Inflammatory cytokines TNF-α and IL-17 enhance the efficacy of cystic fibrosis transmembrane conductance regulator modulators

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Without cystic fibrosis transmembrane conductance regulator–mediated (CFTR-mediated) HCO_3^- secretion, airway epithelia of newborns with cystic fibrosis (CF) produce an abnormally acidic airway surface liquid (ASL), and the decreased pH impairs respiratory host defenses. However, within a few months of birth, ASL pH increases to match that in non-CF airways. Although the physiological basis for the increase is unknown, this time course matches the development of inflammation in CF airways. To learn whether inflammation alters CF ASL pH, we treated CF epithelia with TNF-α and IL-17 (TNF-α+IL-17), 2 inflammatory cytokines that are elevated in CF airways. TNF-α+IL-17 markedly increased ASL pH by upregulating pendrin, an apical Cl^-/HCO_3^- exchanger. Moreover, when CF epithelia were exposed to TNF-α+IL-17, clinically approved CFTR modulators further alkalinized ASL pH. As predicted by these results, in vivo data revealed a positive correlation between airway inflammation and CFTR modulator–induced improvement in lung function. These findings suggest that inflammation is a key regulator of HCO_3^- secretion in CF airways. Thus, they explain earlier observations that ASL pH increases after birth and indicate that, for similar levels of inflammation, the pH of CF ASL is abnormally acidic. These results also suggest that a non-cell-autonomous mechanism, airway inflammation, is an important determinant of the response to CFTR modulators.

Introduction

Cystic fibrosis (CF) is the most common life-shortening inherited disorder among White individuals (1). CF is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR), an apical membrane anion channel in human airway epithelia (2). The physiologically relevant CFTR-permeable anions are Cl^- and HCO_3^- (3, 4). Cl^- secretion drives transepithelial water movement and regulates the volume of the airway surface liquid (ASL). HCO_3^- secretion, on the other hand, controls the acid-base balance of the ASL. Loss of CFTR-mediated HCO_3^- secretion leaves H^+ secretion unbalanced and lowers the pH of the ASL (pH_{ASL}), impairing at least two respiratory host defenses (5–9). The abnormally decreased pH_{ASL} alters the biophysical properties of mucus and thus disrupts mucociliary transport. The acidic pH_{ASL} also impairs antimicrobial factor activity against inhaled pathogens. These host defense defects manifest clinically as chronic progressive airway disease with frequent exacerbations and reduced survival (10). The abnormal acidification of the ASL thus links the molecular defect in CF with the clinical disease phenotype (11–13).

In vivo studies in humans and pigs have shown that CF pH_{ASL} is abnormally acidic soon after birth (5, 14, 15). This abnormality is expected given the loss of CFTR-mediated HCO_3^- secretion. However, a few months after birth, pH_{ASL} in individuals with CF increases to match the pH_{ASL} in non-CF individuals (14–16). This latter observation is puzzling because CFTR activity does not return with time. We considered that inflammation might increase CF pH_{ASL} with time. CF airways lack inflammation in the newborn period but quickly develop inflammation within weeks to months after birth (17–19). Previous reports suggest that inflammatory cytokines increase HCO_3^- secretion (20, 21) and that raising pH_{ASL} may enhance host defense (22).

To assess the relevance of inflammation to CF pH_{ASL} regulation, we considered two main hypotheses. The first of these is that inflammatory cytokines will increase pH_{ASL} in CF epithelia. To test this hypothesis, we treated primary cultures of differentiated human CF airway epithelia with a combination of TNF-α and IL-17 (TNF-α+IL-17). These cytokines are key drivers of neutrophilic inflammation (23–25), a hallmark of CF airway disease, and their levels are elevated in vivo (26–29). Studies in non-CF epithelia indicate that TNF-α+IL-17 alkalinize pH_{ASL}, and increases in both CFTR and pendrin were responsible (30). We

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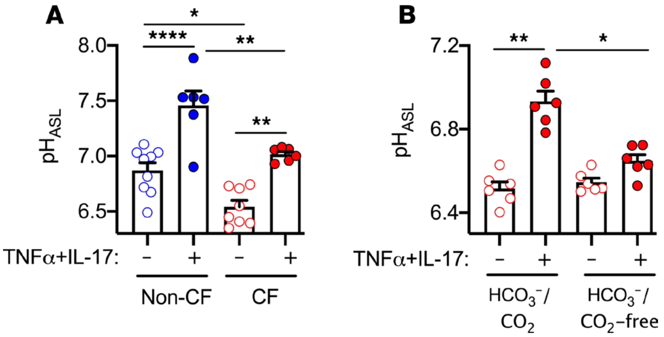

Figure 1. CF ASL is abnormally acidic at baseline but alkalinizes with TNF-α+IL-17. Primary cultures of differentiated human airway epithelia were derived from multiple donors. pH$_{\text{ASL}}$ was measured using SNARF-1-conjugated dextran on an inverted confocal microscope. (**A**) Non-CF (blue) versus CF (red) pH$_{\text{ASL}}$ at baseline and after treatment with TNF-α (10 ng/ml) and IL-17 (20 ng/ml) for 48 hours (n = 6–9). (**B**) CF epithelia treated with TNF-α+IL-17 for 24 hours. pH$_{\text{ASL}}$ was measured in the presence of HCO$_3^-$/CO$_2$ as well as after removing CO$_2$ from the environment and replacing HCO$_3^-$ with HEPES (n = 6). Each data point represents an epithelium from a different donor. Data are shown as the mean ± SEM. Statistical significance was tested using ANOVA with post test Tukey's. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$.

predicted that, even in the absence of CFTR, an increase in pendrin would increase CF pH$_{\text{ASL}}$.

The second considered hypothesis is that restoring CFTR function in inflamed CF epithelia will further increase pH$_{\text{ASL}}$. This hypothesis is in part based on the notion that directly comparing pH$_{\text{ASL}}$ in inflamed CF epithelia to pH$_{\text{ASL}}$ in noninflamed, non-CF epithelia may not be appropriate; that is, two variables are involved: the presence or absence of CFTR and the presence or absence of inflammation. Finding that pH$_{\text{ASL}}$ further alkalinizes when CFTR activity increases would be consistent with the observation that both CFTR and non-CFTR HCO$_3^-$ secretion mechanisms, perhaps pendrin, contribute to the increased pH$_{\text{ASL}}$. The availability of FDA-approved drugs that modify CFTR function (CFTR modulators) allowed us to test this hypothesis. CFTR modulators include ivacaftor, which increases CFTR open-state probability and can be used to increase function of CFTR-G551D and CFTR-R117H, and a triple drug combination that includes elexacaftor and tezacaftor, which enhance CFTR-ΔF508 processing, and ivacaftor to increase the open-state probability of the channels that reach the cell surface (31, 32).

Results

TNF-α+IL-17 alkalinize CF ASL. Because TNF-α+IL-17 altered ion transport in non-CF epithelia (30), we investigated their effect on pH$_{\text{ASL}}$ in CF. We used the pH-sensitive indicator SNARF-1 conjugated to 70 kD dextran and measured pH$_{\text{ASL}}$ under physiologic conditions (25 mM HCO$_3^-$/ 5% CO$_2$ and 37°C). Consistent with our earlier observations (30), applying TNF-α (10 ng/ml) and IL-17 (20 ng/ml) for 48 hours increased pH$_{\text{ASL}}$ of non-CF epithelia (Figure 1A). As previously reported (11), pH$_{\text{ASL}}$ was lower in CF epithelia. However, TNF-α+IL-17 increased CF pH$_{\text{ASL}}$, albeit to a lower level than in non-CF epithelia. Substituting HEPES for HCO$_3^-$/CO$_2$ prevented CF ASL alkalinization (Figure 1B). These results indicate that CF pH$_{\text{ASL}}$ is relatively acidic under basal conditions but alkalinizes with TNF-α+IL-17 by increasing HCO$_3^-$ secretion. They also suggest that for similar exposures to TNF-α+IL-17, CF ASL remains abnormally acidic.

Combined TNF-α+IL-17 increase CF pH$_{\text{ASL}}$ in a time-dependent, durable, and reversible manner. We focused on the response to TNF-α+IL-17 because both cytokines are elevated in CF airways (26, 28). Individually, TNF-α and IL-17 applied for 24 hours, increased CF pH$_{\text{ASL}}$, albeit modestly compared with the combination TNF-α+IL-17 (Figure 2A). This result is consistent with previous reports of TNF-α and IL-17 synergy (26). We also tested the effect of acute versus chronic TNF-α+IL-17. With acute treatment, CF pH$_{\text{ASL}}$ did not change at 4 hours, but markedly increased at 24 hours (Figure 2B). With chronic TNF-α+IL-17, pH$_{\text{ASL}}$ peaked by day 2 and persisted up to day 7 (Figure 2C). Because cytokines can produce lasting

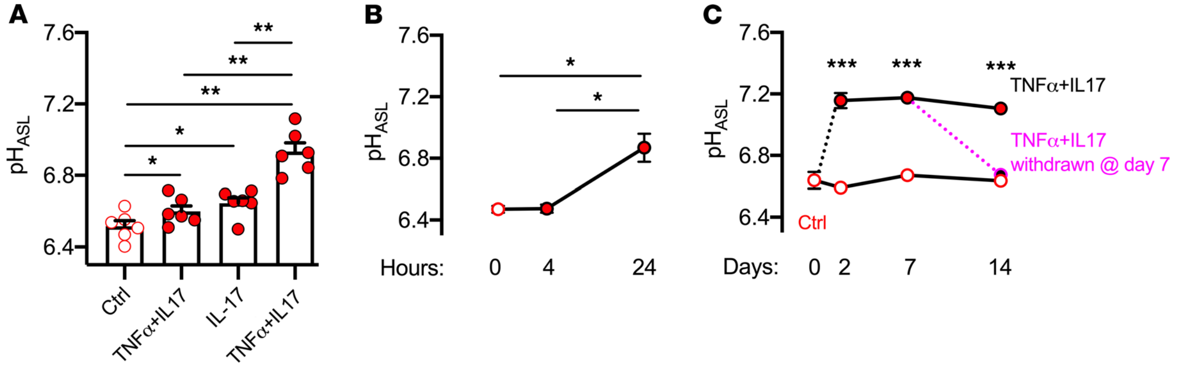

Figure 2. TNF-α+IL-17 combined increase CF pH$_{\text{ASL}}$ in a time-dependent, durable, and reversible manner. (**A**) Human CF airway epithelia were treated with TNF-α (10 ng/ml), IL-17 (20 ng/ml), or both for 24 hours, and pH$_{\text{ASL}}$ measured using SNARF-1-dextran (n = 6). (**B** and **C**) Time-dependent changes in CF pH$_{\text{ASL}}$ after exposure to combined TNF-α+IL-17 (n = 5). Each data point represents epithelium from a different donor. Data are shown as the mean ± SEM. Statistical significance was tested using repeated-measures ANOVA with post test Tukey's for **A** and **B** and paired Student's t test for **C**. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

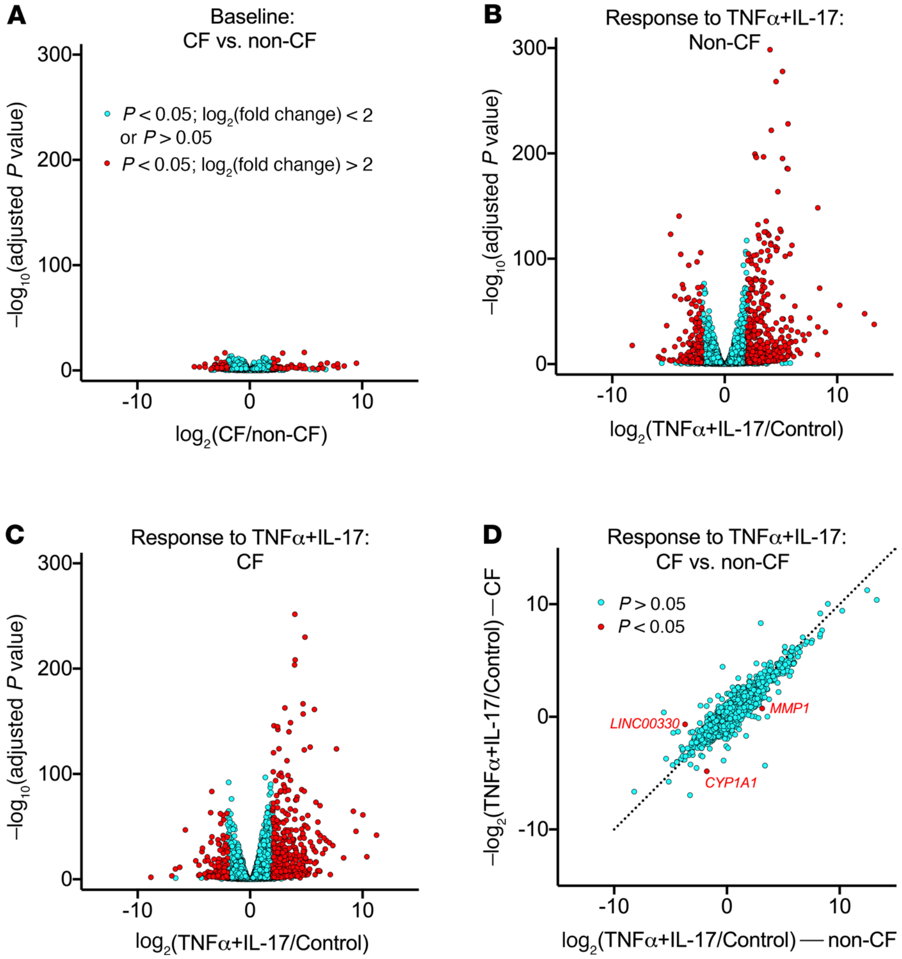

Figure 3. TNF-α+IL-17 induce highly similar transcriptomic changes in CF and non-CF epithelia. Human airway epithelia were treated with vehicle or TNF-α+IL-17 for 48 hours, and RNA-Seq was performed (n = 6 CF or non-CF donors). Differential gene expression was plotted as volcano plots. (**A**) Baseline CF versus non-CF epithelia; (**B**) response to TNF-α+IL-17 in non-CF epithelia; (**C**) response to TNF-α+IL-17 in CF epithelia; and (**D**) response to TNF-α+IL-17 in non-CF versus CF epithelia. Each data point corresponds to a gene. Statistically different transcripts are identified and marked in red. Analysis for differential expression was performed using DESeq2 and *P* values derived using Wald test.

changes, for example, epigenetic modifications or changes in cell types, we asked whether TNF-α+IL-17–induced alkalinization was reversible. We exposed CF epithelia to TNF-α+IL-17 for 7 days, stopped treatment, and measured pH_{ASL} 7 days later. Withdrawal of cytokines allowed pH_{ASL} to return to control levels, whereas continued exposure maintained alkalinization up to 14 days. These results indicate that TNF-α and IL-17 synergistically increase pH_{ASL}, and this response is time-dependent, long-lasting, and reversible.

TNF-α+IL-17 induce profound yet highly similar transcriptomic changes in CF and non-CF epithelia. Some previous reports have proposed that CFTR loss may alter the responses evoked by inflammatory stimuli (33, 34). To further evaluate this possibility, we compared the TNF-α+IL-17–induced response between CF and non-CF epithelia. We performed RNA-Seq and studied differential gene expression, defined as $\log_2$ fold change equal to or greater than 2 and a false discovery rate of less than 0.05. The results are displayed as a volcano plot (Figure 3A). At baseline, few transcripts differed between the CF and non-CF epithelia (Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/JCI150398DS1). Of note, airway-relevant acid-base transporters (e.g., *CFTR*, *ATP12A*) were not differentially expressed genes.

To assess CFTR dependence of inflammatory pathways, we compared responses to TNF-α+IL-17. In both CF and non-CF epithelia, TNF-α+IL-17 had a robust effect and altered the expression of hundreds of genes (Figure 3, B and C). After testing for the interaction between genotypes and treatment effects, the CF response was highly similar to the non-CF response (Figure 3D). The vast majority of transcripts clustered close to the line of identity, indicating relatively small or no differences attributable to genotypes. Three transcripts met criteria for differential response in CF versus non-CF: *CYP1A1*, *LINC00330*, and *MMP1*. However, previous literature does not specify a role for these transcripts in acid-base homeostasis. Overall, these results suggest that CF and non-CF responses to TNF-α+IL-17 are highly similar when measured at transcriptional level.

Calcium-activated Cl^- channels are not involved in CF ASL alkalinization. The TNF-α+IL-17–induced response required HCO_3^-, and CF epithelia lack a functional CFTR. Previous reports indicate that calcium-activated Cl^- channels (CaCCs) also conduct HCO_3^- (35, 36). CaCCs include anoctamins (ANO1 and ANO2), bestrophins (BEST1–BEST4), and chloride channel accessory proteins (CLCA1–CLCA4; ref. 37). In RNA-Seq studies, CaCC-related genes showed no clear change with TNF-α+IL-17 (Figure 4A). To test for electrogenic anion secretion mediated by CaCCs, we mounted epithelia in Ussing chambers with symmetric Krebs-HCO_3^- solution and recorded short-circuit current (I_{SC}; Figure 4, B and C). We inhibited ENaC with amiloride and noted similar ΔI_{SC} in vehicle and TNF-α+IL-17–treated epithelia. Next, we added uridine triphosphate to activate CaCCs, followed by 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) to inhibit CaCCs. We observed modest responses to these agents; however, ΔI_{SC} was not altered in epithelia treated with TNF-α+IL-17. To further assess the role of CaCCs in the pH_{ASL} response, we applied DIDS to the apical side and measured pH_{ASL} 2 hours later. Apical DIDS did not alter pH_{ASL} in either control or TNF-α+IL-17–treated epithelia (Figure 4D). We concluded that CaCCs were not the source of TNF-α+IL-17–mediated CF ASL alkalinization.

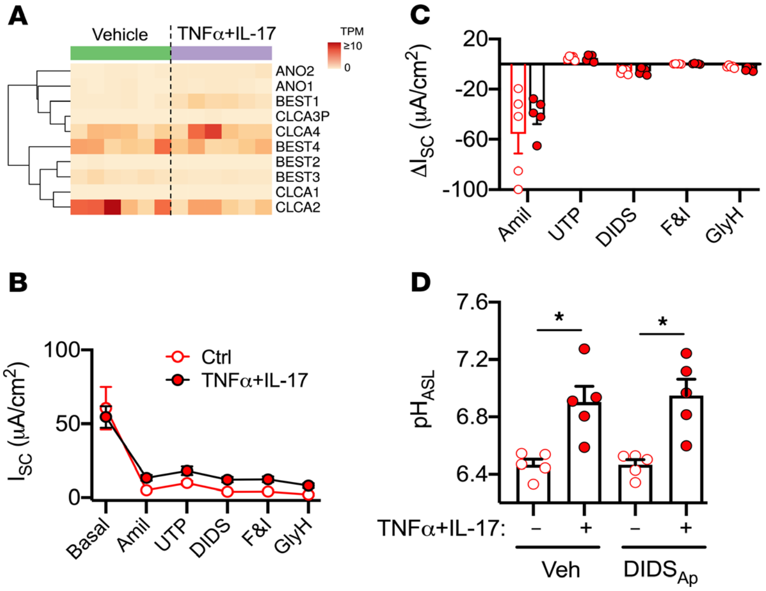

Figure 4. CaCCs are not involved in CF ASL alkalinization. (**A**) Differential expression of 10 CaCC-related genes in CF airway epithelia measured by RNA-Seq and displayed as a heatmap of raw transcripts per million (TPM). Columns represent epithelia from different CF donors (*n* = 6). The columns to the left are from 6 separate cultures under baseline conditions, and the columns to the right are from the same 6 donors treated with TNF-α+IL-17 for 48 hours and are displayed in the same sequence as that for the baseline results. Rows represent individual CaCC-related genes. (**B** and **C**) CF epithelia were treated with TNF-α+IL-17 for 24 hours and mounted in Ussing chambers with symmetric Krebs-HCO_3^- solution gassed with 5% CO_2. Epithelia were voltage clamped, followed by continuous recording of short-circuit current (I_{SC}), as pharmacologic agents were sequentially added to the apical chamber (*n* = 5). (**D**) 100 μM DIDS was suspended in a volatile solvent perfluorocarbon, Fluorinert FC-72 (3M), and applied apically. pH_{ASL} was measured 2 hours later using SNARF-1-dextran (*n* = 5 different donors). Data are shown as the mean ± SEM. **P* < 0.05 by repeated-measures ANOVA and Tukey's multiple comparison test.

TNF-α+IL-17 alkalinize CF ASL by upregulating pendrin. In addition to CFTR and CaCC anion channels, SLC26 transporters may also contribute to epithelial HCO_3^- secretion (38). We used RNA-Seq data to study SLC26 transporters in CF epithelia and displayed results as a heatmap (Figure 5A). The isoform *SLC26A4* showed low level expression at baseline, but this expression increased markedly with TNF-α+IL-17. We validated this result with qRT-PCR and noted a >100-fold increase in expression (Figure 5B). *SLC26A4* encodes pendrin, an electroneutral, DIDS-insensitive, Cl^-/ HCO_3^- exchanger (39, 40). Using immunocytochemistry, we localized pendrin at the apical pole (Figure 5C). To study the role of pendrin, we performed siRNA-mediated knockdown (Figure 5, D and E). Though no effect was observed under control conditions, pendrin knockdown reduced the already elevated pH_{ASL} in TNF-α+IL-17–treated epithelia. These data suggest that pendrin alkalinizes CF ASL and might be particularly relevant to inflamed CF airways.

A triple combination of CFTR modulators further increases pH_{ASL} in TNF-α+IL-17–treated CF epithelia. CFTR-ΔF508 is the most common disease-causing CFTR mutation (1). It results in deletion of a single phenylalanine at position 508 and produces a misfolded protein that is prematurely degraded. This defect can be modulated with a triple combination of drugs (elexacaftor, tezacaftor, and ivacaftor; ref. 41). Because airway inflammation is ubiquitous in individuals with CF who take modulators, we studied pH_{ASL} in CFTR-ΔF508 epithelia exposed to the triple combination and TNF-α+IL-17. In TNF-α+IL-17–treated epithelia with an already elevated pH_{ASL}, the triple combination further increased pH_{ASL} (Figure 6A).

The CFTR-ΔF508 allele is detected in individuals with CF as either homozygous (ΔF508/ΔF508) or compound heterozygous (ΔF508/other) genotypes. In clinical studies, both groups derived similar improvements (increased lung function, reduced pulmonary exacerbations, and reduced sweat [Cl^-]) with the triple combination (41). We predicted that this might be reflected in pH_{ASL} responses. With the triple combination, pH_{ASL} did not vary between ΔF508/ΔF508 and ΔF508/other epithelia (Figure 6B).

We performed several studies to understand how TNF-α+IL-17 increase the pH_{ASL} response to CFTR modulators. CFTR mediates transepithelial electrogenic anion secretion, which can be assayed in Ussing chambers by measuring responses to interventions that increase or inhibit CFTR activity. We mounted epithelia in Ussing chambers with symmetric Krebs-HCO_3^- solution and recorded I_{SC} and transepithelial conductance (G_t). We inhibited ENaCs with amiloride and CaCCs with DIDS, thereby primarily isolating changes in I_{SC} and G_t to CFTR. We added forskolin to activate CFTR, followed by $CFTR_{inh}$-172 to inhibit CFTR, and analyzed ΔI_{SC} and ΔG_t as measures of CFTR activity (Figure 7, A–C). Without modulators, TNF-α+IL-17 modestly increased ΔI_{SC}-CFTR but not ΔG_t-CFTR. The triple combination significantly increased ΔI_{SC}- and ΔG_t-CFTR in vehicle-treated controls as well as TNF-α+IL-17–treated epithelia. Remarkably, both measures of CFTR activity were 4 times larger in the presence of TNF-α+IL-17. These results suggested that TNF-α+IL-17 increased the amount of CFTR or its activity. Consistent with an increase in CFTR expression, TNF-α+IL-17 increased *CFTR* mRNA by 3.5-fold (Figure 7D) and CFTR protein by 2-fold (Figure 7, E and F). Overall, these results indicated that TNF-α+IL-17 increased the CF epithelial response to CFTR modulators by increasing CFTR amount, biosynthesis, and function.

TNF-α+IL-17 increase CFTR activity in G551D CF epithelia. ΔF508 is a class II mutation that produces a trafficking defect. As a result, very little to no functional CFTR reaches the apical membrane (1). In contrast, a class III mutation (e.g., G551D) produces a gating defect and results in a channel with reduced open state probability. We asked whether TNF-α+IL-17 enhanced CFTR-G551D activity. This is relevant because (a) G551D is the third-most common disease-causing mutation and it produces severe airway disease (42); (b) ivacaftor restores anion secretion and has been in clinical use for several years (43, 44); (c) patients with G551D have substantial airway inflammation, even after long-term ivacaftor use (45–47); (d) as opposed to correctors (elexacaftor, tezacaftor,

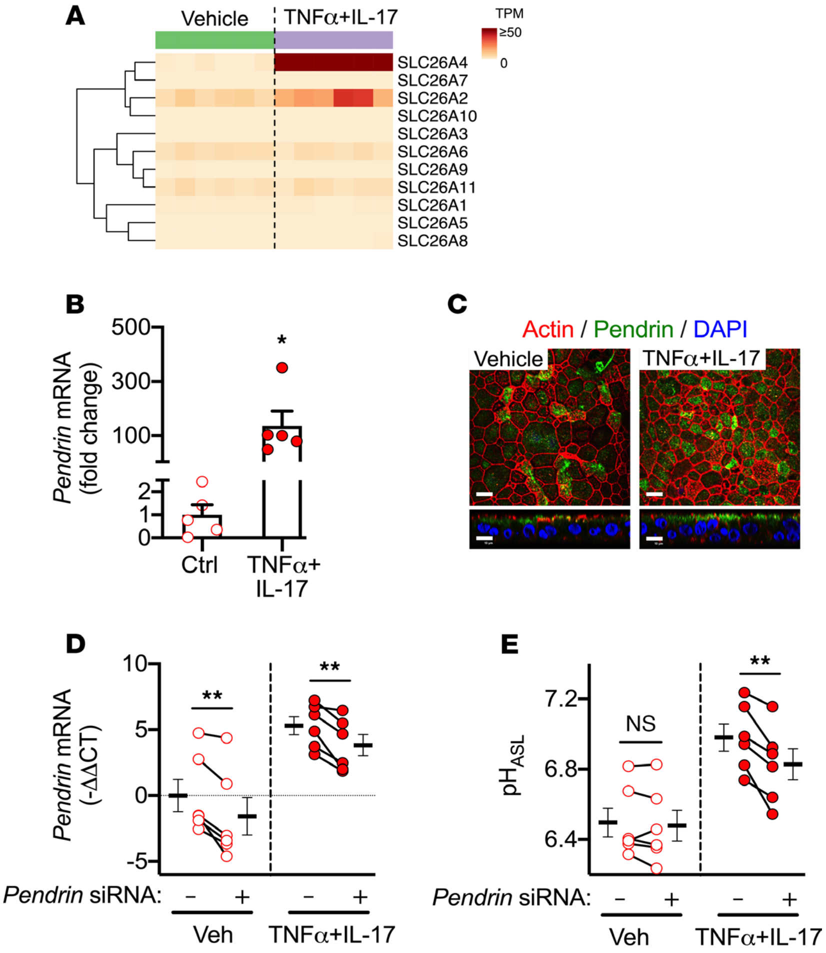

Figure 5. TNF-α+IL-17 alkalinize CF ASL by upregulating pendrin. (**A**) Differential expression of *SLC26* family genes in CF airway epithelia by RNA-Seq displayed as a heatmap of raw transcripts per million (TPM). Columns represent epithelia from different CF donors ($n = 6$). The columns to the left are from 6 separate cultures under baseline conditions, and the columns to the right are from the same 6 donors treated with TNF-α+IL-17 for 48 hours and are displayed in the same sequence as for the baseline results. Rows represent individual *SLC26* family genes. (**B**) *SLC26A4* (also known as pendrin) expression, as measured in qRT-PCR ($n = 5$). (**C**) Pendrin immunolocalization in CF airway epithelia. Scale bar: 10 μm. (**D** and **E**) siRNA directed against pendrin was used to knockdown expression in CF epithelia. TNF-α+IL-17 were applied for 24 hours. pH_{ASL} was measured using SNARF-1-dextran ($n = 6$). Each data point represents epithelium from a different CF donor. Data are shown as the mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$ by paired Student's *t* test.

etc.), ivacaftor works acutely and permits experimental designs that sidestep possible interactions between correctors and cytokines; and (e) chronic ivacaftor may limit corrector efficacy in vitro (48, 49). In G551D epithelia, assessments can be made independent of correctors.

We assayed G551D CF epithelia in Ussing chambers with symmetric Krebs-HCO_3^- solution, and measured I_{SC} and G_t. We minimized non-CFTR channel activity by applying amiloride and DIDS apically. To assess CFTR, we sequentially added forskolin and ivacaftor, followed by the inhibitor GlyH-101. TNF-α+IL-17 increased CFTR-G551D activity, as evidenced by greater ΔI_{SC} and ΔG_t in response to CFTR activators as well as the inhibitor (Figure 8, A–C). We also tested pH_{ASL} responses under open-circuit conditions at the air-liquid interface. Applied for 1 hour, the combination of forskolin and ivacaftor further alkalinized ASL in TNF-α+IL-17–treated epithelia (Figure 8D). These results indicate that TNF-α+IL-17 enhancement of modulator efficacy is not specific to a particular class of mutations or modulators.

However, there were some differences in the response to TNF-α+IL-17 in CFTR-G551D and CFTR-ΔF508 epithelia. In the CFTR-G551D epithelia, TNF-α+IL-17 did not alter basal Cl^- and HCO_3^- current after amiloride, but the response to forskolin, the response to ivacaftor, and the total Cl^- and HCO_3^- current were increased consistent with increased CFTR production and acute activation by forskolin and ivacaftor. In CFTR-ΔF508 epithelia, TNF-α+IL-17 increased basal Cl^- and HCO_3^- current after amiloride and total Cl^- and HCO_3^- current, but the response to forskolin was small. These results suggest that, in addition to increasing CFTR production, these inflammatory cytokines may have complex effects on CFTR biology. This is consistent with previous studies reporting that airway inflammation increased CFTR-ΔF508 biosynthesis and function (50, 51). Enhancing CFTR-ΔF508 biosynthesis requires time, and it is possible that the 48-hour treatment with the triple combination might also have contributed to differences between CFTR-G551D and CFTR-ΔF508 epithelia studied in Ussing chambers.

Airway inflammatory markers correlate with ivacaftor-induced lung function improvements. Our in vitro studies indicated that CFTR modulators may have enhanced efficacy in inflamed CF airways. However, in vivo response may differ due to additional factors, including infection, airway remodeling, and drug biodistribution. As an indirect test of the effect of modulators in inflamed airways, we asked if markers of airway inflammation would correlate with modulator-induced early lung function improvements. We analyzed data from an earlier clinical trial of a cohort of individuals with at least 1 G551D or R117H allele, i.e., mutations amenable to ivacaftor potentiation (15, 45, 52, 53). Just before beginning ivacaftor, all individuals had inflammatory markers, IL-1β, IL-8, and neutrophil elastase, measured in sputum. Lung function was assessed at baseline and at day 2 after starting ivacaftor, a time when the effect of any modulator-induced changes in infection and inflammation would be minimal. Supplemental Table 2 shows baseline clinical characteristics of the study participants. We found a positive correlation between baseline inflammation, measured as a combined score of the 3 inflammatory markers, and lung function improvement, measured as % ($\Delta ppFEV_1$/ $ppFEV_1$) where $ppFEV_1$ is the percentage predicted forced expiratory volume in 1 second (Figure 9A). As for individual markers, we noted significant positive correlations for

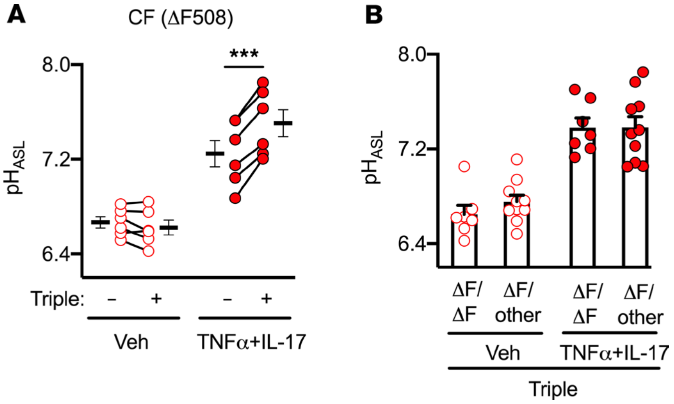

Figure 6. A triple combination of CFTR modulators further increases pH$_{ASL}$ in TNF-α+IL-17–treated CF epithelia. (**A**) Human airway epithelia from CFTR-ΔF508 donors were treated for 48 hours with a combination of elexacaftor (3 μM), tezacaftor (18 μM), and ivacaftor (1 μM), either alone or in the presence of TNF-α+IL-17. pH$_{ASL}$ was measured using SNARF-1-dextran ($n = 6$). (**B**) pH$_{ASL}$ from CF donors homozygous (ΔF508/other [ΔF/other]) or heterozygous (ΔF508/other [ΔF/ΔF]) for ΔF508 allele treated with the triple combination and TNF-α+IL-17 (n = 7-10). Each data point represents epithelium from a different CF donor. Data are shown as the mean ± SEM. Statistical significance was tested using paired (**A**) or unpaired (**B**) Student's t test. $^{***}P < 0.001$.

IL-1β and IL-8 but not neutrophil elastase (Figure 9, B–D). Importantly, TNF-α+IL-17, which we studied in vitro, directly induce *IL8* and *IL1B* expression in CF airway epithelia (Figure 9E).

As a negative control, we also tested for a correlation between airway inflammatory markers and the change in sweat [Cl$^-$], a standard biomarker of CFTR activity in vivo. The sweat gland environment is markedly different from the airway epithelium, in that CF sweat glands do not develop local neutrophil-predominant inflammation. All individuals had decreased sweat [Cl$^-$] at day 2 after starting ivacaftor, indicating CFTR potentiation. However, unlike the improvement in lung function, we observed no correlation between the inflammation index and ivacaftor-induced change in sweat [Cl$^-$] (Figure 9F).

Discussion

CF epithelia lack CFTR-mediated HCO_3^- secretion, and baseline CF pH$_{ASL}$ is abnormally acidic. Here, we found that the inflammatory cytokines TNF-α+IL-17 have a profound impact on CF pH$_{ASL}$. These cytokines induce HCO_3^- secretion by upregulating pendrin, an apical Cl^-/HCO_3^- exchanger. Strikingly, TNF-α+IL-17 increased the response to CFTR modulators and further alkalinized CF pH$_{ASL}$. Consistent with these findings, analysis of in vivo data from individuals with CF revealed a positive correlation between airway inflammation and CFTR modulator–induced lung function improvements. Collectively, these results suggest that inflammation is a key regulator of HCO_3^- secretion in CF airways and that it enhances the efficacy of CFTR modulators.

Previous studies have shown that the loss of CFTR function decreases HCO_3^- secretion in CF airway epithelia. Nonetheless, whether loss of CFTR decreases pH$_{ASL}$ in vivo has been debated (16, 54). Our results, together with those of previous studies (11, 30), identify at least two important factors that change CF pH$_{ASL}$: loss of CFTR-mediated HCO_3^- secretion and inflammation. In the absence of inflammatory cytokines, loss of CFTR-mediated HCO_3^- secretion decreases pH$_{ASL}$ in cultured airway epithelia. Likewise, in neonates tested before the results of genetic tests were known, and, thus, presumably before substantial airway inflammation, pH$_{ASL}$ was abnormally acidic in CF (14). Studies in newborn CF pigs also revealed an abnormally acidic pH$_{ASL}$ (5). However, inflammatory cytokines modified pH$_{ASL}$ in cultured CF airway epithelia: TNF-α+IL-17 increased pH$_{ASL}$ by increasing pendrin-mediated HCO_3^- secretion. Concordantly, in vivo studies of CF in 3-month-old babies, older children, and adults showed that pH$_{ASL}$ had increased so that it no longer differed from that in non-CF controls. These results predicted that if CFTR activity were introduced to inflamed CF epithelia, their pH$_{ASL}$ would further increase. The use of CFTR modulators allowed us to test that prediction, and CFTR modulators increased pH$_{ASL}$ even further in CF epithelia treated with TNF-α+IL-17. These results help explain earlier observations and suggest that for similar levels of inflammation CF pH$_{ASL}$ is abnormally low.

Enhancement of CFTR modulator efficacy by 2 cytokines known to be elevated in CF airways suggests that inflammation might have a profound effect in vivo. This prediction is supported by results that showed a significant positive correlation between baseline inflammatory markers and ivacaftor-induced improvements in lung function. Previous work has sought to uncover predictors of response to modulators and therapeutic outcomes (31, 55). In several studies, population-level effects are obvious but are difficult to reconcile with individual-level variability (55–58). Specifically, why some patients derive greater benefit from modulators than others is not known. We speculate that airway inflammation might be a key determinant. To advance personalized CFTR modulation, it may be relevant to study the airway inflammatory milieu and its interactions with approved modulators.

This study has limitations. First, we studied the response to 2 cytokines relevant to CF-like inflammation. However, inflammation is a complex, heterogeneous process (59), and other mediators may modify HCO_3^- secretion and modulator responses differently. For instance, TGF-β may reduce CFTR expression (60). Second, we did not study the impact of infection, but it remains highly relevant. Some reports have suggested that bacterial factors (e.g., *Pseudomonas* toxins) may suppress CFTR expression (61, 62). Both inflammation and infection persist despite long-term modulator therapy, and both represent suitable targets to improve CF outcomes. Third, we studied acute improvements in lung function with ivacaftor, as most of the improvement is early and is followed by stabilization of lung function. With chronic treatment, airway inflammatory markers either remain stable (46, 47) or decline but remain abnormally elevated (45). Future studies may define long-term relationships between airway inflammation and the response to modulators.

This study has several implications for in vitro and in vivo studies. First, sweat [Cl$^-$] is routinely employed as a CFTR biomarker in translational studies and clinical care. In previ-

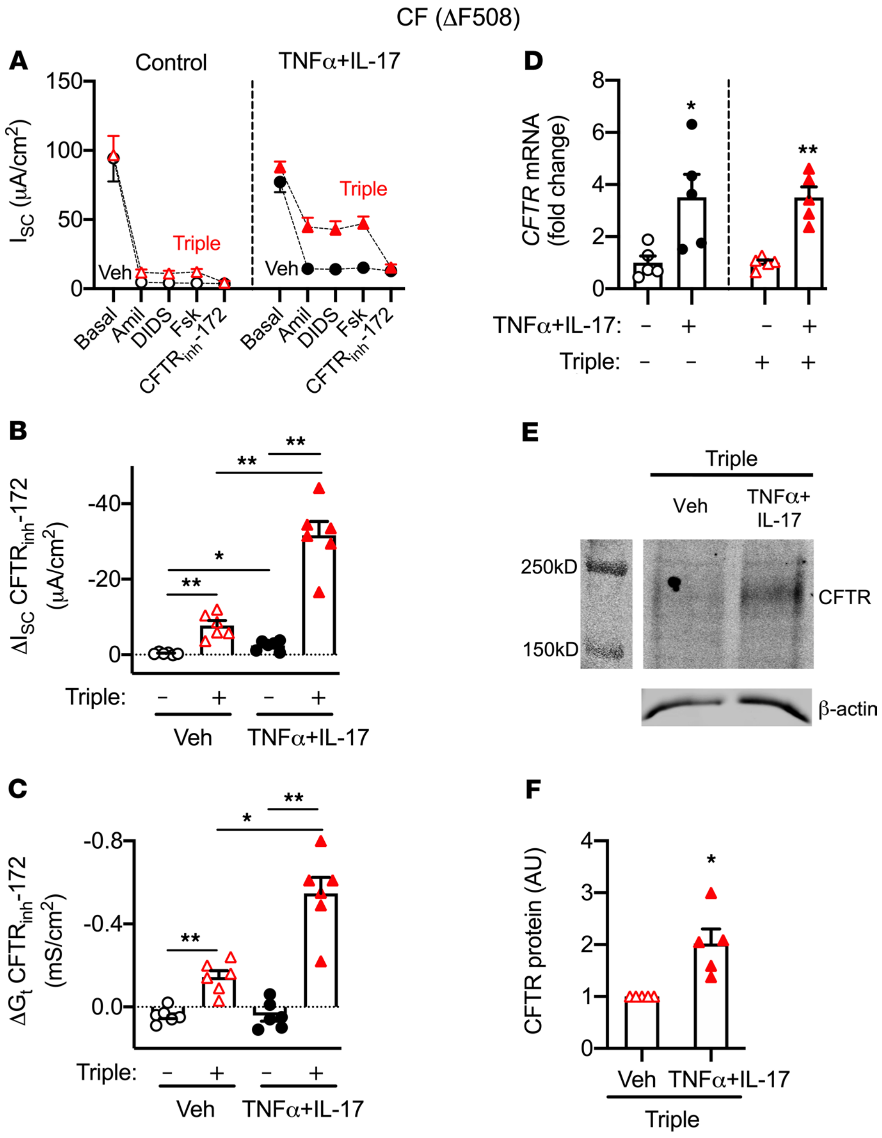

Figure 7. TNF-α+IL-17 increase the response of CFTR-ΔF508 epithelia to the triple combination of CFTR modulators. Human airway epithelia from CFTR-ΔF508 donors were treated for 48 hours with a combination of elexacaftor (3 μM), tezacaftor (18 μM), and ivacaftor (1 μM), either alone or in the presence of TNF-α+IL-17. (**A**–**C**) Epithelia were mounted in Ussing chambers with symmetric Krebs-HCO_3^- solution gassed with 5% CO_2. Epithelia were voltage clamped, followed by recording of I_{sc} and G_t, as pharmacologic agents were sequentially added to the apical chamber (n = 6 different donors). CFTR channel activity was assayed as the response to CFTR$_{inh}$-172, shown in **B** as ΔI_{sc} and in **C** as ΔG_t. (**D**) *CFTR* gene expression measured using qRT-PCR (n = 5). (**E**) Example of Western blot of CFTR and β-actin in CF airway epithelia treated with the triple combination and either vehicle or TNF-α+IL-17 for 48 hours. (**F**) Quantification of CFTR protein expression normalized to β-actin (n = 5). Each data point represents epithelium from a different CF donor. Data are shown as the mean ± SEM. Statistical significance was tested using repeated-measures ANOVA and post test Tukey's test for **B** and **C**, and paired Student's *t* test for **D** and **F**. $^*P < 0.05$, $^{**}P < 0.01$.

ous studies, the modulator-induced change in sweat [Cl$^-$] (a biomarker of CFTR activity in vivo) did not correlate consistently with the change in percentage FEV1 (a measure of airway response; refs. 56, 57). This study suggests that non-cell-autonomous mechanisms, such as local inflammation, may control the response to modulators. Further advancement of CF research and care may benefit from the development of airway-specific CFTR biomarkers, and pH$_{ASL}$ is an emerging candidate.

Second, the airway inflammation that persists after initiation of CFTR modulators is a potential therapeutic target, and several agents are under evaluation (63). Our results suggest that inflammatory pathways may intersect with CFTR biogenesis pathways. Suppressing inflammation nonspecifically might thus involve a trade-off with modulator efficacy. Some studies have suggested that ivacaftor may reduce the efficacy of correctors when used in combination (48, 49). Whether this is also the case in the presence of inflammation is not known. This study underscores the need to assess interactions between candidate antiinflammatory therapies and CFTR corrector/potentiator combination regimens.

Third, in vitro models are routinely employed to assess CFTR modulators (64). As inflammation is ubiquitous in vivo and modifies the response to modulators, prediction of response based on in vitro assays (theratyping) may be optimized by incorporating assessments of airway inflammatory phenotype and its effect on modulator efficacy.

In summary, the inflammatory milieu in CF airways regulates pH$_{ASL}$ and increases the response to CFTR modulators.

Methods

Cell culture. Primary cultures of differentiated airway epithelia were obtained without passage from multiple human donors as previously reported (65). Briefly, donor tracheae and/or proximal bronchi were enzymatically digested. Epithelial cells were isolated and seeded onto collagen-coated inserts (Costar, 3470 and 3413; Falcon 353180). Epithelia were differentiated at the air-liquid interface for 3 weeks or more prior to assay. To assess cytokine-induced responses, epithelia were treated on the basolateral side with 10 ng/ml TNF-α (R&D Systems), 20 ng/ml IL-17 (R&D Systems), or both.

Pharmacologic reagents. Elexacaftor was purchased from MedChemExpress, and tezacaftor and ivacaftor were purchased from Selleckchem. GlyH-101 was a gift from the Cystic Fibrosis Foundation Therapeutics Lab and Robert Bridges (Rosalind Franklin University, Chicago, Illinois, USA). Other reagents were purchased from MilliporeSigma.

pH$_{ASL}$ measurement. The protocol for pH$_{ASL}$ measurements has previously been reported (11). Briefly, we used a ratiometric pH indicator, SNARF-1, conjugated to 70 kD dextran (Thermo Fisher Scientific). SNARF-1 is a single-excitation (514 nm), dual-emission

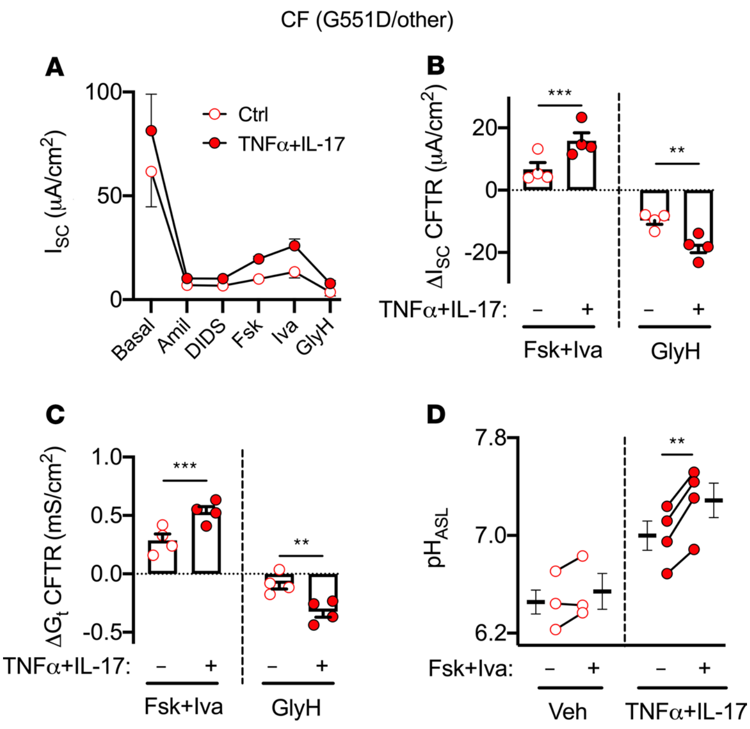

Figure 8. TNF-α+IL-17 increase CFTR activity in CF epithelia with a CFTR-G551D mutation. Human airway epithelia from G551D CF donors were treated with either vehicle or TNF-α+IL-17 for 48 hours. (**A**–**C**) Epithelia were assayed in Ussing chambers containing symmetric Krebs-HCO_3^- solution, with continuous recording of I_{SC} and G_t ($n = 4$). (**D**) pH_{ASL} in G551D CF epithelia, stimulated for 2 hours with forskolin 10 μM and ivacaftor 10 μM ($n = 3$-4). Each data point represents epithelium from a different CF donor. Data are shown as the mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ by paired Student's t test.

(580 nm and 640 nm) fluorescence pH indicator, with optimal range near physiologic pH. SNARF-1–conjugated dextran was delivered as a powder to the apical side and allowed to distribute into ASL for 1 hour. Fluorescence ratios were obtained on a laser-scanning confocal microscope (Zeiss LSM 880) and converted to pH values using calibration curves constructed from colorless standard pH solutions. The microscope chamber housing epithelia maintained a humidified environment at 37°C. 5% CO_2 was added to the chamber atmosphere whenever the basolateral side was immersed in a HCO_3^- containing buffer solution but removed when a HCO_3^--free (HEPES) buffer solution was used.

Electrophysiologic studies. Airway epithelia were mounted in modified Ussing chambers (Physiologic Instruments) and bathed in symmetric Krebs-HCO_3^- buffer solution containing 118.9 mM NaCl, 25 mM $NaHCO_3$, 2.4 mM K_2HPO_4, 0.6 mM KH_2PO_4, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 5 mM dextrose, at 37°C and adjusted to pH 7.4 in the presence of 5% CO_2. Epithelia were voltage clamped, followed by recording of I_{SC} and G_t. The following agents were added apically: 100 μM amiloride, 50 μM uridine triphosphate, 100 μM 4,4′-DIDS, 10 μM forskolin and 100 μM 3-isobutyl-2-methylxanthine, and 100 μM GlyH-101 or 10 $CFTR_{inh}$-172.

Real-time PCR. Total RNA was isolated from airway epithelia using the RNeasy Lipid Tissue Mini Kit (QIAGEN). Genomic DNA was removed through DNase I (QIAGEN) treatment. Quality of RNA isolation was verified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), and samples with a 260:280 ratio ≥ 1.8 were carried forward. RNA was reverse transcribed with SuperScript VILO MasterMix (Invitrogen). cDNA thus obtained was amplified using gene-specific primers and Fast SYBR Green Master Mix (Applied Biosystems) on the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). The gene-specific primer pairs used were as follows: *SLC26A4* (pendrin), 5′-CTCCCCAAATACCGAGTCAA-3′ and 5′-CCATATCCGACAGGAACTGC-3′; *CFTR*, 5′- CACCCAGCCATTTTTGGC-3′ and 5′-AGGAGCGATCCACACGAA-3′; β-actin, 5′- AGAGCTACGAGCTGCCTGAC-3′ and 5′-AGCACTGTGTTGGCGTACAG-3′; and *SFRS9*, 5′-TGCGTAAACTGGATGACACC-3′ and 5′-CCTGCTTTGGTATGGAGAGTC-3′. All reactions were performed in triplicates, and gene expression was quantitated using –ΔΔCT method.

siRNA knockdown. Gene knockdown in primary airway epithelia was achieved as reported previously (66). siRNAs were obtained from Integrated DNA Technologies (negative control, IDT DS NC 1; pendrin: IDT hs.Ri.SLC26A4.13.2) and transfected into dissociated primary airway epithelial cells using Lipofectamine RNAiMax (Invitrogen). Epithelia were seeded onto collagen-coated inserts (Costar, 3470) and differentiated at the air-liquid interface. pH_{ASL} was measured at day 6 or 7 after seeding. The efficiency of gene knockdown was assessed with RT-PCR.

RNA-Seq protocol and analysis. RNA-Seq was performed in collaboration with the University of Iowa Genomics Division using the manufacturer's recommended protocols. Briefly, 500 ng DNase I–treated total RNA was enriched for polyA-containing transcripts using beads coated with oligo(dT) primers. The enriched RNA pool was fragmented, converted to cDNA, and ligated to sequencing adaptors using the TruSeq stranded mRNA sample preparation kit (Illumina, RS-122-2101). The molar concentrations of the indexed libraries were measured using the 2100 Bioanalyzer (Agilent) and combined equally into pools for sequencing. The concentrations of the pools were measured with the Illumina Library Quantification Kit (KAPA Biosystems) and sequenced on the Illumina HiSeq 4000 genome sequencer using 75 bp paired-end SBS chemistry.

Pseudoalignment of raw sequencing reads and quantification of transcript-level expression were obtained using Kallisto version 0.45.0 and human transcriptome reference GRCh38. p12 (67). Gene counts were imported into R, and differential expression tests were performed using DESeq2 version 1.22.2 (68). Further, gene expression modeling in DESeq2 accounted for the experimental design, acknowledging and correcting for paired control and treated samples for each donor. Changes in HCO_3^- transporters were visualized as heatmaps generated using the Clustvis tool (https://biit.cs.ut.ee/clustvis/) (69). RNA-Seq data are available in the NCBI's GEO database (GEO GSE 176121).

Immunocytochemistry. Airway epithelia were washed 3 times with PBS, fixed with 4% paraformaldehyde for 15 minutes, and permeabilized with 0.3% Triton-X for 20 minutes. To minimize nonspecific staining, epithelia were treated with SuperBlock (Thermo Fisher Scientific) containing 0.5% normal goat serum for 1 hour at room temperature. Primary antibodies were diluted in SuperBlock and added apically for 3 hours at 37°C. Epithelia were washed and incubated for

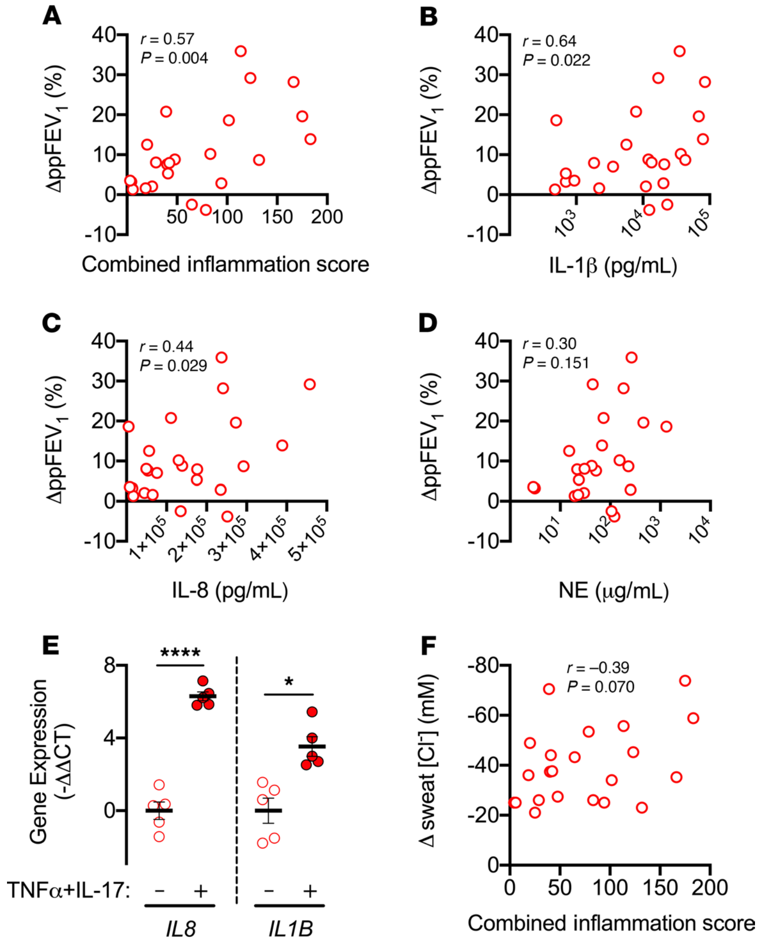

Figure 9. Airway inflammation correlates with ivacaftor-induced lung function improvements. Induced sputum samples were obtained from individuals with CF with a CFTR-G551D or a CFTR-R117H mutation immediately before starting ivacaftor. Sputum inflammatory markers IL-1β, IL-8, and neutrophil elastase (NE) were measured using ELISA. Lung function was evaluated immediately before and 2 days after starting ivacaftor. Data are from previously reported studies (15, 45, 53). (**A**–**D**) Relationship between baseline airway inflammation and changes in lung function, measured as %(ΔppFEV$_1$/ppFEV$_1$) where ppFEV$_1$ is the percentage predicted forced expiratory volume in 1 second ($n = 23$). (**E**) Changes in *IL8* and *IL1B* gene expression in human CF airway epithelia treated with TNF-α+IL-17 for 48 hours ($n = 5$ different donors). (**F**) Relationship between baseline airway inflammation and changes in sweat Cl$^-$ concentration 2 days after starting ivacaftor ($n = 22$). Statistical significance was tested using Pearson's *r* test for **A**–**D** and **F** and paired Student's *t* test for **E**. Bars in **E** indicate mean ± SEM. *$P < 0.05$, ****$P < 0.0001$.

45 minutes with appropriate secondary antibodies diluted in PBS. Pendrin was detected using mouse anti-SLC26A4 (1:200; Abnova) and goat anti-mouse secondary antibody conjugated to Alexa Flour 488 (1:1000; Thermo Fisher Scientific). Actin cytoskeleton was stained with Alexa Fluor 633 phalloidin (1:300; Thermo Fisher Scientific) added at the same time as secondary antibodies. Epithelia were mounted on glass slides, and Vectashield with DAPI (Vector Laboratories) was used to secure glass coverslips. Imaging was performed on the Olympus Fluoview FV 3000 confocal microscope. *Z*-stack images were processed with the Olympus Fluoview program.

Western blot. Airway epithelia were washed 3 times with ice-cold PBS and scraped into 35 μl lysis buffer supplemented with protease inhibitors. The resulting mixture was passed through small bore (27-gauge) needles and centrifuged at 1200*g* for 5 minutes. The supernatants were collected and ultracentrifuged at 174,000*g* for 30 minutes. The pellets were resuspended in 1% Triton X-100 in lysis buffer. Samples were run on 7% gels with high-molecular-mass standards (Bio-Rad, 161-0374). Electrophoresed gels were transferred to PVDF membranes (Millipore, IPFL00010) overnight. Membranes were blocked in 0.1% casein in PBS; immunostained with antibodies for CFTR (1:500, no. 596; CFTR Antibody Distribution Program, Cystic Fibrosis Foundation Therapeutics Lab) and β-actin (1:3000, Abcam, ab8227) and secondary antibodies (1:10,000, LI-COR, 926-32212 and 926-32213); and visualized on an Odyssey IR imager (LI-COR). Quantification for CFTR was performed on ImageJ (NIH) after normalizing to β-actin.

Human study participants. The analysis of data from humans was from previously published studies (15, 45, 52, 53). Adult participants with CF who had at least 1 G551D or R117H allele were enrolled at a single CF center, the National Referral Centre for Adult Cystic Fibrosis, St. Vincent's University Hospital, and University College Dublin School of Medicine, Dublin, Ireland. Supplemental Table 2 summarizes the clinical characteristics of participants. Inflammatory markers (IL-1β, IL-8, and neutrophil elastase) were measured in induced sputum using ELISA immediately before starting ivacaftor and were used to derive a "combined inflammation score." For each marker, the highest measurement was assigned a value of 100, and other measurements were expressed as a fraction of 100. In the end, the values for all 3 markers were added to obtain a combined score. Sweat [Cl$^-$] and spirometry were obtained at baseline and 2 days after starting ivacaftor.

Statistics. Testing for statistical significance was performed on GraphPad Prism 8 Software. Tests included unpaired or paired 2-tailed Student's *t* test for comparing 2 groups, repeated-measures 1-way ANOVA with Tukey's multiple comparison test for comparing more than 2 groups, and Pearson's *r* test for assessing correlations. A *P* value of less than 0.05 was considered significant.

Study approval. All studies were approved by the University of Iowa Institutional Review Board.

Author contributions

TR, DAS, EFM, PKS, and MJW conceived and designed studies. PHK and PT prepared epithelia for experiments. TR performed experiments, and BJG contributed to Western blots. TR, AAP, ALT, SLD, and MJW analyzed data. IMT and MED provided insightful discussions about study design and results. TR and MJW wrote the manuscript. All authors approved the manuscript.

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