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Recurrent *Pseudomonas aeruginosa* infections coupled with robust, damaging neutrophilic inflammation characterize the chronic lung disease cystic fibrosis (CF). The proresolving lipid mediator, 15-epi lipoxin A_4 (15-epi LXA_4), plays a critical role in limiting neutrophil activation and tissue inflammation, thus promoting the return to tissue homeostasis. Here, we show that a secreted *P. aeruginosa* epoxide hydrolase, cystic fibrosis transmembrane conductance regulator inhibitory factor (Cif), can disrupt 15-epi LXA_4 transcellular biosynthesis and function. In the airway, 15-epi LXA_4 production is stimulated by the epithelial-derived eicosanoid 14,15-epoxyeicosatrienoic acid (14,15-EET). Cif sabotages the production of 15-epi LXA_4 by rapidly hydrolyzing 14,15-EET into its cognate diol, eliminating a proresolving signal that potently suppresses IL-8–driven neutrophil transepithelial migration in vitro. Retrospective analyses of samples from patients with CF supported the translational relevance of these preclinical findings. Elevated levels of Cif in bronchoalveolar lavage fluid were correlated with lower levels of 15-epi LXA_4, increased IL-8 concentrations, and impaired lung function. Together, these findings provide structural, biochemical, and immunological evidence that the bacterial epoxide hydrolase Cif disrupts resolution pathways during bacterial lung infections. The data also suggest that Cif contributes to sustained pulmonary inflammation and associated loss of lung function in patients with CF.

Pseudomonas aeruginosa | epoxide hydrolase | inflammation | lipoxin | cystic fibrosis

Chronic pulmonary inflammation and persistent bacterial infections are pathological hallmarks of the genetic disease cystic fibrosis (CF) (1). CF is caused by mutations that impair the function of the cystic fibrosis transmembrane conductance regulator (CFTR), an ion channel that controls epithelial fluid and ion homeostasis. The resulting failure of mucociliary clearance in the CF lung allows microorganisms to repeatedly infect the respiratory tract (2). These bacterial infections incite robust inflammatory responses, dominated by elevated proinflammatory cytokines and continued accumulation of neutrophils in the CF airway (1). However, these responses are ineffective at clearing pathogenic microbes in the CF lung (3), instead creating a hyperinflammatory cycle that leads to host tissue damage, respiratory failure, transplant, or death.

Most airways of adult patients with CF are chronically infected by the opportunistic bacterial pathogen *Pseudomonas aeruginosa*, which is a major cause of morbidity and mortality. *P. aeruginosa* thrives in the hyperinflammatory CF lung, forming biofilms that are mechanically robust and resistant to clinically achievable levels of antibiotics (2). *P. aeruginosa* also persists in the airways by interfering with host defense via secreted bacterial virulence factors and small molecules (2). We recently showed that *P. aeruginosa* secretes the CFTR inhibitory factor (Cif), an epoxide hydrolase that triggers the degradation of ABC transporter family members, including CFTR (4–8). Cif transcripts have been observed in sputum from patients with CF, and longitudinal studies of clinical isolates from individual patients confirm that *P. aeruginosa* maintains Cif expression for up to 15 y (7, 9). Nonetheless, Cif's role in CF pathogenesis and the identity of possible host epoxide substrates have remained unclear.

Following a pathogenic insult, the host rapidly releases polyunsaturated fatty acids from cell membranes and converts them into various lipid mediators that either stimulate or inhibit inflammation. The correct balance of these signals is required to optimize clearance while minimizing collateral damage to host tissues, and perturbations in either direction can be deleterious (10). Among these lipid mediators, arachidonic acid-derived eicosanoids, including epoxides, play important roles. Although many eicosanoids induce

Significance

***Pseudomonas aeruginosa* pulmonary infections cause prolonged and destructive inflammation for cystic fibrosis patients. Despite vigorous neutrophilic responses, *P. aeruginosa* persists in a chronic hyperinflammatory environment. We show that the *P. aeruginosa* virulence factor, cystic fibrosis transmembrane conductance regulator inhibitory factor (Cif), promotes sustained airway inflammation by reducing host pro-resolving lipid mediators. Cif hydrolyzes epithelial-derived 14,15-epoxyeicosatrienoic acid, disrupting transcellular production of the proresolving lipid 15-epi lipoxin A_4 (15-epi LXA_4) by neutrophils. Clinical data from cystic fibrosis patients revealed that Cif abundance correlated with increased inflammation, decreased 15-epi LXA_4, and reduced pulmonary function. Our study and the recent identification of Cif homologs in *Acinetobacter* and *Burkholderia* species suggest that bacterial epoxide hydrolases represent a novel virulence strategy shared by multiple respiratory pathogens.**

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Conflict of interest statement: C.D.B., C.M., B.D.H., and D.R.M. are coinventors of patent-pending Cif inhibitor compounds. B.D.L. is an inventor on patents (resolvins) licensed by Brigham and Women's Hospital to Resolvyx Pharmaceuticals, a company that seeks to develop Resolvin therapeutics for inflammatory diseases. B.D.L. also owns equity in the company. B.D.L.'s interests were reviewed and are managed by the Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict of interest policies.

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Data deposition: For the crystallographic structure reported in this paper, atomic coordinates and structure factors have been deposited in the Protein Data Bank (PDB), www.pdb.org (PDB ID code 5JYC).

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proinflammatory cascades, recent studies have also identified immunomodulatory and proresolving functions (11, 12). In particular, lipoxins decrease neutrophil extravasation and enhance macrophage efferocytosis, thus promoting the resolution of inflammation and a return to tissue homeostasis (13, 14). In the CF lung, the concentration of lipoxin A_4 (LXA_4) is significantly reduced, suggesting that a failure to activate proresolving mechanisms contributes to excessive inflammation in the airway (15). In this study, we show that Cif selectively converted an endogenous epoxide-containing eicosanoid 14,15-epoxyeicosatrienoic acid (14,15-EET) to its corresponding diol, destroying the signal that triggers increased biosynthesis of the specialized proresolving mediator 15-epi LXA_4. The translational relevance of this unexpected biochemical virulence activity to clinical CF was investigated by retrospective analysis of bronchoalveolar lavage fluid (BALF) samples and suggested that Cif contributes to the hyperinflammatory environment of the chronically infected CF lung.

Results

Cif Specifically Hydrolyzed the Mammalian Epoxyeicosatrienoic Acid 14,15-EET. Airway epithelial cells (AECs) produce numerous lipid mediators. Via a major cytochrome P450 monooxygenase-catalyzed pathway, they convert arachidonic acid into epoxide-containing eicosanoids called EETs (Fig. S1*A*) (10). To assess the ability of Cif to hydrolyze these candidate epoxide substrates, we incubated the enzyme with each of the four mammalian EET regioisomers, 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET, and assayed for production of a vicinal diol through a colorimetric assay (5). Cif hydrolyzed 14,15-EET, but did not exhibit detectable epoxide hydrolase activity for any of the other three regioisomers (Fig. 1*A*), suggesting selective conversion of 14,15-EET by Cif to the corresponding vicinal diol 14,15-dihydroxyeicosatrienoic acid (DHET) (Fig. 1*B*). An immunoassay selective for the diol product confirmed conversion of 14,15-EET to 14,15-DHET by Cif, but not by the structurally conserved mutant Cif-D129S (Fig. S1*B*), which is enzymatically inactive (16).

Previous crystal structures show a limited steric volume for the Cif active site (5), and almost all previously reported Cif substrates are terminal epoxides, which can enter deep into the active site. In contrast, lipid mediators typically carry epoxide groups in the middle of an extended carbon chain and were thus thought unlikely to serve as Cif substrates. To investigate how the enzyme catalyzes 14,15-EET hydrolysis, we incubated the compound with the Cif-E153Q mutant, which can attack substrates with its active-site nucleophile, but cannot catalyze the secondary attack required for product release (17). We then crystallized the resulting complex for X-ray diffraction analysis. The refined structure (Table S1) showed the expected adduct of the catalytic Asp^{129} (D129) nucleophile with the 20-carbon EET chain attached at the C^{15} moiety (Fig. 1*C* and Fig. S2). Unexpectedly, it also showed that the active site could expand at both ends, opening a tunnel through the enzyme to accommodate the extended eicosanoid substrate (Fig. 1*D*). The strong preference for 14,15-EET presumably reflects the shorter distance in the other regioisomers between the epoxide moiety and the terminal carboxylate, which would thus have to be unfavorably sequestered within the active site during hydrolysis.

***P. aeruginosa* Can Trigger Hydrolysis of Epithelial 14,15-EET Produced in Response to Inflammation.** To determine whether Cif's regioselectivity matches the biological epoxides present in the airway, we first tested whether polarized immortalized CF AECs (CFBE41o-, hereafter called CFBE cells) produce 14,15-EET in response to inflammatory signals. We treated CFBE cells with tumor necrosis factor-α (TNFα) (18) and performed lipid extractions on apical supernatants. Eicosanoid levels were determined in parallel by immunoassay (Fig. 2) and by mass spectrometry (Fig. S3*A*). Supernatants from CFBE cells exposed to TNFα showed a substantial increase in 14,15-EET levels, compared with untreated cells (Fig. 2*A* and Fig. S3*A*).

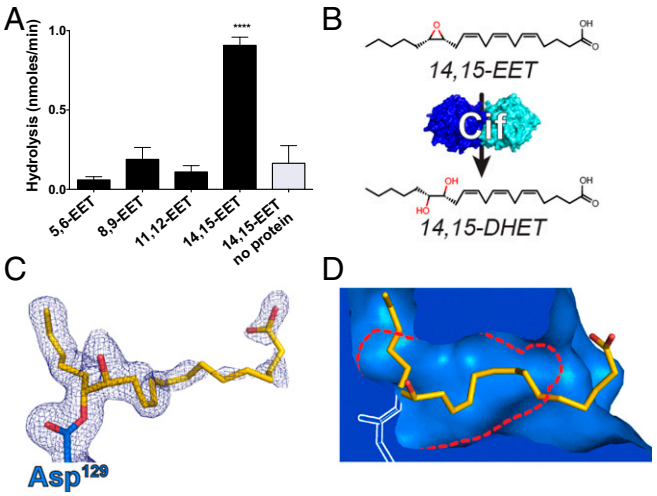

Fig. 1. The *P. aeruginosa* enzyme Cif hydrolyzes the epoxyeicosatrienoic acid 14,15-EET. (*A*) Incubation of recombinant Cif with each of the EET regioisomers demonstrates its ability to selectively hydrolyze 14,15-EET (black bars). ****$P <$ 0.0001 for 14,15-EET compared with all other experimental conditions, one-way ANOVA with Tukey's post hoc test; $n \geq 3$; mean ± SD. (*B*) Cif hydrolyzes the epoxide moiety of 14,15-EET, converting it to the vicinal diol 14,15-DHET. (*C*) Experimental electron density ($2mF_O$-DF_C; blue mesh, contoured at 1σ) shows the refined position of the covalent enzyme–substrate intermediate formed by nucleophilic attack of Asp^{129} (D129; blue carbons; chain D) on 14,15-EET (yellow carbons). (*D*) In the covalently linked structure, several residues shift, opening a tunnel through the active site (blue surface area representation), allowing for accommodation of the 14,15-EET (yellow carbons) adduct. The active site of the apo enzyme (PDB ID 3KD2) does not contain this tunnel. Its boundaries are shown as red dotted lines. Noncarbon atoms are colored by atom type (N, blue; O, red).

Furthermore, when CFBE cells were exposed to TNFα in the presence of purified Cif-WT protein, the apical level of 14,15-EET was reduced to that of unstimulated controls, whereas the catalytically inactive Cif-D129S protein had no significant impact on 14,15-EET levels (Fig. 2*A*). We next sought to determine whether *P. aeruginosa* could use Cif activity to hydrolyze CFBE-derived 14,15-EET. We first showed that presence of 14,15-EET did not affect the growth rate of *P. aeruginosa* strain PA14, which expresses Cif (Fig. S4). CFBE cells were treated with TNFα and exposed to strain PA14 or to the corresponding *cif* deletion strain PA14Δ*cif* (7). Apical supernatant concentrations of 14,15-EET were significantly reduced in the presence of the Cif-producing strain PA14, compared with the TNFα-only control (Fig. 2*B*). Similar results were observed for well-differentiated primary CF human bronchial epithelial cells (CF HBEs) (Fig. 2*C*) and also for the non-CF Calu3 cell line (Fig. S3*B*). In each case, PA14Δ*cif* had no statistically significant effect on the 14,15-EET levels. The Cif effect is not associated with differences in overall *P. aeruginosa* CF AEC cytotoxicity. Both PA14 and PA14Δ*cif* showed minimal cytotoxicity at the 5-h time point used in our experiments (Fig. S3*C*). Interestingly, we detected comparable levels of 14,15-EET secreted by CF and non-CF AECs following TNFα treatment, suggesting that both CF and non-CF epithelial cells were similarly capable of secreting 14,15-EET in response to an inflammatory stimulus. Taken together, our data demonstrate that Cif enables *P. aeruginosa* to promote the hydrolysis of 14,15-EET secreted by AECs and that Cif is necessary for efficient hydrolysis.

Cif-Mediated 14,15-EET Hydrolysis Reduced 15-epi LXA_4 Production and Disinhibited Transepithelial Migration. AEC-derived 14,15-EET acts as a transcellular signal and stimulates neutrophil generation of the proresolving mediator 15-epi LXA_4, whereas other EET regioisomers do not (18, 19). Thus, we next examined whether Cif-mediated hydrolysis of 14,15-EET impacts the transcellular

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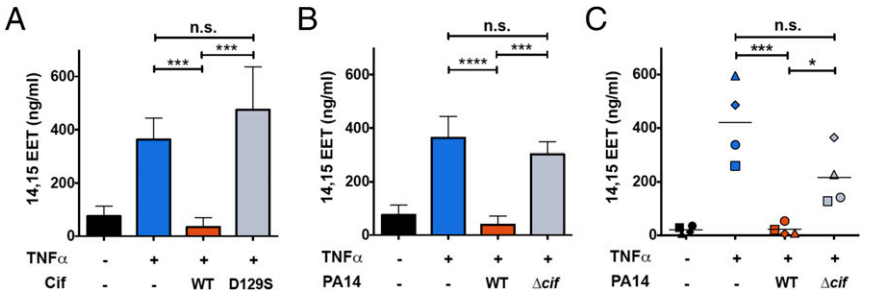

Fig. 2. Cif hydrolyzes 14,15-EET derived from CF AECs. (*A–C*) Treatment of polarized CF AECs with TNFα (1 ng/mL) for 24 h stimulates increased apical secretion of 14,15-EET (blue bars or symbols) compared with untreated cells (black bars or symbols). (*A*) Recombinant Cif protein hydrolyzes 14,15-EET produced by TNFα-treated CFBE cells. CFBE cells were apically treated with either recombinant Cif-WT or Cif-D129S (1 μM) for 45 min (red or gray bars, respectively). Apical secretions were collected in cold MeOH, solid-phase lipid extracted, and analyzed for 14,15-EET concentrations by ELISA. ***$P < 0.001$; one-way ANOVA with Tukey's post hoc test; $n \geq 3$; mean ± SD. (*B*) Cif secreted by *P. aeruginosa* hydrolyzes 14,15-EET produced by TNFα-treated CFBE cells. CFBE cells were inoculated apically with either PA14 or deletion mutant PA14Δ*cif* (MOI = 25) for 5 h (red bars and gray bars, respectively) and analyzed for 14,15-EET concentrations by ELISA. ***$P < 0.001$ and ****$P < 0.0001$; one-way ANOVA with Tukey's post hoc test; $n \geq 3$; mean ± SD. (*C*) *P. aeruginosa* hydrolyzes 14,15-EET produced by primary CF airway epithelial cells. Primary CF HBEs were apically exposed to either PA14 or deletion mutant PA14Δ*cif* (MOI = 25) for 5 h (red and gray symbols). Apical secretions were collected and analyzed for 14,15-EET concentrations by ELISA. Each symbol represents one individual primary donor. *$P < 0.05$ and ***$P < 0.001$; one-way ANOVA with Tukey's post hoc test; $n \geq 3$; mean ± SD.

biosynthesis of 15-epi LXA_4. Neutrophils obtained from healthy donors or subjects with CF were exposed to 14,15-EET alone or in combination with either Cif-WT or Cif-D129S. Following incubation, lipids were extracted from supernatants and quantified by ELISA for 15-epi LXA_4. As expected, neutrophils generated 15-epi LXA_4 when incubated with 14,15-EET, and 15-epi LXA_4 levels were significantly reduced in the presence of Cif-WT (Fig. 3*A*). Similar results were observed for neutrophils from donors with CF (Fig. S5*B*). In a neutrophil–CFBE coculture model, CFBE cells were treated with TNFα to induce the production of 14,15-EET and then incubated with neutrophils. Apical supernatants from the neutrophil–CFBE coculture showed robust production of 15-epi LXA_4 following TNFα stimulation. The addition of Cif to the neutrophil–CFBE coculture substantially reduced transcellular generation of 15-epi LXA_4 to near baseline levels (Fig. 3*B*). The 15-epi LXA_4 is the 15(R) epimer of LXA_4, yet these epimers have distinct biosynthetic pathways (11). As a control, we also used the coculture system to assess transcellular generation of LXA_4. As expected, Cif had no significant effect on LXA_4 levels (Fig. S5*A*), but instead appeared to be specific for the 15-epi LXA_4 biosynthetic pathway.

The 15-epi LXA_4 is a potent inhibitor of neutrophil transepithelial migration (14), so we next modified our coculture system to examine whether Cif affects neutrophil transepithelial migration. Polarized CFBE cell monolayers were grown on the bottom of Transwell permeable membrane supports to physiologically model neutrophil transepithelial migration in the basolateral-to-apical direction. The neutrophil chemoattractant IL-8 was added in the apical compartment to drive neutrophil migration (20). Exposure of neutrophils to 14,15-EET (100 nM, 45 min) or to 15-epi LXA_4 (100 nM, 15 min) significantly decreased transepithelial migration by 74% and 84%, respectively (Fig. 3*C*). Cif-WT, but not Cif-D129S, significantly abrogated the inhibition of neutrophil transepithelial

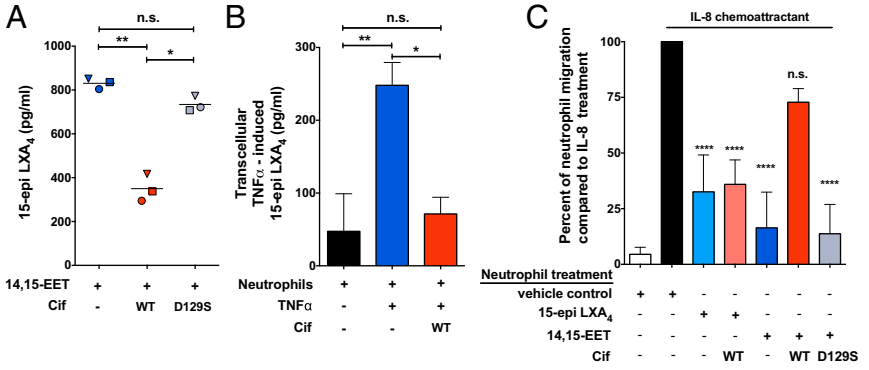

Fig. 3. Cif-mediated 14,15-EET hydrolysis suppresses neutrophil-derived 15-epi LXA_4 and restores neutrophil transepithelial migration. (*A*) Neutrophil generation of 15-epi LXA_4 in the presence of 14,15-EET (blue symbols) is reduced in the presence of recombinant Cif-WT (red symbols) but not by the catalytic mutant Cif-D129S (gray symbols). Freshly isolated human neutrophils from healthy donors (10^6) were incubated with 14,15-EET (1 μM) in the presence of either Cif-WT (1 μM) or Cif-D129S (1 μM). The reactions were stopped with cold MeOH, lipid extracted, and analyzed for 15-epi LXA_4 concentrations by ELISA. *$P < 0.05$, **$P < 0.01$; one-way ANOVA with Tukey's post hoc test; $n \geq 3$; mean. (*B*) Cif hydrolysis of 14,15-EET suppresses the production of 15-epi LXA_4 in CFBE–neutrophil coculture. Polarized CFBEs stimulated with TNFα (1 ng/mL) for 24 h (blue bar) were treated apically with Cif-WT (1 μM) for 45 min (red bar), before the apical addition of 1.5×10^6 of freshly isolated neutrophils for an additional 15 min. The apical supernatants from the cocultures were collected and analyzed for 15-epi LXA_4 by ELISA. Data are normalized to CFBEs treated with TNFα. *$P < 0.05$, **$P < 0.01$; one-way ANOVA with Tukey's post hoc test; $n \geq 3$; mean ± SD. (*C*) Cif hydrolysis of 14,15-EET abrogates the reduction of neutrophil transepithelial migration mediated by 15-epi LXA_4. Freshly isolated human neutrophils (5×10^5) were calcein-AM loaded and treated either with 15-epi LXA_4 alone (light blue bar) or in the presence of Cif-WT (light red bar) or with 14,15-EET alone (blue bar) or in the presence of Cif-WT (red bar) or Cif-D129S (gray bar) for 15–45 min, as noted in *Materials and Methods*. The treated neutrophils were applied to the basolateral side of polarized CFBEs following the apical addition of the chemoattractant IL-8 (black bar, IL-8 positive control; white bar, no IL-8 negative control). After 2 h, calcein-AM fluorescence was measured in the apical compartment to assess neutrophil transepithelial migration. Results are shown for the comparison of each experimental condition to the migration measured in the IL-8 positive control condition. ****$P < 0.0001$; n.s., not significant; one-way ANOVA with Tukey's post hoc test; $n \geq 4$; mean ± SD.

migration mediated by the addition of 14,15-EET (Fig. 3*C*). However, Cif-WT had no effect on migration when added in the presence of 15-epi LXA_4, consistent with our proposal that its inhibitory effect on 15-epi LXA_4 is not direct, but instead mediated by hydrolysis of 14,15-EET. These data demonstrate that Cif can disrupt 15-epi LXA_4 regulation of neutrophil transepithelial migration in the presence of IL-8.

Cif Expression in Patients with CF Is Widespread and Correlates with Elevated IL-8, Loss of 15-epi LXA_4, and Reduced Pulmonary Function. To assess the extent of patient exposure to Cif, we tested serum samples from a collection of adult donors with CF and donors without CF for the presence of Cif-specific antibodies by ELISA (Table S2 and Fig. 4*A*). Patients with CF uniformly exhibited high levels of α-Cif antibodies, suggesting widespread exposure to Cif protein during the natural history of disease. Based on our in vitro preclinical data (Figs. 1–3), we hypothesized that Cif levels in patients with CF would be positively correlated with lung inflammation and inversely correlated with BALF levels of 15-epi LXA_4 and with several measures of lung function. To test these hypotheses, we obtained BALF from a random cross-sectional cohort of pediatric subjects with CF who had undergone bronchoscopy (Table S3). Using newly developed Cif-specific antisera (Fig. S6), we quantified Cif abundance in BALF samples that tested positive for the presence of *P. aeruginosa* lipopolysaccharide. We also quantified the levels of IL-8 and 15-epi LXA_4 in these samples by ELISA. A Pearson cross-correlation matrix of available data demonstrated patterns clearly consistent with our predictions (Fig. 4*B*). Pulmonary function tests (Fig. 4*B*, solid outline) showed strong positive correlations with each other, consistent with CF airway disease. The 15-epi LXA_4 levels positively correlated with lung function (Fig. 4*B*, dashed outline), consistent with the idea that proresolution signals are protective against inflammatory lung damage in CF. As predicted, Cif inversely correlated with both 15-epi LXA_4 and pulmonary function measures (Fig. 4*B*, dotted outline), and IL-8 correlated inversely with 15-epi LXA4 and positively with Cif (Fig. 4*B*, red line). To investigate these patterns in more detail, we divided patients into "Cif low" and "Cif high" groups (Fig. 4*C*), using 10 ng Cif per milligram of total protein to delineate groups. Individual analyses confirmed significantly reduced levels of 15-epi LXA_4 and poor FEV_1/FVC, as well as elevated levels of IL-8, for patients with higher Cif (Fig. 4 *D*, *F*, and *G*). IL-8 and 15-epi LXA_4 are strongly inversely correlated (Fig. 4*E*). Additionally, in the high-Cif patient cohort FEV_1 values were reduced compared with the low-Cif group (Fig. S7*A*). Overall, among subjects with *P. aeruginosa* infections, higher Cif levels are associated with worse obstructive lung disease.

Discussion

A major hallmark of the disease cystic fibrosis is vigorous and persistent pulmonary inflammation, which damages host lung tissue, eventually causing respiratory failure and death. Chronic *P. aeruginosa* infections often accompany robust airway inflammatory processes, provoking continuous immune cell infiltration into the lung. In the current study, we identified the epithelial-derived eicosanoid 14,15-EET as an endogenous substrate for the *P. aeruginosa* virulence factor Cif. We also showed that the Cif-mediated reduction of 14,15-EET disrupted paracrine signaling to neutrophils to produce 15-epi LXA_4, blocking its proresolving functions (Fig. S8). Consistent with the Cif-mediated effect of blocking the generation of proresolving mediators, we demonstrated that higher Cif levels in the bronchoalveolar lavage fluid of patients with CF correlated with reduced 15-epi LXA_4, increased inflammatory marker IL-8, and reduced pulmonary function. Taken together, our results provide evidence for an unanticipated role of the bacterial epoxide hydrolase Cif in obstructing normal resolution pathways in the airway and promoting pulmonary inflammation in patients with CF colonized with *P. aeruginosa*.

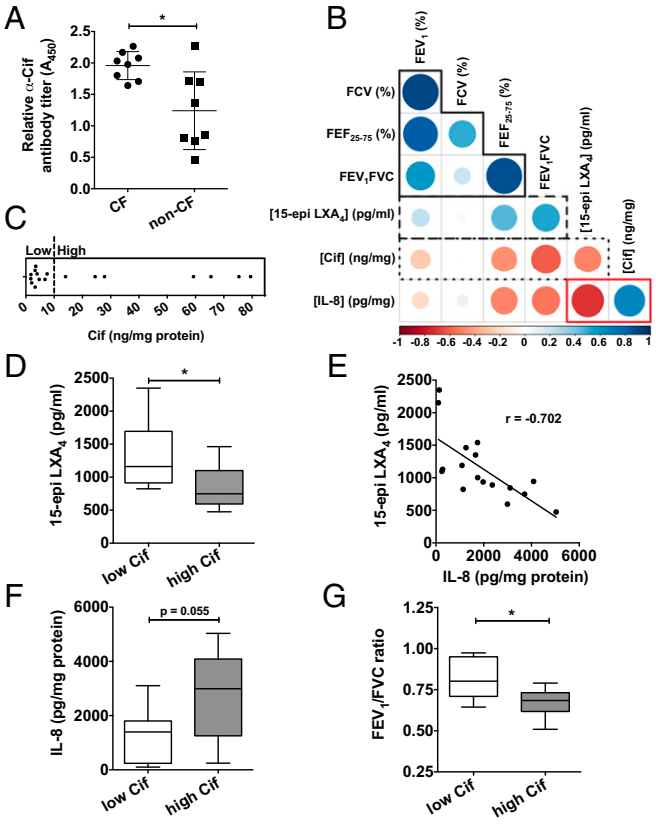

Fig. 4. Elevated levels of Cif in CF patient bronchial lavage fluid result in lower 15-epi LXA_4 and higher IL-8 concentrations and worsening pulmonary function. (*A*) Anti-Cif serum antibodies are generated in adult patients with CF. Sera collected from a CF and a non-CF cohort were probed for anti-Cif antibodies via ELISA. Patients with CF have significantly increased levels of anti-Cif serum antibodies compared with a non-CF cohort. $*P \leq 0.05$, Wilcoxon rank-sum test. (*B*–*G*) BALF was obtained from pediatric patients with CF who received bronchoscopy. $n = 17$. (*B*) A visualization of a Pearson correlation matrix depicts positive correlations in blue and negative correlations in red. Color intensity and the size of the circle are proportional to the correlation coefficients. Below the correlogram, the scale shows the correlation coefficients and the corresponding colors. (*C*) Cif expression in BALF from patients with CF was determined by Western blot analysis and binned into "low" and "high" categories. (*D*) Concentrations of 15-epi LXA_4 are significantly reduced in patients with CF with elevated levels of Cif. CF BALF samples were lipid extracted and analyzed for 15-epi LXA_4 concentrations by ELISA. $*P < 0.05$, Wilcoxon rank-sum test. (*E*) Increased IL-8 concentrations strongly correlate with lower 15-epi LXA_4 concentrations in BALF from patients with CF. $P = 0.0016$, Pearson correlation. (*F*) Patients with CF with elevated Cif have increased IL-8 concentrations in the airways. IL-8 concentrations in CF BALF were determined by sandwich ELISA and normalized to total protein in each sample. $P = 0.055$, Wilcoxon rank-sum test. (*G*) Patients with CF with high Cif have worse lung obstruction. Patient FEV_1/FVC ratio was measured at time of bronchoscopy, stratified by high- or low-Cif groups. $*P < 0.05$, Wilcoxon rank-sum test.

In contrast to the proinflammatory effects of Cif, several pathogens manipulate lipid mediators to suppress the immune response and evade detection. *Mycobacterium tuberculosis* and *Toxoplasma gondii* both enhance the generation of lipoxins (21, 22), whereas the fungal pathogen *Candida albicans* synthesizes resolvin E1 (23). *M. tuberculosis* can also inhibit the formation of the proinflammatory lipid LTB_4, further shifting the host lipid environment toward an antiinflammatory state (24). *P. aeruginosa* produces the 15-lipoxygenase LoxA (25, 26), as well as the phospholipase ExoU (27), both of which can potentially generate antiinflammatory signals. However, unlike many airway pathogens, *P. aeruginosa* survives in a hyperinflammatory environment, particularly in the context of chronic lung disease (2). Consistent with the notion that *P. aeruginosa*

actively manipulates the inflammatory environment in the airway, other studies have demonstrated that i.v. antibiotic treatments increase LXA_4 levels, while decreasing proinflammatory chemokine concentrations in BALF of patients with CF (28). As a result, *P. aeruginosa* may be uniquely poised to exploit both pro- and antiinflammatory strategies to thwart host defense mechanisms.

It was not expected that 14,15-EET could serve as a host substrate for *P. aeruginosa* Cif. Previous crystallization studies identified Cif as a member of the α/β-hydrolase family of proteins, with epoxide hydrolase activity focused on small, monosubstituted substrates (29). Thus, it was surprising to discover that Cif could accommodate the extended carbon chain of an eicosanoid such as 14,15-EET. Our crystal structure revealed an unexpected rearrangement of specific residues that define the boundaries of the active site. Small-molecule inhibitors were recently reported to drive conformational changes in an epoxide hydrolase active site (30), but our study represents an example of a substrate-associated shift in this class of enzymes. Furthermore, whereas our work shows that Cif cannot hydrolyze the other EET regioisomers, its active-site flexibility may enable it to hydrolyze other members of the broad network of regulatory lipids (31).

Among its multiple antiinflammatory functions (32, 33), 14,15-EET plays a particularly critical role as a paracrine stimulus for the production of the proresolving lipid 15-epi LXA_4 by neutrophils (19). In addition to enzymatically targeting 14,15-EET, our study also demonstrated that Cif indirectly reduced levels of the proresolving lipid mediator 15-epi LXA_4. Previous studies have shown that CF airways exhibit substantially reduced levels of its epimer LXA_4 (15), although the infection status of the patients was not reported. Our data provide evidence that the capacity to generate 15-epi LXA_4 is fundamentally intact in the context of CF, yet susceptible to Cif-mediated reductions when *P. aeruginosa* is present in the CF airway. Specifically, the production of 14,15-EET and the associated modulation of neutrophil behavior were both observed with primary cells derived from patients with CF. Consistent with this proposal, elevated levels of Cif in BALF from patients with CF correlated with reduced concentrations of 15-epi LXA_4, increased levels of the inflammatory cytokine IL-8, and worsened pulmonary function. Our study suggests that by inhibiting normal host resolution programs, the secreted *P. aeruginosa* virulence factor Cif contributes to the characteristic hyperinflammatory environment of the CF airway.

These results have important implications for our understanding of CF disease pathogenesis. The targeting of the proresolution 14,15-EET/15-epi LXA_4 axis represents an unexpected bacterial virulence strategy. As is demonstrated by decades-long chronic infections in the inflamed lungs of patients with CF, *P. aeruginosa* thrives in a hyperinflammatory environment (34). Longitudinal clinical isolates of *P. aeruginosa* collected over a decade express *cif* (35) and our survey of serum titers from a group of adult subjects with CF shows that all had generated an immune response to Cif protein, suggesting that it is widely present in CF airway infections. Because our results show that Cif enables *P. aeruginosa* to manipulate the host inflammatory environment, it may play a significant role in defining the nature of these ongoing infections, which ultimately lead to lung tissue damage and respiratory failure. Cif homologs have been identified in other airway pathogens, including *Acinetobacter nosocomialis* and *Burkholderia cepacia* (4, 36). As a result, these bacterial epoxide hydrolases may represent a distinct class of therapeutic targets in CF and other airway diseases in which hyperinflammatory responses lead to accelerated tissue damage.

Our observations also suggest additional therapeutic approaches, as increased Cif levels correlated with more severe obstructive lung disease, as measured by FEV_1/FVC ratios, in patients with CF. As increasing FEV_1/FVC ratio remains a key therapeutic objective of clinical therapy, one strategy may be direct replacement of the absent proresolving lipid mediator. Multiple studies have demonstrated that exogenous administration of specialized proresolving mediators improves morbidity and mortality outcomes following infection (11). In particular, the administration of a LXA_4 analog in mice challenged intratracheally with *P. aeruginosa* led to reduced neutrophil infiltration, weight loss, and bacterial burden, resulting in overall lessening of disease severity (15). An alternative strategy may be to inhibit Cif activity with a targeted small-molecule approach (16, 37). In addition to blocking the enzymatic degradation of lipid mediators, inhibitors would also block the ability of Cif to subvert rescue of CFTR by recently approved clinical correctors (38). Finally, because Cif represents a key link between chronic infections and the damaging, hyperinflammatory environment present in the CF airway, it may serve as a valuable biomarker of airway disease and treatment options in CF.

Materials and Methods

Protein Expression and in Vitro Hydrolysis Assay. Wild-type Cif protein (Cif-WT) and Cif-D129S and Cif-E153Q mutants were expressed as described previously (16, 17, 29). Hydrolysis of the epoxyeicosatrienoic acids by Cif was measured using a modified adrenochrome assay (36, 39).

Crystallographic Structure of Cif with 14,15-EET. Cif-E153Q protein incubated with 14,15-EET was crystallized, as described previously (17, 29). Oscillation data were collected and were processed and scaled with the XDS package (v. December 6, 2010). Phases were calculated by molecular replacement with the Cif-WT structure [Protein Data Bank (PDB) ID 3KD2] in the Phenix suite (v. 1.10.2055) (40–42). Standard iterative refinement was performed with Phenix and WinCoot (v. 0.7), yielding models for two dimers in the asymmetric unit. The ligand was added after the second round, before the addition of water. We deposited coordinates to the PDB (PDB ID 5JYC) and used Pymol (www.pymol.org) to render images of chain D.

Epithelial Cell Culture and Bacterial Strains. The immortalized human CF bronchial epithelial cell line CFBE41o– (referred to here as CFBE cells) was seeded on 0.4-μM polyester Transwell filters (Corning) coated with collagen and fibronectin and grown at air–liquid interface for at least 7–10 d, as previously described (43). Primary human CF AECs (CF HBEs) acquired from the University of Pittsburgh Airway Cell and Tissue Core were cultured from explanted lungs of patients with CF, using an Institutional Review Board approved protocol at the University of Pittsburgh (IRB no. 11070367) (44). *P. aeruginosa* strains PA14 and PA14Δ*cif* were gifts from George O'Toole, Geisel School of Medicine at Dartmouth (7, 45).

Quantification of 14,15-EET. Polarized CFBE cells were treated with TNFα (R&D Systems) for 24 h prior to treatment with either Cif-WT or PA14 [multiplicity of infection (MOI) = 25]. Apical supernatants were collected in methanol, layered with N_2, and solid-phase lipid extracted using C18 Sep-Pak cartridges (Waters) as previously described (19, 46). The 14,15-EET concentrations were quantified by ELISA (Detroit R&D) using prostaglandin B2 (100 ng) as an internal standard and monitored by HPLC to control for variances in extraction recovery. The 14,15-EET concentrations in apical supernatants were confirmed by liquid chromatography mass spectrometry, using previously described techniques (47, 48).

Neutrophil Isolation, 15-epi LXA_4 Quantification, and Transepithelial Migration. Neutrophils were obtained by venipuncture from volunteers who had given written informed consent according to a protocol approved by the University of Pittsburgh IRB Committee (IRB no. 14070447). Neutrophils isolated as previously described (49) were incubated with 14,15-EET (Cayman Chemical). In some conditions 14,15-EET was previously incubated with Cif-WT or Cif-D129S before neutrophil treatment. All reactions were collected in methanol and solid-phase lipid extracted, and 15-epi LXA_4 concentrations were determined by ELISA (Neogen) (50). For neutrophil–AEC coculture experiments, CFBE cells were treated with TNFα for 24 h and exposed to Cif before the addition of neutrophils. For transepithelial neutrophil migration, CFBE cells were seeded on inverted 3-μM pore polycarbonate Transwell filters (51). Calcein AM (Life Technologies)-labeled neutrophils were treated with 15-epi LXA_4 or 14,15-EET in the presence of Cif-WT or Cif-D129S and applied to the basolateral side of CFBE cells. IL-8 (100 pg/mL) applied to the apical compartment stimulated neutrophil transepithelial migration (20), which was measured by Calcein AM fluorescence following a 2-h incubation.

Antibody Detection in Human Sera. Deidentified human serum samples were obtained from the Translational Research Core at Geisel School of Medicine, in accordance with the approved IRB protocol (IRB no. 22781). An indirect ELISA, with purified Cif-WT protein as bait, was developed to detect human antibodies against Cif from a 1:5,000 dilution of the serum.

Analysis of BALF from Subjects with CF. BALF was collected prospectively over time in a pediatric population followed for management of CF at Children's Hospital of Pittsburgh, in accordance with a protocol approved by the University of Pittsburgh IRB Committee (IRB no. 504067). ELISAs were used to determine 15-epi LXA_4 (Neogen) and IL-8 (R&D Systems) concentrations in BALF of patients with CF. Cif protein quantification was determined by Western blot analysis detected by Cif-specific rabbit antisera (Cocalico Biologicals). Cif and IL-8 values were normalized and expressed per milligram of total BALF protein. Pulmonary function testing (PFT) was performed on all patients as part of their CF management and the most recent PFT data in relation to the bronchoscopy were collected.

Statistics. Experimental differences for in vitro assays were evaluated for statistical significance by one-way analysis of variance (ANOVA) with a Tukey's post hoc test (GraphPad 6.0). For the human studies, a Pearson correlation matrix was created using the cor package in R from measurements of Cif, 15-epi LXA_4, IL8, and spirometry values for the 16 patients for whom a complete set of values was available. The corrplot package in R was used to visualize the results. Significance was determined using the nonparametric Wilcoxon rank-sum test (GraphPad 6.0) for serum antibody titers and differences between high and low Cif cohorts.

Additional methods can be found in *SI Materials and Methods*.

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