Post-Transcriptional Regulation of Cystic Fibrosis Transmembrane Conductance Regulator Expression and Function by MicroRNAs

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MicroRNAs (miRNAs) are increasingly recognized as important posttranscriptional regulators of gene expression, and changes in their actions can contribute to disease states. Little is understood regarding miRNA functions in the airway epithelium under normal or diseased conditions. We profiled miRNA expression in well-differentiated primary cultures of human cystic fibrosis (CF) and non-CF airway epithelia, and discovered that miR-509–3p and miR-494 concentrations were increased in CF epithelia. Human non-CF airway epithelia, transfected with the mimics of miR-509–3p or miR-494, showed decreased cystic fibrosis transmembrane conductance regulator (CFTR) expression, whereas their respective anti-miRs exerted the opposite effect. Interestingly, the two miRNAs acted cooperatively in regulating CFTR expression. Upon infecting non-CF airway epithelial cells with *Staphylococcus aureus*, or upon stimulating them with the proinflammatory cytokines TNF-α or IL-1β, we observed an increased expression of both miRNAs and a concurrent decrease in CFTR expression and function, suggesting that inflammatory mediators may regulate these miRNAs. Transfecting epithelia with anti-miRs for miR-509–3p and miR-494, or inhibiting NF-κB signaling before stimulating cells with TNFα or IL-1β, suppressed these responses, suggesting that the expression of both miRNAs was responsive to NF-κB signaling. Thus, miR-509–3p and miR-494 are dynamic regulators of CFTR abundance and function in normal, non-CF airway epithelia.

Keywords: ATP binding cassette protein; cystic fibrosis transmembrane conductance regulator; 3′ UTR; epithelial fluid and electrolyte transport

MicroRNAs (miRNAs) comprise an evolutionarily conserved class of small (~21–24 nucleotides), noncoding RNA molecules that regulate post-transcriptional gene expression by binding to the 3′ untranslated region (UTR) of target mRNAs (1, 2). The majority of human genes are under posttranscriptional regulation by miRNAs (1). As such, miRNAs are essential for multiple biological processes such as tissue development, cell-cycle checkpoints, DNA repair, senescence, organogenesis, and innate immunity (3, 4). Interestingly, although a single miRNA can target anywhere from 3–300 genes, distinct miRNAs can act independently or cooperatively, in a tissue-specific manner, to repress the expression of a single gene by binding to their target sequences in the 3′ UTR of the mRNA (2, 4, 5). MiRNAs play an important role in lung development (6, 7) and in several facets of pulmonary biology (7–10). However, the roles of miRNAs in the regulation of fluid and electrolyte transport in the airways are not well understood.

CLINICAL RELEVANCE

We demonstrate the regulation of cystic fibrosis transmembrane conductance regulator (CFTR) expression and function by microRNAs (miRNAs). The results highlight the ability of miRNAs to influence airway epithelial cell function in response to environmental stimuli. We speculate that changes in CFTR abundance and function might acutely affect airway surface liquid volume and composition, host defense, and mucociliary clearance.

The cystic fibrosis transmembrane conductance regulator (*CFTR*) gene encodes an anion channel, which when mutated causes the disease cystic fibrosis (CF) (11). Chronic pulmonary infection and the associated inflammation contribute to the decline in respiratory function in patients with CF. The increased expression of proinflammatory cytokines (IL-1, IL-8, IL-6, and TNF-α), along with the decreased expression of anti-inflammatory cytokines and an altered protease/antiprotease balance, has been reported in the airways of patients with CF (12). Similar proinflammatory signals occur in the context of many pulmonary diseases caused by bacteria, viruses, and environmental stimuli. Toll-like receptors on epithelia, macrophages, and other cells recognize pathogen-associated molecular patterns such as bacterial LPS, flagellin, lipoteichoic acid, and additional microbial products. Signal transduction from these receptors, in turn, mediates inflammatory responses, in part by activating the transcription factor NF-κB. NF-κB regulates cellular pathways that induce the production of the inflammatory proteins and cytokines that influence the onset and resolution of numerous pulmonary diseases (13, 14).

Although extensive study of *CFTR* and its protein product has proceeded since its discovery in 1989 (11), the complex regulation of its expression remains poorly understood (15, 16). *CFTR* constitutes a low-abundance mRNA in airway epithelia (17), and miRNAs have been suggested to silence low-abundance

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mRNA targets more efficiently than highly abundant mRNAs (2, 18). Thus, *CFTR* mRNA concentrations may be tightly controlled post-transcriptionally by miRNAs. Indeed, evidence indicates that miRNAs may influence *CFTR* expression (19, 20), but the functional effects of these interactions have not been investigated. Furthermore, our understanding of the role miRNAs play in CF and other lung diseases associated with inflammation and infection is nascent. We recently discovered that miR-138 positively influences CFTR expression, post-translational biosynthesis, and function indirectly by inhibiting the transcriptional regulatory protein SIN3A (21). In this study, we hypothesized that miRNAs directly regulate the production of CFTR protein and hence its function in epithelia.

MATERIALS AND METHODS

RNA Isolation

Total RNA from human primary airway epithelial cells and Calu-3 cells was isolated using the *mir*Vana miRNA Isolation Kit or the TRIzol Reagent (both from Life Technologies, Carlsbad, CA) (22). Total RNA was tested for quality on an Agilent Model 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only samples with an RNA integrity number greater than 7.0 were selected for downstream processing.

Primary Human Airway Epithelia

Airway epithelia from human tracheas and primary bronchi removed from organs donated for research were cultured at the air–liquid interface (23).

Detailed materials and methods are available in the online supplement.

RESULTS

MicroRNA Profiling Identifies Candidate Regulators of *CFTR*

Primary air–liquid interface (ALI) cultures of human airway epithelia replicate many features of *in vivo* airways (23, 24). We investigated miRNA expression in well-differentiated primary cultures of human non-CF and CF (homozygous for the *CFTR*-ΔF508 mutation) airway epithelia via quantitative PCR. Using a *P*-value cutoff of < 0.05, 18 miRNAs were found to be significantly differentially expressed in CF samples (*see* Table E1 in the online supplement). Of these, miR-509–3p and miR-494 were remarkable for a single predicted binding site each (Targetscan) within the *CFTR* 3′ UTR (Figure E1), suggesting that these miRNAs might cooperate to regulate CFTR expression posttranscriptionally.

Both miR-509–3p and miR-494 exhibited increased expression in CF epithelia (Table E1). To validate this change in expression, we harvested RNA from well-differentiated primary cultures of human non-CF and CF (homozygous for *CFTR*-ΔF508) airway epithelia, and from freshly dissociated tracheobronchial airway epithelial cells from human non-CF and CF (homozygous for *CFTR*-ΔF508) donors. Using miRNA-specific quantitative RT-PCR, we confirmed that both miR-509–3p and miR-494 showed significantly greater expression in CF epithelia (Figure 1).

CFTR Is a Target of miR-509–3p and miR-494

The increased expression of miR-509–3p and miR-494 in CF epithelia led us to hypothesize that *CFTR* is regulated by both miRNAs. To test whether miR-509–3p and miR-494 repress *CFTR* expression by binding to its 3′ UTR, we performed a dual-luciferase reporter assay. The *CFTR* 3′ UTR was cloned into the psiCHECK-2 vector and transfected into HEK293T

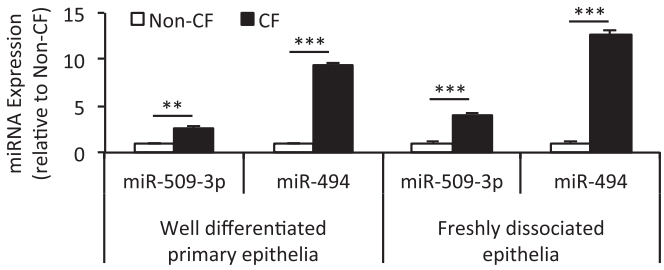

Figure 1. Cystic fibrosis (CF) epithelia show increased expression of microRNA (miR)–509–3p and miR-494. RNA harvested from well-differentiated primary epithelia (six donors per genotype) and freshly dissociated tracheobronchial epithelia (five donors per genotype) were profiled for microRNA (miRNA) expression using quantitative RT-PCR. *Error bars* indicate mean ± SE. Statistical significance was determined by the Student *t* test. $^{**}P < 0.01$. $^{***}P < 0.001$.

cells with increasing concentrations of either the miR-509–3p mimic (Figure E2A) or the miR-494 mimic (Figure E2B). The results demonstrated a dose-dependent repression of luciferase expression for each mimic. Mutation of the miR-509–3p (Figure E2A) or the miR-494 (Figure E2B) binding site relieved repression by the miRNAs *in vitro*, indicating that the repression was site-specific.

We measured the effects of these miRNAs on endogenous *CFTR* concentrations in human airway epithelial cells and Calu-3 epithelial cells that express CFTR abundantly (25). We note that in contrast to the expression of recombinant CFTR, endogenous CFTR expression in native tissue and primary airway epithelia produces mainly the band C form of the protein and little band B (26, 27). We have not used radioactive phosphorylation techniques to amplify the signal, and for this reason the band B abundance is often at the lower limits of detection. Transfecting cells with the miR-509–3p mimic caused a significant decrease in CFTR mRNA and protein concentrations in both airway epithelial cells (Figures 2A and 2B) and Calu-3 cells (Figures E3A and E3B). The opposite effects were observed with the miR-509–3p anti-miR, wherein CFTR mRNA and protein expression increased in both airway epithelial cells (Figures 2A and 2B) and Calu-3 cells (Figures E3A and E3B). Similar results were observed with the miR-494 mimic and anti-miR (airway epithelia, Figures 2A and 2B; Calu-3 cells, Figures E3A and E3B), indicating that both miRNAs are potent post-transcriptional repressors of *CFTR*. Because CFTR creates ion permeability, its function can be assayed by measuring trans-epithelial electrical conductance in polarized epithelial cells. Transfection with either miR mimic resulted in a significant reduction in CFTR-mediated Cl^- conductance (G_t) and current (I_t) in both polarized ALI primary airway epithelial cell cultures (Figures 2C, 2D, and E4) and polarized ALI Calu-3 epithelial cell cultures (Figures E3C and E3D). The opposite effect was observed after transfecting Calu-3 cells with the anti-miR for each miRNA (Figures E3C and E3D). In polarized ALI primary airway epithelial cell cultures, despite an increase in G_t, the anti-miRs did not increase I_t (Figures 2D and E4), consistent with the presence of other rate-limiting steps for Cl^- secretion in airway epithelia (28). Furthermore, both miRNAs worked cooperatively, because transfecting cells with the mimics of both miRNAs together caused an even greater decrease in CFTR mRNA, protein, G_t, and I_t in primary airway epithelia (Figures 2A–2D and E4) and in Calu-3 cells (Figures E3A–E3D), compared with either intervention alone. A similar enhanced effect was seen with the combined anti-miR transfection, wherein an increase in CFTR mRNA, protein, G_t, and I_t

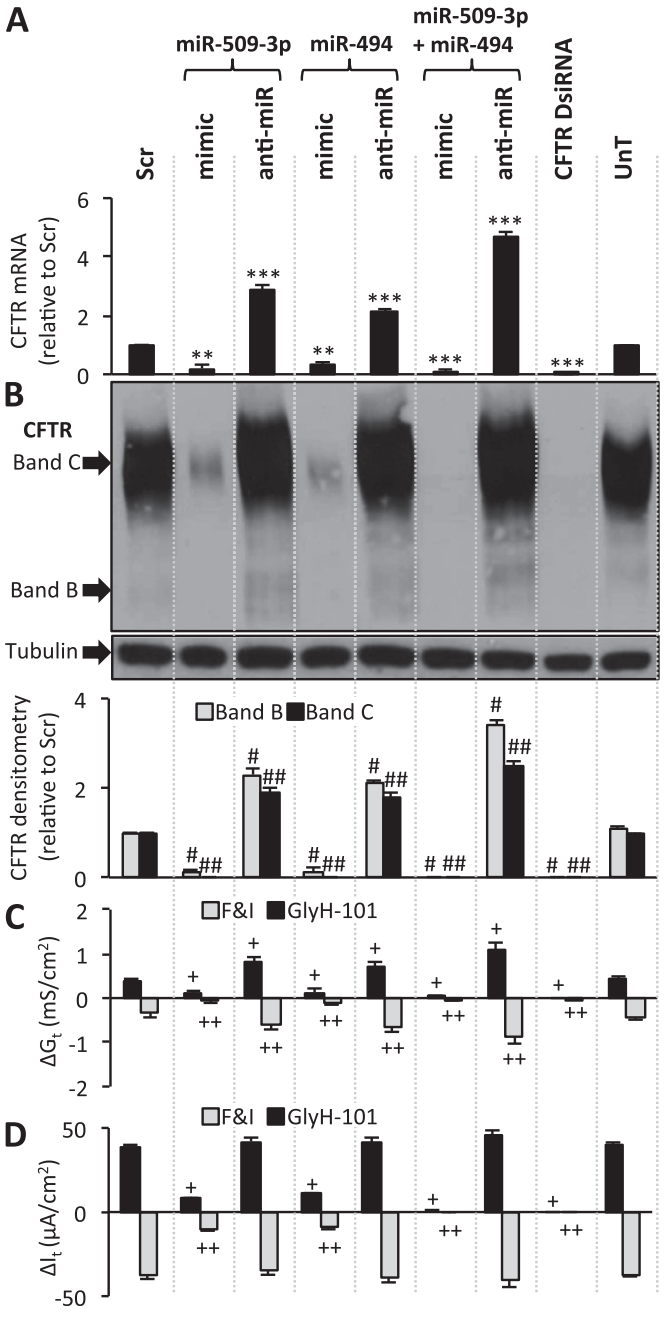

Figure 2. miR-509–3p and miR-494 regulate cystic fibrosis transmembrane conductance regulator (*CFTR*) gene expression and function in human non-CF airway epithelial cells. (*A*) *CFTR* mRNA abundance in primary airway epithelia, 24 hours after indicated transfections ($n = 6$ donors). (*B*) CFTR protein abundance in primary airway epithelia, 72 hours after transfection. Densitometry and relative fold change of CFTR protein abundance in primary airway epithelia were determined in six different human donors (eight replicates each). (*C* and *D*) Changes in (*C*) conductance (G_t) and (*D*) transepithelial current (I_t) with indicated treatments. Each *bar* represents data from four different human donors (six replicates each). Basal transepithelial resistance range, 311–383 ohms · cm^2; current (I_t) range, 24–58 microamperes · cm^2. DsiRNA, Dicer substrate siRNA; UnT, untransfected. *Error bars* indicate the mean ± SE. Statistical significance was determined by the Student *t* test. $^{**}P < 0.01$ and $^{***}P < 0.001$, relative to scrambled control (Scr). $^{\#}P < 0.01$, relative to Scr CFTR band B. $^{\#\#}P < 0.01$, relative to Scr CFTR band C. $^{+}P < 0.01$ and $^{++}P < 0.01$, relative to ΔG_t or ΔI_t in Scr-transfected samples upon forskolin and IBMX (F&I) or CFTR inhibitor GlyH-101 treatment, respectively.

was observed compared with either single intervention in primary airway epithelia (Figures 2A–2D) and in Calu-3 cells (Figures E3A–E3D). We note that the transfection of primary cells with anti-miRNAs, miRNA mimics, or Dicer substrate siRNA (DsiRNA) did not appreciably change the morphology of epithelia studied 4 weeks later (Figure E5). These findings provide functional evidence in primary human airway epithelia that CFTR-mediated anion transport is under cooperative posttranscriptional regulation by miRNAs.

Infection and Inflammation Increase miR-509–3p and miR-494 Expression

A characteristic feature of CF airway disease involves recurrent and chronic infection and inflammation (12). Exacerbations in other respiratory diseases, such as chronic obstructive pulmonary disease (COPD) and asthma, are also linked to infection and inflammation. We hypothesized that either or both of these stimuli might influence miR-509–3p and miR-494 concentrations, possibly explaining their increased expression in CF epithelia. To test this hypothesis, polarized cultures of Calu-3 epithelia and well-differentiated primary cultures of human non-CF airway epithelia were stimulated with an inoculum of exotoxin A–deficient *Staphylococcus aureus*. We observed a significant increase in miR-509–3p and miR-494 expression in both cell types (Figure 3A). *S. aureus* is a frequent pathogen in patients with CF, particularly during the first decade of life (29). Whereas *S. aureus* may elicit innate immune responses through multiple mechanisms, the increased expression of miR-509–3p and miR-494 was notable and raises questions about how complex infectious stimuli may alter CFTR expression in the airways.

We next asked whether a proinflammatory cytokine stimulus might influence the expression of miR-509–3p and miR-494. Treatment with either TNF-α or IL-1β caused a significant increase in miR-509–3p and miR-494 expression in both cell types (Figure 3B). These results suggest that miR-509–3p and miR-494 are regulated in part by a common pathway that responds to bacterial and proinflammatory stimuli.

TNF-α and IL-1β Regulate CFTR Expression

Previous studies suggested a role for proinflammatory cytokines in the regulation of CFTR expression (30–37). To test the possibility that the TNF-α–mediated or IL-1β–mediated increases in miR-509–3p and miR-494 expression influence CFTR abundance, we measured CFTR expression in well-differentiated primary cultures of human non-CF airway epithelia and polarized ALI cultures of Calu-3 airway epithelia after TNF-α or IL-1β treatment. The increased expression of miR-509–3p and miR-494 (Figure 3B) was associated with concurrent decreases in CFTR mRNA and protein concentrations in primary airway epithelia (Figures 3C and 3D) and Calu-3 cells (Figures E6A and E6B). Furthermore, TNF-α and IL-1β significantly diminished cyclic adenosine monophosphate (cAMP)–stimulated G_t, and I_t in primary airway epithelia (Figures 3E, 3F, and E7) and in polarized Calu-3 cultures (Figures E6C and E6D).

These results suggest the possibility that the repression of CFTR expression by TNF-α or IL-1β occurs via the action of miR-509–3p and miR-494. To test this hypothesis, we transfected Calu-3 and primary human non-CF airway epithelial cells with a scrambled control oligonucleotide (Scr oligo), or with the anti-miRs for both miR-509–3p and miR-494. Notably, anti-miR transfections did not interfere with the induction of miRNA expression in Calu-3 cells (Figure E8) or in primary airway epithelia (Figures E9A and E9B). After these procedures, we treated the cells with TNF-α or IL-1β. TNF-α

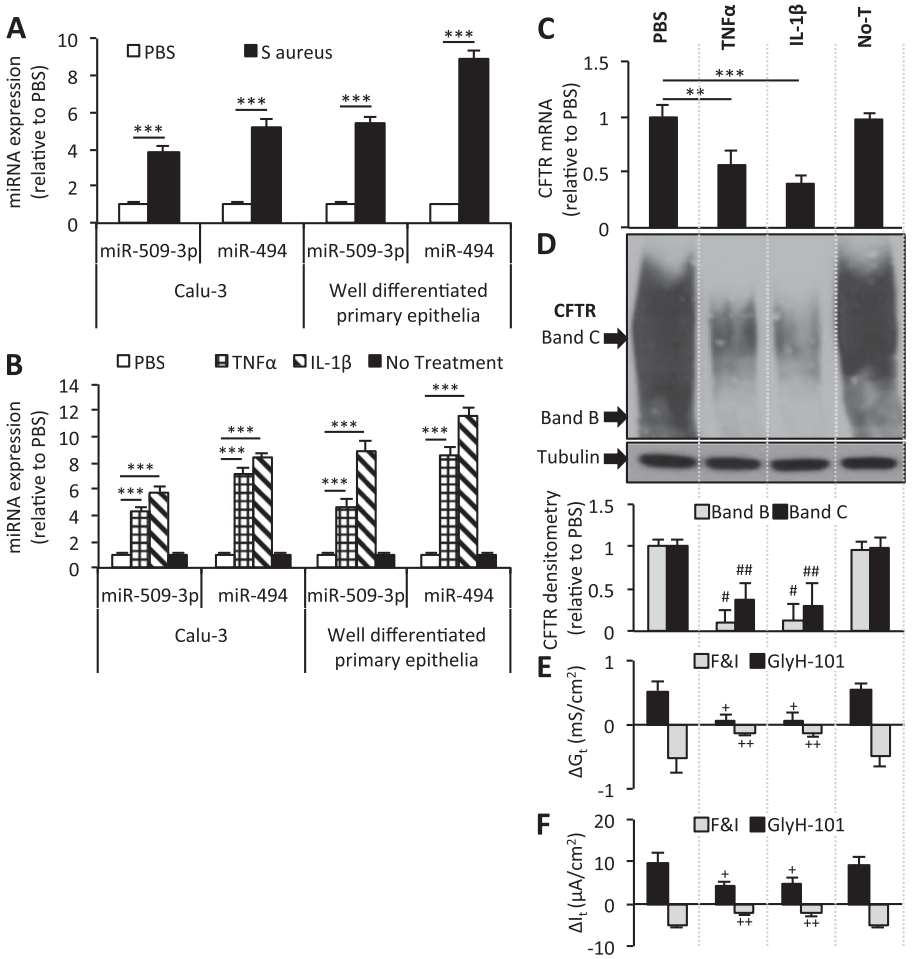

Figure 3. Bacteria stimuli and TNF-α and IL-1β regulate CFTR expression. (*A*) Calu-3–polarized cultures ($n = 10$) and well-differentiated primary epithelia (eight donors) were apically stimulated with an inoculum of *Staphylococcus aureus* (USA300Δ*hla* mutant) suspended in PBS (1.0×10^7 colony-forming units/millicell). RNA was harvested 48 hours after stimulation, and profiled for miRNA expression by quantitative RT-PCR. (*B*) Calu-3–polarized cultures ($n = 10$) and well-differentiated primary epithelia (eight donors) were basolaterally stimulated with 100 ng/ml of the indicated cytokines. RNA was harvested 8 hours after stimulation and profiled for miRNA expression by quantitative RT-PCR. (*C*) *CFTR* mRNA concentrations were determined according to quantitative RT-PCR in well-differentiated primary epithelia. ($n = 8$). (*D*) CFTR protein abundance 8 hours after stimulation in well-differentiated primary epithelia. Densitometry and relative fold change of CFTR protein abundance ($n = 3$, three replicates each) were ascertained. Changes in (*E*) conductance (G_t) and (*F*) transepithelial current (I_t) were measured in well-differentiated primary epithelia, 8 hours after the indicated treatments ($n = 6$, two replicates each). Basal transepithelial resistance range, 191–264 ohms · cm^2; I_t range, 9–28 microamperes · cm^2. No-T, no treatment. *Error bars* indicate the mean ± SE. Statistical significance was determined by the Student *t* test. $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{\#}P < 0.01$, relative to PBS CFTR band B. $^{\#\#}P < 0.01$, relative to PBS CFTR band C. $^{+}P < 0.01$ and $^{++}P < 0.01$, relative to ΔG_t or ΔI_t in PBS-treated samples upon forskolin and IBMX (F&I) or CFTR inhibitor GlyH-101 treatment, respectively.

or IL-1β stimulation alone (pretransfected with Scr oligo) decreased CFTR expression and function in Calu-3 cells (Figures 4A–4D), compared with the PBS control. However, the TNF-α–treated or IL-1β–treated Calu-3 cultures that were pretransfected with the anti-miRs showed a significantly reduced effect on CFTR mRNA, protein, G_t, and I_t (Figures 4A–4D). Similar results were observed in primary cultures of human non-CF airway epithelia, where the absence of miRNA activity significantly reduced the effects of TNF-α or IL-1β on *CFTR* mRNA concentrations (Figure E9C).

To examine the influence of miRNA activity on cytokine-mediated CFTR repression, we performed two-way ANOVA. The results indicate that the repression of CFTR expression by TNF-α and IL-1β is greater when miR-509–3p and miR-494 bind to the *CFTR* 3′ UTR (Calu-3 cells, Table E2A; primary airway epithelia, Table E2B). Interestingly, the effect of IL-1β on CFTR was almost entirely abrogated by blocking both miRNAs (Figures 4 and E9C), suggesting that the IL-1β–stimulated repression of CFTR is more dependent on miR-509–3p and miR-494 than is that of TNF-α. These results indicate that the binding of miR-509–3p and miR-494 to the 3′ UTR of *CFTR* influences TNF-α–mediated and IL-1β–mediated regulation of CFTR expression in airway epithelia.

Expression of miR-509–3p and miR-494 Is Regulated by NF-κB Signaling

The increase in miRNAs in response to *S. aureus* or proinflammatory cytokines suggests the possibility that common transcriptional machinery is activated in response to these stimuli. A number of bacterial products are known to activate NF-κB signaling (38). Similarly, both TNF-α and IL-1β signal in part via NF-κB (39, 40). Because the promoter regions of miR-509–3p and miR-494 have not been characterized, we looked for NF-κB binding sites matching the general consensus binding sequence GGGRNNYYCC (R-purine and Y-pyrimidine) (41). We identified three predicted binding sites in the 5′ flanking sequence of each miRNA (Figure E10), suggesting that NF-κB may interact with the promoters of miR-509–3p and miR-494. Therefore, we hypothesized that NF-κB might contribute to the transcriptional activation of miR-509–3p and miR-494 in response to these cytokines.

To test this hypothesis, we first transduced polarized ALI cultures of Calu-3 cells and well-differentiated primary cultures of human non-CF airway epithelia with either an empty adenovirus (Ad-e) or an adenovirus expressing a dominant-negative IκB-α (Ad.IkBaS32/36A or Ad-dnIκB-α) (42) under the control of a cytomegalovirus (CMV) promoter. Three days later, we treated the cells with cytokines. Cells expressing the dominant-negative IκB-α failed to increase miR-509–3p and miR-494 concentrations significantly after stimulation with TNF-α or IL-1β, in contrast to control samples (Ad-e–transduced) (Calu-3 cells, Figures E11A and E11B; airway epithelia, Figures E12A and E12B). Hence, an activated NF-κB complex is needed for miR-509–3p and miR-494 induction by TNF-α or IL-1β, suggesting that the NF-κB transcription factor complex regulates the expression of miR-509–3p and miR-494.

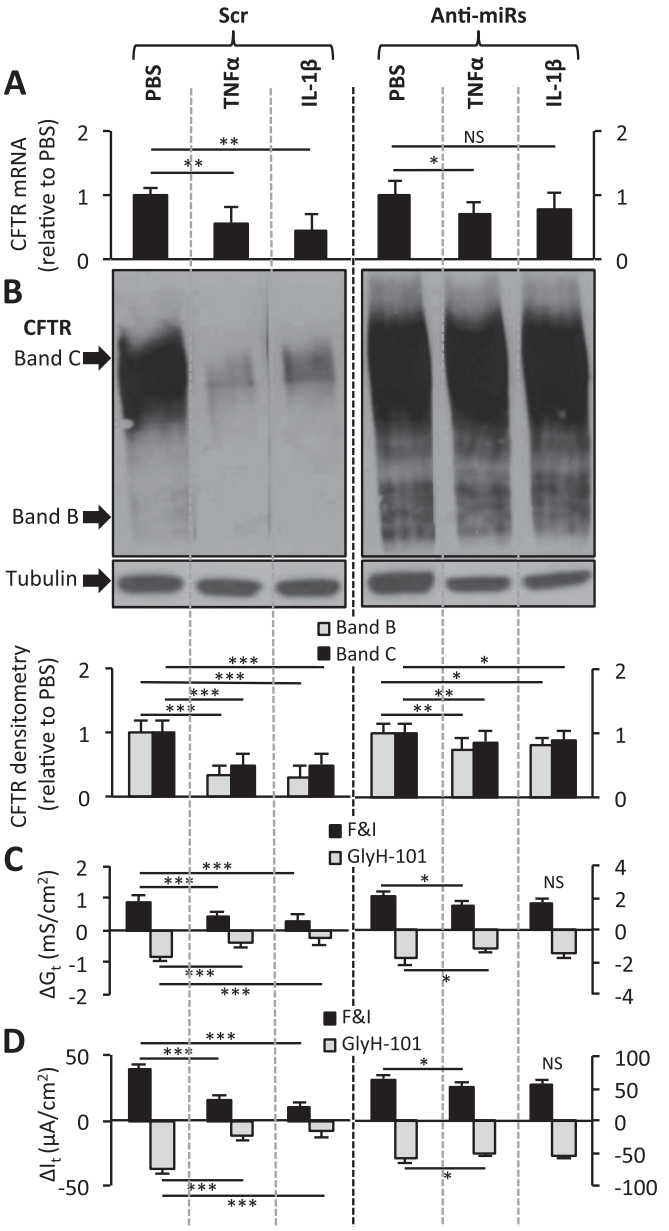

Figure 4. TNF-α and IL-1β regulate CFTR expression via the actions of miR-509–3p and miR-494. (*A*) *CFTR* mRNA concentrations were determined by quantitative RT-PCR in polarized Calu-3 cells, 8 hours after cytokine stimulation. Cultures were transfected with oligonucleotides 10 days before cytokine stimulation ($n = 4$, four replicates each). (*B*) CFTR immunoblot in pretransfected Calu-3 cells, 8 hours after PBS or TNF-α or IL-1β stimulation. Densitometry and relative fold change of CFTR protein abundance ($n = 4$, four replicates each) were determined. Immunoblotting was rearranged to suit the presentation order. Changes in (*C*) conductance (G_t) and (*D*) transepithelial current (I_t) were measured in polarized Calu-3 cells with the indicated treatments ($n = 4$). Basal transepithelial resistance range, 294–415 ohms · cm^2; I_t range, 39–63 microamperes · cm^2. All *error bars* indicate the mean ± SE. Statistical significance was determined by the Student *t* test. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. NS, not statistically significant.

To address whether blocking NF-κB activity diminishes the effect of cytokines on CFTR, we compared the effect of cytokines on CFTR in the presence or absence of a dominant-negative inhibitor of NF-κB in Calu-3 cells (Figure 5). TNF-α or IL-1β stimulation alone (transduced with Ad-e) decreased CFTR expression and function (Figures 5A–5D), and increased miRNA concentrations (Figures E11A and E11B), compared with the PBS control samples. However, Calu-3 epithelia pretreated with the dominant-negative inhibitor of NF-κB and then treated with TNF-α or IL-1β showed almost no induction of miR-509–3p and miR-494 concentrations (Figures E11A and E11B), concurrent with a significantly diminished effect on CFTR mRNA, protein, G_t, and I_t (Figures 5A–5D). Similar results were observed in well-differentiated primary cultures of human non-CF airway epithelia, where the inhibition of NF-κB led to a reduced induction of miR-509–3p and miR-494 expression in response to TNF-α or IL-1β (Figures E12A and E12B), concurrent with significantly diminished effects on *CFTR* mRNA expression (Figure E12C).

To examine the influence of NF-κB activity on cytokine-mediated CFTR repression, we performed two-way ANOVA. The results indicate that the repression of CFTR expression by TNF-α and IL-1β is greater in the presence of a functional NF-κB complex (Calu-3 cells, Table E3A; primary airway epithelia, Table E3B). Thus, a dominant-negative IκB-α suppressed both the induction of miR-509–3p and miR-494, and the repression of CFTR in response to a proinflammatory stimulus. These data provide evidence that the cytokine-mediated repression of CFTR is regulated in part via the NF-κB–dependent induction of miR-509–3p and miR-494 and their actions on the *CFTR* 3′ UTR.

DISCUSSION

Here we show that miR-509–3p and miR-494 influence *CFTR* expression in human airway epithelia. Both miRNAs repress CFTR mRNA, protein abundance, and function in a site-specific manner. Interestingly, the binding sites of both miRNAs lie within 100 base pairs of each other in the *CFTR* 3′ UTR. This finding, along with the enhanced effect seen when the mimic or anti-miR of both miRNAs is cotransfected, strongly suggests that miR-509–3p and miR-494 may act cooperatively to regulate CFTR expression and function in the airway epithelium. The expression of both miRNAs was increased in epithelia isolated from patients with CF. In addition, bacterial infection and first-response cytokines increased miR-509–3p and miR-494 concentrations. We show for the first time that TNF-α and IL-1β increase miR-509–3p and miR-494 concentrations, in part via the action of the NF-κB transcriptional activator complex, and that the increased abundance of miR-509–3p and miR-494 results in a correlative decrease of CFTR expression and function. These findings are significant because they implicate inflammatory responses, and in particular TNF-α or IL-1β, in miRNA-mediated CFTR repression.

Our findings are consistent with previous results indicating that the abundance of CFTR mRNA (17) and protein is similar in CF and non-CF airway epithelia (43). Although we did not detect a reduced abundance of *CFTR* mRNA in CF epithelia (Figure 6A), we note that the regulation of CFTR expression is complex, and that counterregulatory effectors may exert an impact (21, 35, 44–46). For example, NF-κB has been reported to interact directly with the *CFTR* promoter, positively regulating its transcription (35). Moreover, we previously showed that miR-138 positively regulates CFTR transcription and expression (21), increasing *CFTR* mRNA concentrations by de-repressing transcription. When we profiled for miRNA expression in well-differentiated primary cultures of human non-CF and CF (homozygous for *CFTR*-ΔF508) airway epithelia, we found miR-138 to be increased in CF airway epithelia (Table E1). These results suggest that counterregulatory effectors in CF airway epithelia could increase *CFTR* mRNA abundance.

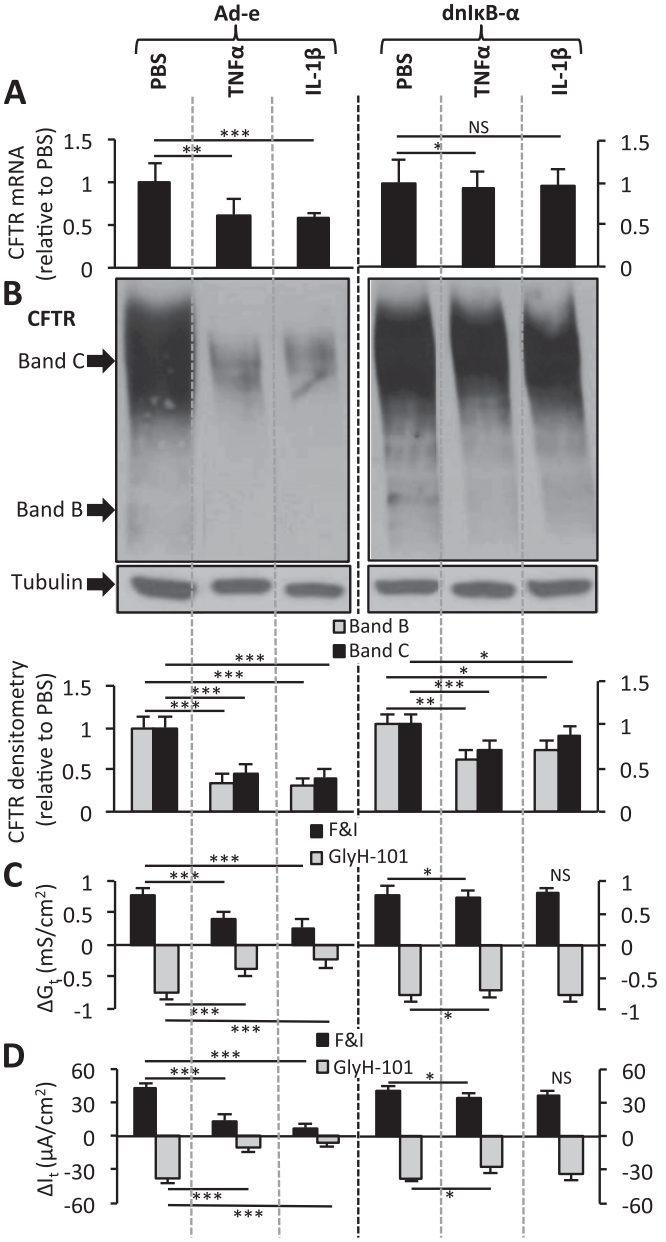

Figure 5. TNF-α and IL-1β require a functional NF-κB complex to regulate CFTR. (*A*) *CFTR* mRNA concentrations were ascertained according to quantitative RT-PCR in Calu-3–polarized cultures, 8 hours after cytokine stimulation ($n = 4$, four replicates each). Cultures were transduced 3 days before cytokine stimulation. (*B*) CFTR immunoblot in pretransduced Calu-3 cells, 8 hours after PBS or TNF-α or IL-1β stimulation. Densitometry and relative fold change of CFTR protein abundance ($n = 4$, three replicates each) were determined. Immunoblotting was rearranged to suit the presentation order. Changes in (*C*) conductance (G_t) and (*D*) transepithelial current (I_t) were measured 8 hours after the indicated treatments in Calu-3–polarized cultures ($n = 4$). Basal transepithelial resistance range, 318–479 ohms · cm^2; and I_t range, 32–58 microamperes · cm^2. All *error bars* indicate the mean ± SE. Statistical significance was determined by the Student *t* test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. Ad-e, empty adenovirus; dnIκB-α, dominant-negative IκB-α; NS, not statistically significant.

Such counterregulatory mechanisms may also vary between genotypes. To test this, well-differentiated primary airway epithelia from three CF donors (homozygous for *CFTR*-ΔF508) were treated with PBS, TNF-α, or IL-1β. The abundance of both miR-509–3p and miR-494 increased (Figure 6B), although the magnitude of increase was lower compared with non-CF primary epithelia treated with cytokines (Figure 3B). This genotype-dependent difference could be attributable to the unavailability of the NF-κB transcription factor complex or a saturation of miRNA expression. *CFTR* mRNA concentrations remained unchanged after treatment (Figure 6C). This result suggests that *CFTR* mRNA is subject to regulatory pathways that may differ between CF and non-CF airway epithelia. In addition, the effects of chronic inflammatory stimuli may modify responses in CF epithelia.

The miRNA binding sites on the mutant *CFTR* 3′ UTR may possess an altered secondary structure, thereby preventing or reducing miRNA binding. The ΔF508 mutation was reported to change the secondary structure of *CFTR* mRNA (47). This

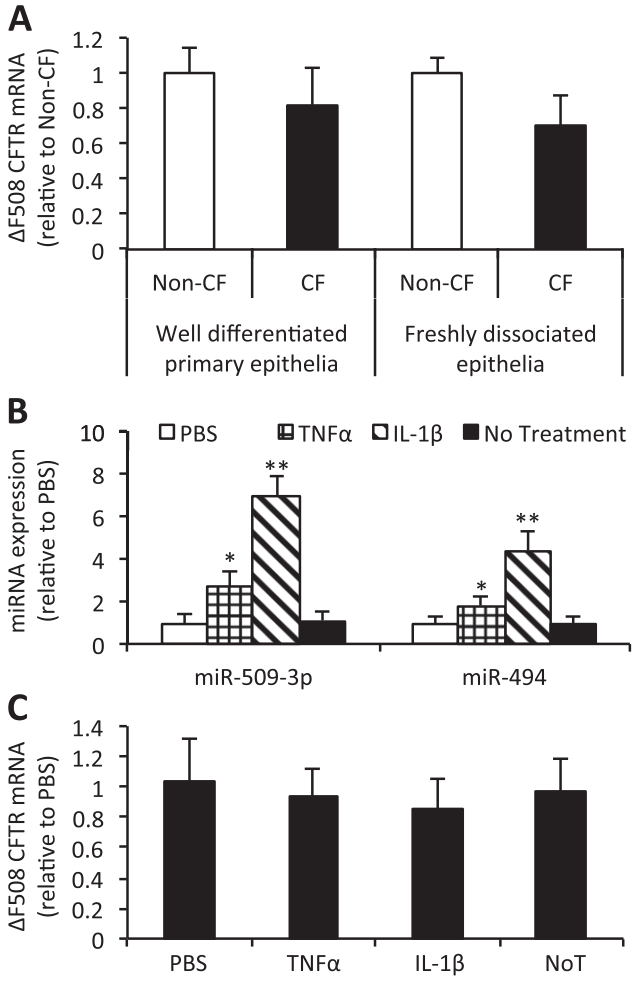

Figure 6. *CFTR* mRNA expression is not significantly different in CF airway epithelia. (*A*) RNA harvested from well-differentiated primary epithelia (six donors per genotype) and from freshly dissociated tracheobronchial airway epithelia from donors (five donors per genotype) were profiled for *CFTR* mRNA concentrations by quantitative RT-PCR. CF donors were homozygous for CFTR-ΔF508. (*B* and *C*) Well-differentiated primary epithelia (three donors, with three cultures each) were basolaterally stimulated with 100 ng/ml of the indicated cytokines. RNA was harvested 8 hours after stimulation, and profiled for (*B*) miRNA expression and (*C*) *CFTR* mRNA concentrations according to quantitative RT-PCR. All *error bars* indicate the mean ± SE. Statistical significance was determined by the Student *t* test. *$P < 0.05$. **$P < 0.01$. NoT, no treatment.

might influence the site availability or its thermodynamics, factors known to influence miRNA binding (48–50). CFTR expression and biosynthesis are subject to complex regulatory mechanisms involving multiple pathways (16, 21, 46, 51), some interacting with the *CFTR* 3′ UTR (19, 30). These scenarios point to the possibility that the miRNA-mediated regulation of CFTR expression may be transient, dynamic, or subject to counterregulatory effectors that can increase *CFTR* transcription in CF airway epithelia (21, 35).

Oglesby and colleagues previously reported an increase in miR-494 concentrations in bronchial epithelial brushings from human CF airways (52). The repression of *CFTR* by miR-494 has also been reported previously (19, 20), without investigation of the functional effects on anion transport. Our results confirm an increase in miR-494 expression in human CF airway epithelia, and provide new evidence that miR-494 and miR-509–3p regulate both CFTR expression and anion transport. MiR-509–3p is a primate-specific small RNA transcript expressed from three distinct genomic loci within a 2-kb region on chromosome Xq, and has not previously been reported to play a functional role in airway epithelia.

The TNF-α–mediated and IL-1β–mediated regulation of CFTR expression was addressed in previous studies (30–37). TNF-α was reported to decrease CFTR expression (33) and reduce *CFTR* mRNA stability via the influence of mitogen-activated protein kinase cascades (30, 33), and may be more effective in reducing *CFTR* mRNA concentrations in combination with IFN-γ (35). IL-1β, on the other hand, exhibits a biphasic effect on CFTR expression (31, 37). Concentrations greater than 1 to 10 ng/ml decrease CFTR expression, whereas concentrations below 1 ng/ml exert the opposite effect. In experiments performed using human bronchial epithelial cells, low concentrations of IL-1β (with prolonged exposure) increased CFTR expression and function (37), whereas TNF-α exerted no effect on cAMP-dependent current (36). These studies emphasize the dose-dependent and time-dependent effects of cytokines on CFTR expression and function (30–37). Here we provide evidence that at higher concentrations and with acute exposure, TNF-α and IL-1β both repress CFTR via the induction of miRNAs that target the *CFTR* 3′ UTR, and coincidently alter CFTR function. Under the experimental conditions used in primary human airway epithelia and Calu-3 cells, we observed the TNF-α–mediated and IL-1β–mediated repression of CFTR function in airway epithelia. Although the genomic elements controlling the expression of miR-494 and miR-509–3p remain poorly understood, our findings indicate that inflammatory stimuli and NF-κB signaling play a role in regulating their abundance. The findings that both miRNAs increase in response to inflammatory stimuli suggest the possibility of a cooperative partnership between miR-509–3p and miR-494 in regulating ion and liquid transport.

miRNAs regulate multiple biological processes through their action on the 3′ UTR of target mRNA. Our discovery that miR-494 and miR-509–3p regulate both CFTR expression and function has implications for airway biology in health and disease. Because the overexpression of ΔF508-CFTR exerts little or no effect on rescuing Cl^- transport in native epithelia (21, 53), increasing ΔF508-CFTR concentrations with anti-miRs will be unlikely to pose therapeutic benefit for CF (caused by ΔF508-CFTR). However, in other settings, influencing CFTR abundance via miRNAs may have therapeutic implications. One example involves the overexpression of miR-138, which we demonstrated can rescue ΔF508-CFTR maturation and function in CF airway epithelia (21). Our findings highlight the potential for miRNAs to regulate airway epithelial cell function acutely in response to environmental stimuli. The dynamic modulation of airway surface liquid (ASL) volume and composition is an important homeostatic function in the respiratory tract. We speculate that changes in CFTR abundance might acutely affect ASL volume and composition, host defense, and mucociliary clearance (54). Further studies are needed to better define the role of miRNAs in airway epithelial cell biology and pulmonary disease pathogenesis.

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