization domain (NOD)-like receptor families. There are currently 12 known mammalian TLRs and more than 20 NOD-like receptors (6, 7).

Our understanding of TLR-mediated signaling has progressed rapidly in the last few years. Following dimerization, TLRs bind different adaptor molecules through their Toll/IL-1 receptor domain (7). The best characterized adaptor is MyD88 (myeloid differentiating factor 88), which was shown to, through the sequential recruitment of IL-1R-associated protein kinases (8, 9), TRAF6 (TNF-receptor-associated factor 6) (10),

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and TAK1 (transforming growth factor-β-activated kinase) (11, 12), serve as the template for the activation of four major signaling pathways: the NF-κB pathway and the three MAPK pathways (ERK1/ERK2, JNK, and p38 MAPK). The NOD receptors bind through their caspase recruitment domains to a protein kinase termed RIP2 (receptor-interacting protein 2; also called RICK or CARDIAK) (13), which will initiate downstream signaling via the recruitment of TAK1 and activation of the IkB protein kinase complex (14, 15).

p38 MAPK was initially recognized for its role regulating the biosynthesis of proinflammatory cytokines, namely IL-1 and TNFα, in endotoxin-stimulated monocytes (16). Subsequently, it was found to be involved in regulating the production of CXCL8 (also known as IL-8) in response to IL-1 (17) and the production of IL-6 in response to TNFα (18). p38α is thought to be the main isoform involved in mediating cytokine production because mice lacking p38β, the most closely related isoform, show no defect in cytokine production or immune function (19). In view of its critical role in the synthesis of TNFα, IL-1, IL-6, and CXCL8, p38 MAPK is a major contributor to inflammation and may play a critical role in the inflamed lung of cystic fibrosis patients.

Therefore, we investigated the role of p38α MAPK in mediating proinflammatory cytokine production in airway epithelial cells expressing the most common mutation found in CF, CFTRΔF508 (20). Patients harboring the CFTRΔF508 mutation lack CFTR expression at the membrane due to misfolding, which leads to the protein being degraded instead of transported to the cell surface (21). In this paper, we describe unexpected findings in CFTRΔF508 cells leading to enhanced IL-6 synthesis in response to P. aeruginosa diffusible material.

EXPERIMENTAL PROCEDURES

Materials—SB203580 was obtained from InvivoGen (San Diego, CA). PD184352 was bought from USBiological (Swampscott, MA). BIRB0796 was kindly provided by Professor Sir Philip Cohen (Medical Research Council Protein Phosphorylation Unit, University of Dundee, UK). All chemicals were bought from Fisher. Pam3CSK4, flagellin, lipopolysaccharide from P. aeruginosa, and C12-IE-DAP were bought from Invivogen. Recombinant human IL-17A was purchased from Biovision (catalog no. 4176-25). Recombinant human IL-6 was obtained from R&D Systems (Minneapolis, MN). N-Acetyl-l-cysteine (NAC), GSH, and 2-vinylpyridine were bought from Sigma. The diffusible P. aeruginosa material was prepared as previously reported (22). Briefly, filtrates of late stationary phase P. aeruginosa (mucoid strain 508) were heat-inactivated for 10 min at 95°C before being used. P. aeruginosa 508 is a stable mucoid clinical isolate from the sputum of a patient with CF (Hôpital Sainte-Justine, Montréal, Canada).

Antibodies—Neutralizing antibodies against TLR2 and TLR5 were used at 5 μg/ml and purchased from Invivogen. Anti-phospho-ERK1/ERK2 (Thr202/Tyr204) (AB3826; dilution 1:1000) and anti-ERK1/ERK2 (AB3053; 0.3 μg/ml) were purchased from Chemicon-Millipore (Temecula, CA). Anti-phospho-p38 MAPK (Thr180/Tyr182) (09-272; dilution 1:1000) and anti-p38 MAPK (05–454; 0.4 μg/ml) were bought from Upstate Biotechnology, Inc. (Lake Placid, NY). Neutralizing antibody against IL-6 was bought from Bender MedSystems (BMS130, Burlingame, CA). Goat anti-rabbit IgG DyLight™ 800 (35571; 1:15,000) and goat anti-mouse IgG DyLight™ 680 (35518; 1:15,000) were bought from Thermo Scientific (Rockford, IL). IRDye™ 800CW-conjugated Streptavidin (S000-31) was bought from Rockland (Gilbertville, PA).

Immunohistochemistry—Five-micrometer-thick sections from endobronchial biopsy of healthy adult subjects (non-CF) or from explanted lung of subjects with cystic fibrosis undergoing lung transplantation (CF) were graciously provided by Dr. James G. Martin (Meakins-Christie Laboratories, Montréal, Canada). All patients gave written informed consent, as approved by the hospital ethics committee. Slides were deparaffinized in xylene and rehydrated in decreasing concentrations of alcohol. For staining, heat-induced epitope retrieval was performed using EDTA buffer, pH 8.0. Sections were then permeabilized with 0.2% Triton and incubated with 5% hydrogen peroxide. After blocking with universal blocking solution for 30 min (Dakocytomation, Mississauga, Canada), slides were incubated overnight with 1:50 dilutions of HSP27 phospho-Ser82 antibodies (catalog no. 2401, Cell Signaling Technologies) or MAPKAP-K2 phospho-Thr344 antibodies (catalog no. 3007, Cell Signaling Technologies). Isotype controls were prepared by replacing the primary antibody with a nonspecific Ig at the same concentration. After rinsing, the slides were incubated with a biotinylated secondary antibodies (1:100), followed by a horseradish peroxidase-antibody complex (both from Dakocytomation). Immunoreactivity was developed with dianaminobenzidine chromogen (Dakocytomation), and slides were counterstained with hematoxylin and lithium carbonate.

Inflammation Antibody Array—We used RayBio Human Inflammation Antibody Array 3 to screen a panel of 40 different proteins being secreted in the cell media. One milliliter of cell supernatant was incubated with each membrane according to the manufacturer’s protocol. The signal was revealed using IRDye™ 800CW-conjugated Streptavidin (1 μg/ml), and fluorescence was detected and quantified using a Licor Odyssey imaging system.

Cell Culture—Human airway epithelial cell line NuLi was derived from a normal lung of a 36-year-old male patient by dual retroviral infection to prevent cells undergoing growth arrest in cell culture. The CuFi airway epithelial cell line was derived from lung of a 14-year-old female patient with cystic fibrosis by the same method and is homozygous for the CFTRΔF508 mutation. These cells were purchased from the ATCC or, alternatively, generously provided by Dr. Emmanuelle Brochiero (Centre de Recherche, Hôtel-Dieu du CHUM, Université de Montréal, Canada). They were used until passage 17. The cells were maintained at 37°C, 5% CO2, 100% humidity, in Bronchial Epithelial Growth Medium (Lonza, Walkersville, MD) supplemented with growth factors (SingleQuots (Lonza), except gentamicin), 50 units/ml penicillin G, 50 μg/ml streptomycin, 50 μg/ml genetin, and 2 μg/ml amphotericin B.

Neutrophil Chemotaxis Assay—Neutrophil migration assays were performed using a modified Boyden chamber (Transwell, Fisher) with 5-μm pores. The conditioned medium of treated AECs was added to the lower chamber, whereas the upper chamber was filled with untreated medium. To determine the
role of IL-6, supernatants were first incubated for 1 h at room temperature with neutralizing antibodies against IL-6 (50 µg/ml) before adding supernatants to the lower chamber. Neutrophils (0.5 × 10⁶) suspended in Dulbecco’s modified Eagle’s medium were put into the upper well, and the chamber was placed in a humidified incubator under 5% CO₂ for 4 h at 37 °C. Cells located on the upper surface of the membrane were scraped with a cotton swab, and the cells that crossed the poly-carbonate filter were collected from the lower compartment and counted with a hemacytometer. The same experiment was performed by adding recombinant human IL-6 (200 pg/well) in Dulbecco’s modified Eagle’s medium to the lower chamber instead of conditioned medium.

ELISA—Human IL-6 (DY206) DuoSet ELISA kits was purchased from R&D Systems. 100 µl of supernatant collected after cell stimulation was directly used for IL-6 quantification according to the manufacturer’s protocol.

RNA Extraction and cDNA Synthesis—Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer’s protocol. The RNA was quantified, and 1 µg was treated with DNase I Amp Grade (Invitrogen) and reverse-transcribed using Superscript II reverse transcriptase (Invitrogen), according to the manufacturer’s protocols.

Real-time PCR—Semiquantitative real-time PCR was performed in 96-well plate format using SYBR Green-based detection on a Step-One-Plus machine (ABI) with each 10-µl reaction containing ~50 ng of cDNA, 0.3 µM sense and antisense primers (Table 1), and 1× Quantitect SYBR Green supermix (Qiagen). The plate was sealed and cycled under the following conditions: 95 °C for 10 min, 50 cycles of 95 °C for 10 s, and 60 °C for 45 s. Each reaction was performed in duplicate, mRNA levels of GAPDH were used for normalization, and -fold induction was determined from Ct values using the Pfaffl method (23). PCR efficiencies were determined from the slope of a standard curve generated using a 5-fold dilution series of the DNA template.

mRNA Stability—Measurement of mRNA stability was performed as described previously (24). Briefly, cells were first stimulated with P. aeruginosa filtrates as specified, and transcription was blocked by the addition of 5 µg/ml actinomycin D (380-009-M005; Alexis Biochemicals, San Diego, CA). At the times indicated in the figure legends, total RNA was extracted following the addition of actinomycin D (referred as time 0).

Cell Lysis and Immunoblotting—Following stimulation, cells were lysed in ice-cold buffer A (50 mM Tris-Cl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton x-100, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 0.27 M sucrose, complete miniprotease inhibitor mixture, and 2 mM dithiothreitol). Proteins were quantified using the Bradford method, and 20 µg of lysates were submitted to SDS-PAGE, transferred to nitrocellulose, and immunoblotted with specified antibodies. The signal was detected and quantified using a Licor Odyssey imaging system.

Glutathione Assay—GSH levels were measured according to the manufacturer’s instructions (Arbor Assays). Briefly, cells were stimulated with P. aeruginosa filtrates (3 h), and the supernatant was collected. Total GSH was detected by the color change at 405 nm. GSH levels were quantified using standards of GSSG with and without 2-vinylpyridine and expressed as GSSG equivalents. GSH levels were calculated from the difference between total GSH and GSSG.

Statistical Analysis—Analyses of variance followed by a multiple comparison test (Bonferroni) were used to test differences in mean between groups. p values of <0.05 were considered significant.

RESULTS

p38α MAPK Is Activated in CF Airways and Regulates the Production of Inflammatory Cytokines—We first sought to determine if there was activation of the p38 MAPK pathway in the lungs of CF patients. We therefore stained for the phosphorylated form of the small heat-shock protein 27 (HSP27) (25), a substrate of the p38 MAPK-activated protein kinase MAPKAP-K2. We found positive staining of phosphorylated HSP27 (phospho-Ser82) when compared with IgG control staining in airway epithelial cells of a cystic fibrosis patient (Fig. 1). Moreover, we also observed staining for the phos-

![FIGURE 1. p38 MAPK activation in the airway epithelium of CF patients.](image-url)

Loss of CFTR Enhances MAPK Activation and IL-6 Synthesis

![TABLE 1 Primer sequences](table-url)
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FIGURE 2. p38 MAPK-dependent IL-6 synthesis is greatly enhanced in AECs expressing CFTRΔF508 in response to P. aeruginosa filtrates. NuLi (non-CF) and CuFi (CFTRΔF508) AECs were left untreated (white bars) or exposed to P. aeruginosa (PA) filtrates in the absence (light gray bars) or presence of BIRB0796 (0.1 µM; dark gray bars) or SB203580 (5 µM; black bars) for 24 h. Medium was collected and incubated with a RayBio Human Inflammation Antibody Array 3 to screen for 40 different inflammatory proteins (A). The amount of fluorescence for each spot was quantified using a Licor Odyssey imaging system. The p38 MAPK-dependent genes shown in B, GM-CSF, granulocyte-macrophage colony-stimulating factor; RANTES, regulated on activation normal T cell expressed and secreted. Error bars, S.D.

in response to bacterial infection. In order to identify inflammatory mediators up-regulated by P. aeruginosa filtrates in AECs expressing wild-type CFTR (NuLi) or CFTRΔF508 (CuFi), an array of 40 secreted proteins was screened (Fig. 2A). The role of p38 MAPK was assayed using two structurally distinct and relatively specific inhibitors of this protein kinase, namely SB203580 (16, 27) and BIRB0796 (28). SB203580 potently inhibits p38 MAPK but is also known to inhibit with similar potency RIP2 (29), a protein kinase involved in transmitting signals from the NOD1 and NOD2 pattern recognition receptors (see Introduction). BIRB0796 is an even more potent inhibitor of p38 MAPK and does not inhibit RIP2, making it useful to differentiate the effects mediated by RIP2 from those mediated by p38 MAPK (15, 28). Screening of the array identified IL-6, CXCL8, granulocyte-macrophage colony-stimulating factor, and LTα as p38 MAPK-dependent genes induced by P. aeruginosa filtrates in non-CF or CFTRΔF508 AECs (Fig. 2B). Moreover, in the CFTRΔF508 cells, a striking increase of IL-6 was seen upon stimulation with P. aeruginosa filtrates compared with wild-type

phorylated form of MAPKAP-K2 (phospho-Thr334) (Fig. 1) in the same patient. In accordance with the known predominant locations of phosphorylated MAPKAP-K2 and HSP27, the first was seen in the nucleus, whereas the latter was observed mostly in the cytoplasm (see insets in Fig. 1). In comparison, very little staining for the phosphorylated forms of HSP27 and MAPKAP-K2 was seen on a biopsy of a healthy lung (Fig. 1).

These results indicate that the p38 MAPK pathway is more activated in the airway epithelium of CF patients. As mentioned in the Introduction, these patients suffer from chronic infections, mostly from the Gram-negative bacteria P. aeruginosa. Bacteria present in the lungs of CF patients are found mostly as intraluminal masses distal from airway epithelial cells (26). Therefore, diffusible P. aeruginosa products may represent a more accurate reflection of the pathogenic factors encountered by AECs rather than live organisms. Filtrates of late stationary phase P. aeruginosa (a mucoid clinical isolate) were prepared to stimulate bronchial airway epithelial cells. We first confirmed that p38 MAPK was phosphorylated by exposure of airway epithelial cells to the P. aeruginosa filtrates using immunoblotting (supplemental Fig. 1).

We then checked which inflammatory mediators were synthesized in a p38α MAPK-dependent fashion by AECs CFTR-expressing cells (Fig. 2B).

Airway Epithelial Cells Expressing CFTRΔF508 Have Increased Neutrophil Chemotactic Activity upon Bacterial Challenge—In order to determine whether the observed increase in IL-6 secretion by AECs expressing CFTRΔF508 had a biological impact, we tested the capacity of conditioned medium from these cells to stimulate neutrophil migration. When fresh human neutrophils isolated from peripheral blood were exposed to P. aeruginosa filtrate-stimulated CFTRΔF508 medium, their migration was greater than when they were exposed to the non-CF medium (Fig. 3A). To determine the contribution of IL-6 to this neutrophil chemotactic activity, we blocked its action in the conditioned medium using a neutralizing antibody. We found that blocking IL-6 activity together, these results clearly imply IL-6 as a direct actor of neutrophil recruitment by AECs.
Loss of CFTR Enhances MAPK Activation and IL-6 Synthesis

Higher Levels and More Stable IL-6 mRNA Are Found in CFTRΔF508 AECs—In order to understand why there was such an increased production of IL-6 in cells lacking functional CFTR, we first looked at the mRNA expression of IL-6 in both NuLi and CuFi cells treated with *P. aeruginosa* filtrates. Increased levels of IL-6 mRNA were detected at all times, measured by real-time PCR (Fig. 4A). The induction of IL-6 mRNA by *P. aeruginosa* filtrates was dependent on both the p38 and ERK MAPKs because inhibition of both these pathways impaired this production in the two cell types (Fig. 4B). This is in accordance with previous results obtained for CXCL8 in airway epithelial cells stimulated by TLR agonists (31). This, however, does not preclude a role for other pathways, like that of NF-κB, as previously shown (31). We then determined the secreted levels of IL-6 in the media by ELISA. Much higher basal and induced levels were found in the media of the CFTRΔF508 cells compared with wild-type AECs (Fig. 4C). In accordance with the data obtained with the IL-6 mRNA, inhibition of both p38 and ERK MAPKs pathways led to a reduction of secreted IL-6 in *P. aeruginosa* filtrate-stimulated cells, which was more pronounced in wild-type cells than the CFTRΔF508-expressing AECs (Fig. 4D). Moreover, these two pathways also played a role in regulating the basal levels of IL-6 because their inhibition reduced the amount of IL-6 mRNA present in unstimulated CFTRΔF508 cells (supplemental Fig. 2). A role for p38 MAPK in the post-transcriptional regulation of IL-6 has been previously reported (32). This prompted us to investigate the mRNA stability of IL-6 in *P. aeruginosa* filtrate-stimulated AECs. Strikingly, we found that in CFTRΔF508 cells treated with *P. aeruginosa* filtrates, the mRNA of IL-6 was much more stable than in the non-CF cells (Fig. 4E). These results further explain why there is more IL-6 protein made because the turnover rate of the IL-6 mRNA is much longer in the presence of functional CFTR expression, a very surprising result. We further established that when p38 MAPK activity is blocked with a pharmacological inhibitor, BIRB0796, the IL-6 mRNA instability is partially restored (Fig. 4E).

**p38 and ERK MAPK Activations Are Increased in Cells Lacking Functional CFTR Expression**—The previous results suggested that the p38 MAPK plays a key role in this phenotype. We therefore looked at the...
Loss of CFTR Enhances MAPK Activation and IL-6 Synthesis

Pattern recognition receptor or cytokine receptor stimulation of AECs. Non-CF and CFTRΔF508 cells were stimulated with the following pattern recognition receptor agonists: a triacylated synthetic lipoprotein (Pam3CSK4) that activates TLR1/TLR2; lipopolysaccharide from P. aeruginosa that activates TLR4; flagellin from Salmonella typhimurium that activates TLR5; or C12-ide-DAP an acylated derivative of the dipeptide γ-D-Glu-mDAP, present in the peptidoglycan of bacteria, that activates the intracellular receptor NOD1. The cells were also stimulated with IL-17A, a cytokine driving neutrophil recruitment to inflamed areas via the synthesis of CXCL8 by signaling pathways analogous to TLRs (33). The CFTRΔF508-expressing cells only showed increased secretion of IL-6 in response to pattern recognition receptor activation but not IL-17A stimulation (Fig. 6).

Reactive Oxygen Species Are Key Intermediates in Mediating IL-6 Synthesis in AECs—Because the greatest increases in IL-6 synthesis were seen in response to agonists of TLR2 and TLR5 in Fig. 6, we sought to determine if these two receptors were crucial links to MAPK activation and IL-6 synthesis by P. aeruginosa filtrates. We used neutralizing antibodies against TLR2 and TLR5 proven effective by completely blocking CXCL8 synthesis in response to their respective ligands (Pam3CSK4 for TLR2 and flagellin for TLR5) in airway epithelial cells (supplemental Fig. 3). Blocking TLR2 and TLR5 reduced p38 MAPK phosphorylation in non-CF AECs, whereas blocking TLR5 activation was much more effective in CFTRΔF508 AECs (Fig. 7A). When the two neutralizing antibodies were combined, a further additive effect was seen, but some activity remained (Fig. 7A). In contrast, the ERK MAPKs were essentially unaffected by inhibition of TLR2 and TLR5, demonstrating that another pathway is involved in their activation (Fig. 7B). Surprisingly, neutralizing TLR2 and TLR5 had very little impact on the synthesis of IL-6 by AECs (CFTRΔF508 and non-CF) in response to P. aeruginosa filtrates (Fig. 7C). Moreover, the addition of the p38 MAPK inhibitor BIRB0796 to the neutralizing antibodies reduced IL-6 synthesis in non-CF AECs (Fig. 7C), demonstrating that another signaling pathway was able to activate p38 MAPK in the P. aeruginosa filtrates. Taken together, these results suggest that although the main p38 MAPK activation occurs through TLR5, the activation of ERK MAPK, residual p38 MAPK activity, and IL-6 synthesis are dependent on other pathways.

P. aeruginosa has been suggested to also signal in AECs through the generation of reactive oxygen species (34). Accord-

Activation of p38 MAPK induced by the presence of P. aeruginosa filtrates in both non-CF and CFTRΔF508 AECs. We found that in cells lacking functional expression of CFTR, p38 MAPK activity was increased when compared with cells expressing CFTR at the membrane (Fig. 5A). However, this increased activation was not specific to the p38 MAPK because increased activation in CFTRΔF508 cells was also observed for ERK MAPKs (Fig. 5B). These results suggest that the increased activation is occurring upstream of MAPK activation.

Increased IL-6 Synthesis in CFTRΔF508 Cells Is Linked to Pattern Recognition Receptor Activation and Not Cytokine Receptors—In order to further dissect where this increased activation is occurring, we compared IL-6 synthesis in response to pattern recognition receptor or cytokine receptor stimulation of AECs. Non-CF and CFTRΔF508 cells were stimulated with the following pattern recognition receptor agonists: a triacylated synthetic lipoprotein (Pam3CSK4) that activates TLR1/TLR2; lipopolysaccharide from P. aeruginosa that activates TLR4; flagellin from Salmonella typhimurium that activates TLR5; or C12-ide-DAP an acylated derivative of the dipeptide γ-D-Glu-mDAP, present in the peptidoglycan of bacteria, that activates the intracellular receptor NOD1. The cells were also stimulated with IL-17A, a cytokine driving neutrophil recruitment to inflamed areas via the synthesis of CXCL8 by signaling pathways analogous to TLRs (33). The CFTRΔF508-expressing cells only showed increased secretion of IL-6 in response to pattern recognition receptor activation but not IL-17A stimulation (Fig. 6).

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ingly, preincubation of both non-CF and CFTR/H9004F508 AECs with the antioxidant NAC greatly diminished IL-6 synthesis in response to P. aeruginosa filtrates (Fig. 7D). Taken together, these results demonstrate that ROS intermediates are essential for the inflammatory response to P. aeruginosa.

**CFTRΔF508 AEC Decreased Glutathione Levels Prime These Cells to Hyperresponsiveness**—Interestingly, in CFTR-depleted cells, the antioxidant GSH efflux is lower than in CFTR-replete cells (35). This led us to hypothesize that CFTRΔF508 cells have lower extracellular GSH levels, making them more sensitive to the action of ROS. We therefore measured the extracellular levels of GSH in CFTRΔF508 and non-CF AECs. We found a 6-fold reduction in GSH levels in CFTRΔF508 cells compared with the non-CF cells under basal and treated conditions (Fig. 7E). Because ROS and a pro-oxidative shift are associated with enhanced intracellular signaling (36), we checked whether restoring GSH in the extracellular compartment could reverse the increased IL-6 synthesis observed in CFTRΔF508 cells. Fittingly, the addition of extracellular GSH led to a reduction of IL-6 synthesis in CFTRΔF508 cells back to the levels of their non-CF counterpart (Fig. 7D), whereas extracellular GSH had no detectable inhibition on the non-CF cells (Fig. 7D).

**DISCUSSION**

CF patients suffer from a vicious circle of inflammation that leads to the eventual destruction of their lungs. Therefore, understanding key factors driving this inflammation is crucial to develop therapies aimed at restoring a balanced inflammatory response. In this paper, we have uncovered a mechanism explaining increased IL-6 synthesis in cells expressing the most common mutation found in CF patients, CFTR/H9004F508. We found that in these cells, IL-6 mRNA was more abundant and stable due to increased activation of the MAPK pathways, a situation occurring due to increased sensitivity to extracellular oxidants.

Enhanced IL-6 secretion by CF cells is in accordance with a number of previous studies, which have shown that in many but not all CF models, AECs respond to P. aeruginosa with increased or prolonged IL-6 and CXCL8 synthesis (37, 38). This increase seems to be dependent on the presence of serum in the culture media of the cell models (39). Moreover, freshly harvested cells from inflamed CF lungs show evidence of enhanced cytokine production (40), and IL-6 levels are found to be higher in CF lungs (41). IL-6 actions in the CF lungs are probably numerous. Here we showed that IL-6 is sufficient to increase neutrophil migration in vitro. Moreover, blocking its activity was sufficient to reverse the increased neutrophil-chemotactic activity of conditioned medium obtained from P. aeruginosa filtrate-treated CFTRΔF508 cells. However, in vivo, it has additional and probably more important roles in driving neutrophilic inflammation. IL-6 is an essential cytokine for the differentiation of Th17 lymphocytes.
IL-17, the major product synthesized by Th17 cells, is a potent inducer of CXCL8 and neutrophil recruitment in vivo (43).

We observed increased IL-6 synthesis upon stimulation with P. aeruginosa filtrates, a stimulus frequently encountered by AECs in the CF lung, which is the result of more abundant and stable mRNA in the CFTRF508 cells. The increased synthesis stems in part from greater activation of p38 and ERK MAPKs. These two MAPKs are important transcriptional regulators, leading among others to the activation of the AP-1 transcription factor likely to contribute to increased IL-6 transcription. Moreover, p38 MAPK is a pathway well established to play a role in post-transcriptional regulation (44). Accordingly, blocking its activity partially restored IL-6 mRNA instability. Potential targets of p38 MAPK include the AU-rich element-binding protein KSRP (KH-type splicing regulatory protein) (45) and a number of MAPKAP-K2 substrates, like the AU-rich element-binding proteins tristetraprolin (46) and heterogeneous nuclear ribonucleoprotein A0 (24).

Increased IL-6 synthesis in CFTRΔF508 cells depends on lower GSH levels in the media. This may be explained by reports that CFTR not only acts as an anion chloride channel but also a channel for GSH (35). The important role of low level and localized ROS as a key intermediate in intracellular signaling is becoming more and more apparent. A pro-oxidative shift is associated with enhanced intracellular signaling (36), an observation matched by our current study, whereas decreased antioxidant levels in the extracellular space led to increased activation of MAPK pathways and IL-6 synthesis. This may also explain the basal levels of IL-6 seen in CFTRΔF508 cells compared with non-CF cells, where ambient ROS are more effective at triggering a response. However, there is some selectivity because stimulation of CFTRF508 AECs with IL-17A showed a different profile, with higher IL-6 synthesis in the non-CF cells.

Our study not only defined ROS as facilitators of intracellular signaling in CFTRF508 cells but also identified them as key intermediates in the response of both non-CF and CF AECs to P. aeruginosa filtrates. P. aeruginosa diffusible material contains ligands for both TLR2 and TLR5, as shown by decreased scavenging, leading to inhibition of key intracellular signals (47). Therefore, targeting ROS may be more attractive to prevent inflammation and tissue damage in the CF lung.

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REFERENCES
1. Kelley, T. J., and Drumm, M. L. (1998) J. Clin. Invest. 102, 1200–1207
2. Schwierbert, L. M., Estell, K., and Propp, S. M. (1999) Am. J. Physiol. 276, C700–C710
3. Lyczak, J. B., Cannon, C. L., and Pier, G. B. (2002) Clin. Microbiol. Rev. 15, 194–222
4. Wedzicha, J. A. (2002) Chest 121, 1365–1415
5. Ratner, A. J., Bryan, R., Weber, A., Nguyen, S., Barnes, D., Pitt, A., Gelber, S., Cheung, A., and Prince, A. (2001) J. Biol. Chem. 276, 19267–19275
6. Fritz, J. H., Ferrero, R. L., Philpott, D. J., and Girardin, S. E. (2006) Nat. Immunol. 7, 1250–1257
7. O’Neill, L. A. (2006) Curr. Opin. Immunol. 18, 3–9
8. Muzio, M., Ni, J., Feng, P., and Dixit, V. M. (1997) Science 278, 1612–1615
9. Wescott, H., Henzel, W. J., Shillinglaw, W., Li, S., and Cao, Z. (1997) Immunity 7, 837–847
10. Cao, Z., Xiong, J., Takeuchi, M., Kurama, T., and Goeddel, D. V. (1996) Nature 383, 443–446
11. Lee, J., Mira-Arbeibe, L., and Ulevitch, R. J. (2000) J. Leukoc. Biol. 68, 909–915
12. Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z., and Matsumoto, K. (1999) Nature 398, 252–256
13. Kufer, T. A., Kremmer, E., Banks, D. J., and Philpott, D. J. (2006) Infect. Immun. 74, 3115–3124
14. Inohara Chaumard, McDonald, L., and Nuñez, G. (2005) Annu. Rev. Biochem. 74, 355–383
15. Windheim, M., Lang, C., Peggie, M., Plater, L. A., and Cohen, P. (2007) Biochim. J. 404, 179–190
16. Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R., and Landvatter, S. W. (1994) Nature 372, 739–746
17. Shapiro, L., and Dinarello, C. A. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 12230–12234
18. Beyaert, R., Cuenda, A., Vanden Bergh, W., Plaisance, S., Lee, J. C., Hageman, G., Cohen, P., and Fiers, W. (1996) EMBO J. 15, 1914–1923
19. Bezdorm, V. A., Hinton, H. J., Eftychi, C., Apostolaki, M., Armaka, M., Darragh, J., McIlrath, J., Carr, J. M., Armit, L. J., Clacher, C., Malone, L., Kollias, G., and Arthur, J. S. (2005) Mol. Cell. Biol. 25, 10454–10464
20. White, M. B., Amos, J., Hsu, J. M., Gerrard, B., Finn, P., and Dean, M. (1990) Nature 344, 665–667
21. Cheng, S. H., Gregory, R. J., Marshall, J., Paul, S., Souza, D. W., White, G. A., O’Riordan, C. R., and Smith, A. E. (1990) Cell 63, 827–834
22. Wu, Q., Lu, Z., Verghese, M. W., and Randell, S. H. (2005) Respir. Res. 6, 26
23. Pfaffl, M. W. (2001) Nucleic Acids Res. 29, e45
24. Rousseau, S., Morris, N., Peggie, M., Campbell, D. G., Gaestel, M., and Cohen, P. (2002) EMBO J. 21, 6505–6514
25. Rouse, J., Cohen, P., Trigon, S., Morange, M., Carollo-Llamazares, A., Zamanillo, D., Hunt, T., and Nebreda, A. R. (1994) Cell 78, 1027–1037
26. Baltimore, R. S., Christie, C. D., and Smith, G. J. (1989) Am. Rev. Respir. Dis. 140, 1650–1661
27. Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R., and Lee, J. C. (1995) FEBS Lett. 364, 229–233
28. Kuma, Y., Sabio, G., Bain, J., Shipton, N., Marquez, R., and Cuenda, A. (2005) J. Biol. Chem. 280, 19472–19479
29. Godl, K., Wissing, J., Kuntenbach, A., Habenberger, P., Blencke, S., Gutbrod, H., Salassidis, K., Stein-Gerlach, M., Missio, A., Cotten, M., and Daub, H. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 15434–15439
30. Yang, H. T., Cohen, P., and Rousseau, S. (2008) Cell. Signal. 20, 375–380
31. Bérubé, J., Bourdon, C., Yao, Y., and Rousseau, S. (2009) Cell. Signal. 21, 448–456
32. Neininger, A., Kontoyiannis, D., Kotlyarov, A., Winzen, R., Eckert, R., Volk, H. D., Holtmann, H., Kollia, G., and Gaestel, M. (2002) J. Biol. Chem. 277, 3065–3068
33. Liu, C., Qian, W., Qian, Y., Giltiay, N. V., Lu, Y., Swaidani, S., Misra, S., Deng, L., Chen, Z. J., and Li, X. (2009) Sci. Signal 2, ra63
34. Yan, F., Li, W., Jono, H., Li, Q., Zhang, S., Li J. D., and Shen, H. (2008) Biochem. Biophys. Res. Commun. 366, 513–519
35. Gao, L., Kim, K. J., Yankaskas, J. R., and Forman, H. J. (1999) Am. J. Physiol. 277, L113–L118
36. Allen, R. G., and Tresini, M. (2000) Free Radic. Biol. Med. 28, 463–499
37. Stecenko, A. A., King, G., Torii, K., Breyer, R. M., Dworski, R., Blackwell, T. S., Christman, J. W., and Brigham, K. L. (2001) Inflammation 25, 145–155
38. Kube, D., Sontich, U., Fletcher, D., and Davis, P. B. (2001) Am. J. Physiol. Lung Cell Mol. Physiol. 280, L1493–L1502
39. Becker, M. N., Sauer, M. S., Muhlebach, M. S., Hirsh, A. J., Wu, Q., Verghese, M. W., and Randell, S. H. (2004) Am. J. Respir. Crit. Care Med. 169, 645–653
40. Bonfield, T. L., Konstan, M. W., and Berger, M. (1999) J. Allergy Clin. Immunol. 104, 72–78
41. Bonfield, T. L., Panuska, J. R., Konstan, M. W., Hilliard, K. A., Hilliard, J. B., Ghnaim, H., and Berger, M. (1995) Am. J. Respir. Crit. Care Med. 152, 2111–2118
42. Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., Weiner, H. L., and Kuchroo, V. K. (2006) Nature 441, 235–238
43. Roussel, L., Houle, F., Chan, C., Yao, Y., Bérubé, J., Oulivenstein, R., Martin, J. G., Huot, J., Hamid, Q., Ferri, L., and Rousseau, S. (2010) J. Immunol. 184, 4531–4537
44. Cuenda, A., and Rousseau, S. (2007) Biochim. Biophys. Acta 1773, 1358–1375
45. Briata, P., Forcales, S. V., Ponasi, M., Costa, G., Chen, C. Y., Karin, M., Puri, P. L., and Gherzi, R. (2005) Mol. Cell 20, 891–903
46. Chrestensen, C. A., Schroeder, M. I., Shabanowitz, J., Hunt, D. F., Pelo, J. W., Worthington, M. T., and Sturgill, T. W. (2004) J. Biol. Chem. 279, 10176–10184
47. von Bernuth, H., Picard, C., Jin, Z., Pankla, R., Xiao, H., Ku, C. L., Chrabieh, M., Mustapha, I. B., Ghandil, P., Camcioglu, Y., Vasconcelos, J., Sirvent, N., Guedes, M., Vitor, A. B., Herrero-Mata, M. J., Aróstegui, J. I., Rodrigo, C., Alsina, L., Ruiz-Ortiz, E., Juan, M., Fortuny, C., Yagüe, J., Antón, J., Pascal, M., Chang, H. H., Janniere, L., Rose, Y., Garty, B. Z., Chapel, H., Issekutz, A., Maródi, L., Rodríguez-Gallego, C., Banchereau, J., Abel, L., Li, X., Chaußabel, D., Puel, A., and Casanova, J. L. (2008) Science 321, 691–696