ORIGINAL ARTICLE

miR-31 Dysregulation in Cystic Fibrosis Airways Contributes to Increased Pulmonary Cathepsin S Production

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Abstract

Rationale: Cathepsin S (CTSS) activity is increased in bronchoalveolar lavage (BAL) fluid from patients with cystic fibrosis (CF). This activity contributes to lung inflammation via degradation of antimicrobial proteins, such as lactoferrin and members of the β-defensin family.

Objectives: In this study, we investigated the hypothesis that airway epithelial cells are a source of CTSS, and mechanisms underlying CTSS expression in the CF lung.

Methods: Protease activity was determined using fluorogenic activity assays. Protein and mRNA expression were analyzed by ELISA, Western blotting, and reverse-transcriptase polymerase chain reaction.

Measurements and Main Results: In contrast to neutrophil elastase, CTSS activity was detectable in 100% of CF BAL fluid samples from patients without *Pseudomonas aeruginosa* infection. In this study, we identified epithelial cells as a source of pulmonary CTSS activity with the demonstration that CF airway epithelial cells express and secrete significantly more CTSS than non-CF control cells in the absence of proinflammatory stimulation. Furthermore, levels of the transcription factor IRF-1 correlated with increased levels of its target gene CTSS. We discovered that miR-31, which is decreased in the CF airways, regulates IRF-1 in CF epithelial cells. Treating CF bronchial epithelial cells with a miR-31 mimic decreased IRF-1 protein levels with concomitant knockdown of CTSS expression and secretion.

Conclusions: The miR-31/IRF-1/CTSS pathway may play a functional role in the pathogenesis of CF lung disease and may open up new avenues for exploration in the search for an effective therapeutic target.

Keywords: protease; microRNA; cystic fibrosis; epithelium

A major feature of the cystic fibrosis (CF) airways is the presence of free and active proteases, which are responsible for the destruction of lung tissue and other soluble proteins present in the respiratory tract. In CF, disruption of the protease-antiprotease balance has been demonstrated to be established by the age of 1 year, and is thought to be sustained chronically thereafter (1). Lung remodeling is evident soon after diagnosis even in children diagnosed with CF following newborn screening (2–5). Furthermore, structural lung disease is progressive and is associated with worsening inflammation and pulmonary infection (6, 7). Neutrophil elastase (NE) is regarded as a key driver of lung destruction (3, 8) and inflammation

(Received in original form November 12, 2013; accepted in final form April 28, 2014)

Supported by the Cystic Fibrosis Foundation (TAGGAR12IO), the Department of Education and Learning (C.C.T.), P01 HL-51670 (P.B.M.), P01 HL-091842 (P.B.M.), P30 DK-54759, and the Roy J. Carver Charitable Trust (P.B.M.).

Author Contributions: S.W., C.M.G., and C.C.T. conceived of and designed experiments. S.W., I.K.O., C.L.W.-L., and J.A.B. performed experiments. P.M., D.F.M., C.J.S., N.G.M., C.M.G., J.A.B., and P.B.M. provided samples and/or reagents. S.W., C.M.G., and C.C.T. analyzed the data. S.W. and C.C.T. wrote the manuscript.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 190, Iss 2, pp 165–174, Jul 15, 2014

Originally Published in Press as DOI: 10.1164/rccm.201311-1986OC on June 18, 2014
Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the Subject: Dysregulated inflammation in the cystic fibrosis (CF) lung is caused, in part, by excessive protease activity. We have previously demonstrated the presence of the potent elastase, cathepsin S (CTSS), in the CF lung. However, the reasons for up-regulated CTSS in the CF lung are unclear and its correlation to lung function in CF is unknown.

What This Study Adds to the Field: We now show that CTSS activity and levels are significantly increased in CF regardless of infection status and correlate negatively with lung function. We also show that CTSS expression is significantly up-regulated in CF airway epithelium because of altered miRNA-31 expression. These findings indicate that CTSS is a significant contributor to lung inflammation in the CF lung and may represent a viable therapeutic target in this setting.

especially during chronic *Pseudomonas aeruginosa* infection (9, 10). In contrast to NE, the role of cysteine cathepsins in CF has been relatively unexplored to date.

In the last decade, attention to the role of cathepsin S (CTSS) in cancer and heart disease has greatly increased (11, 12). We and others have previously reported the presence of members of the elastolytic cathepsin family including CTSS in cell-free CF lung secretions (13–16). In contrast to other cysteine cathepsin isoforms, CTSS can retain proteolytic activity after prolonged exposure to neutral pH in the extracellular environment, where it retains nearly as much elastase activity as NE (17–20). Consequently, in addition to pivotal roles in cellular events, such as antigen processing (21), CTSS has the capacity to promote remodeling of the extracellular matrix via its potent elastinolytic activity. A recent report demonstrated the ability of CTSS to activate the epithelial sodium channel, which may be pathophysiologically relevant in inflammatory diseases, such as CF (22). In addition, CTSS can cleave and inactivate key antimicrobials in the CF airways including surfactant protein A (23), lactoferrin (14), and members of the β-defensin family (13). In contrast to NE, the cellular sources and role of CTSS in the CF lung has been relatively unexplored to date.

In this study, we expand our previous investigations into the presence and activity of CTSS in the CF lung with a focus on young patients with CF. A range of epithelial dysfunctions have been implicated in the pathogenesis of CF lung disease, which are ultimately consequences of mutations in the CF transmembrane conductance regulator (*CFTR*) gene. Given that epithelial cells have been reported to express CTSS (21, 24, 25), we investigated the hypothesis that CF airway epithelial cells contribute to the protease burden of the CF lung by releasing increased levels of CTSS compared with non-CF control cells, and explore the mechanism responsible for our observations. Some of the results of these studies have been previously reported in the form of abstracts (26, 27).

Methods

Full details are available in the online supplement.

Bronchoalveolar Lavage Fluid Samples and Study Approval

Group 1 pediatric CF subjects. Bronchoalveolar lavage (BAL) fluid samples were obtained from 26 patients (0.7–18 yr; mean age, 10.84 yr) diagnosed with CF; 11 with *P. aeruginosa* infection (Ps+) and 15 without (Ps−), as described previously (9). We refer readers to (9) and Table E1 in the online supplement for further clinical information, cell counts, and details of pathogens detected in this cohort of patients. Ethical approval was obtained from the institutional review board of the Adelaide and Meath Hospital incorporating the National Children's Hospital with all parents providing written informed consent before participation.

Group 2 preschool CF and non-CF subjects. BAL fluid was obtained from *Pseudomonas*-negative children with CF (n = 43; 0.9–5 yr; mean age, 2.91 yr) through the Study of Host Immunity and Early Lung Disease in CF. In addition, nine children who did not have CF (0.9–3 yr; mean age, 1.73 yr) were included as a non-CF control group. Children in this control group were undergoing clinically indicated BAL for a variety of reasons, most commonly a history of recurrent respiratory infection, hemoptysis, or cough. Details of pathogens detected and cell counts in the CF and non-CF BAL fluid and CF genotype details are presented in Table E2. Ethical approval was obtained from the institutional review board of Our Lady's Children's Hospital Crumlin with all parents providing written informed consent before participation.

Primary Bronchial Epithelial Cell Culture and Study Approval

Primary cultures of well-differentiated human bronchial epithelial cells (PBECs) were obtained from patients with CF (n = 8) and from non-CF (n = 8) healthy volunteers and grown at the air-liquid interface (28). The use of human tissues was approved by the University of Iowa Institutional Review Board.

Bronchial Brush Analysis and Study Approval

Following informed consent under a protocol approved by Beaumont Hospital Institutional Review Board, bronchial brushings were sampled from patients with CF (n = 5) and non-CF (n = 5) volunteers.

Epithelial Cell Lines

The human bronchial epithelial cell line 16HBE14o− (HBE), the CF bronchial epithelial cell line CFBE41o− homozygous for the F508del mutation (CFBE), the human tracheal epithelial cell line 9HTEo− (HTE), and the CF tracheal epithelial cell line CFTE29o− homozygous for the F508del mutation (CFTE) were obtained as a gift from Professor Dieter Gruenert (California Pacific Medical Centre Research Institute, San Francisco, CA) (29, 30). Cell lines were maintained in Minimum Essential Medium (Life Technologies Ltd., Paisley, UK), supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies Ltd.), 2 mM L-glutamine, and 1% penicillin-streptomycin (PAA Laboratories GmbH, Pasching, Austria) at 37°C under 5% CO_2.

Statistical Analysis

All data were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc.,

San Diego, CA) and are reported as mean ± SEM or median (interquartile range) where appropriate. Results are representative of at least n = 3 unless otherwise indicated. Means were compared by unpaired *t* test, Mann-Whitney test, one-way analysis of variance, or Kruskal-Wallis test as appropriate. *P* less than 0.05 was accepted to indicate statistical significance. The relationships between clinical parameters were verified using Spearman correlation.

Results

Increased CTSS in the CF Lung Correlates Negatively with Lung Function in a Pediatric CF Patient Cohort

We previously reported the presence of active CTSS in the CF lung (13, 14). In this study, we expanded these observations by first comparing CTSS activity and levels in BAL fluid from a cohort of pediatric patients with CF stratified on the basis of the absence (Ps−; n = 15) or presence (Ps+; n = 11) of chronic *P. aeruginosa* infection as described previously (9). Details of pathogens detected in this CF cohort are presented in Table E1. In agreement with previous work (16), there was no significant difference in either CTSS activity (Figure 1A) or CTSS levels (Figure 1B) in Ps+ compared with Ps− CF BAL, which supports the suggestion that CTSS may not be not a reliable marker of *P. aeruginosa* infection in CF. However, both CTSS activity (Figure 1C; $r = -0.56$; $P = 0.0069$) and CTSS levels (Figure 1D;

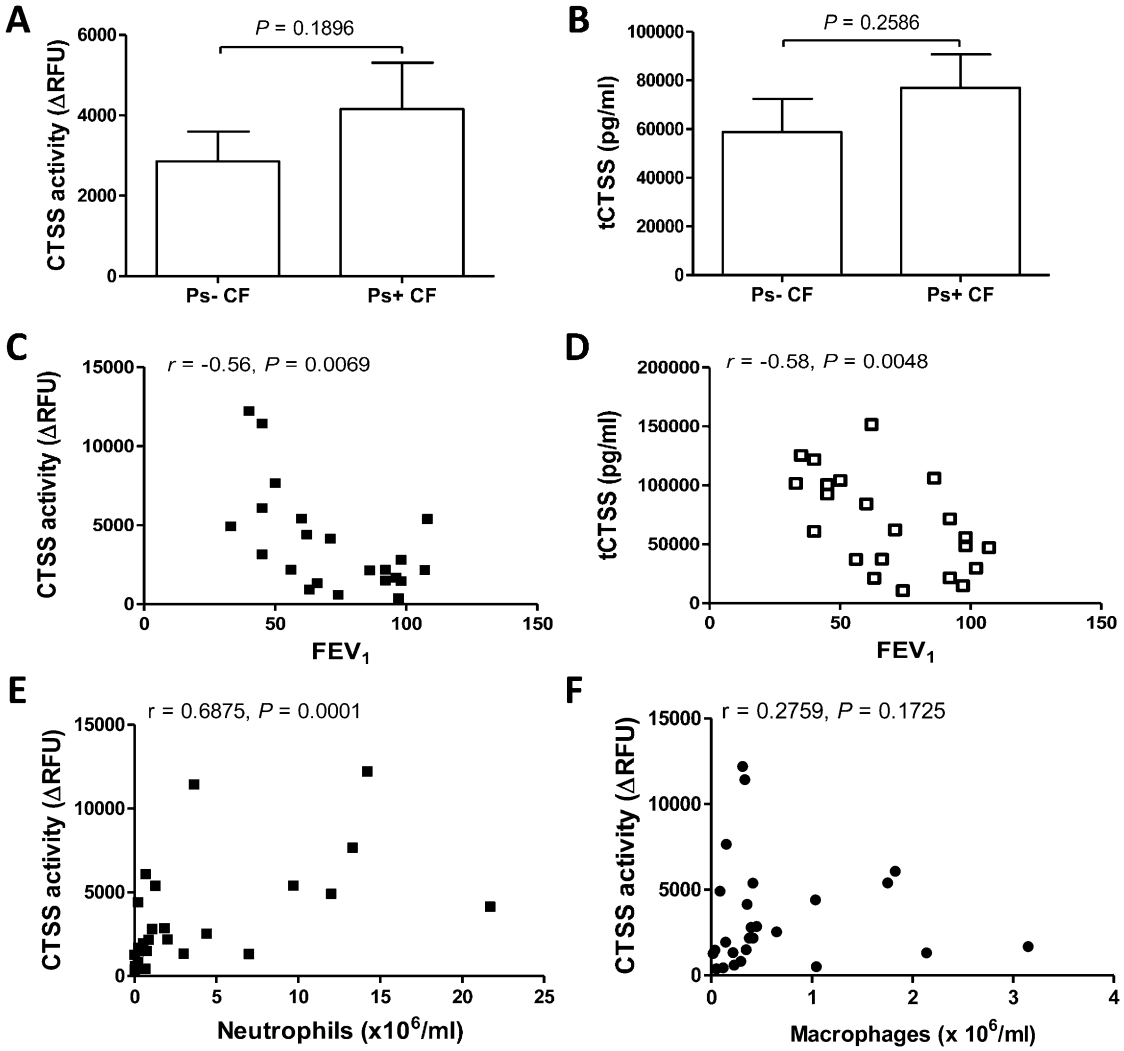

Figure 1. Increased cathepsin S (CTSS) levels and activity in cystic fibrosis (CF) bronchoalveolar lavage (BAL) fluid correlate negatively with lung function. (*A*) CTSS activity in BAL fluid from a cohort of pediatric *Pseudomonas*-infected (Ps+; n = 15) and noninfected (Ps−; n = 11) patients with CF (Group 1) was determined using the fluorogenic FR-AMC substrate (pH 7.5). Results were expressed as the change (Δ) in relative fluorescence units (ΔRFU) over time. (*B*) Total CTSS levels (tCTSS) in Ps− and Ps+ CF BAL fluid were quantified by ELISA. There was no significant difference between Ps− and Ps+ CF BAL fluid for either parameter measured. The correlation between CF BAL fluid (*C*) CTSS activity and (*D*) tCTSS levels and FEV_1 (n = 22) was determined using Spearman correlation. The correlation between CF BAL fluid CTSS activity and (*E*) neutrophils and (*F*) macrophages were determined using Spearman correlation.

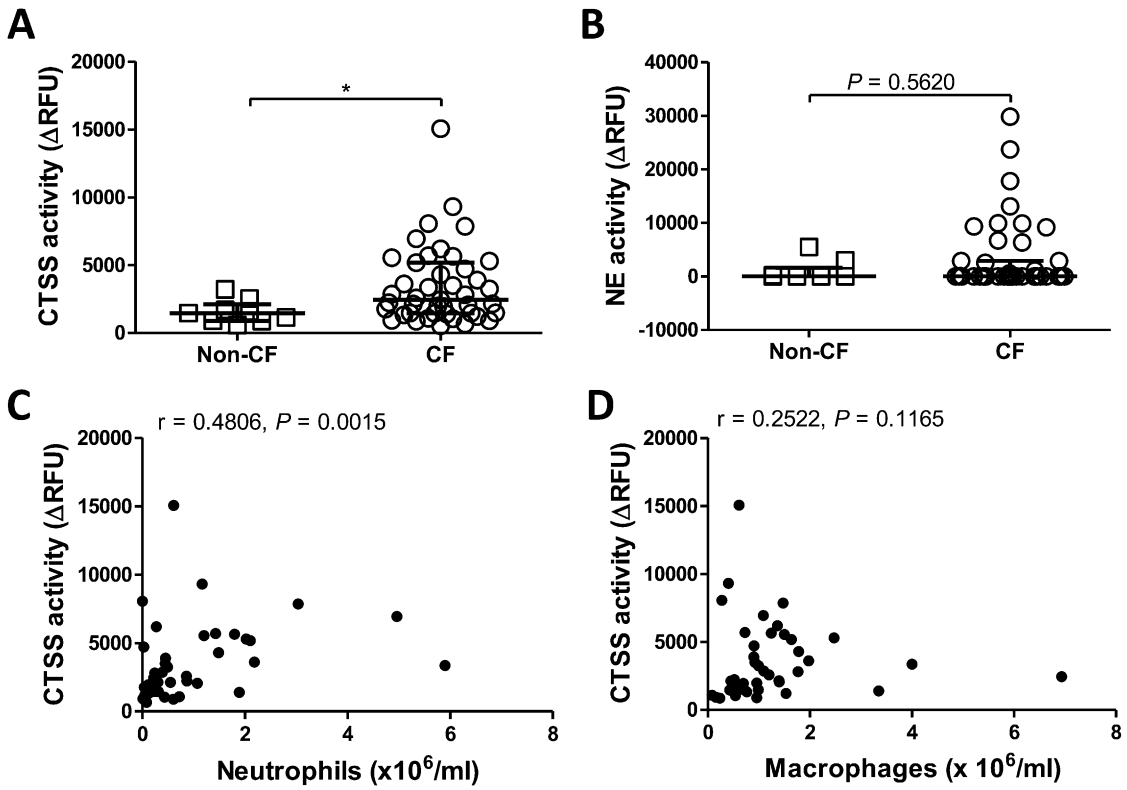

Figure 2. Profile of cathepsin S (CTSS) and neutrophil elastase (NE) activity in bronchoalveolar lavage (BAL) fluid from non–cystic fibrosis (CF) and CF children who tested negative for *Pseudomonas*. (*A*) CTSS and (*B*) NE activity were quantified in BAL fluid from a cohort of preschool, *Pseudomonas*-negative non-CF (n = 9) and CF children (n = 43; Group 2). CTSS and NE activities were detected using the fluorogenic substrates FR-AMC (pH 7.5) and AAPV-AMC, respectively. Results were expressed as the change (Δ) in relative fluorescence units (ΔRFU) over time. CTSS activity was detectable in all 43 CF samples analyzed. However, in contrast, NE activity was undetectable in most samples (>60%). The correlation between CF BAL fluid CTSS activity and (*C*) neutrophils and (*D*) macrophages were determined using Spearman correlation. $^{*}P < 0.05$.

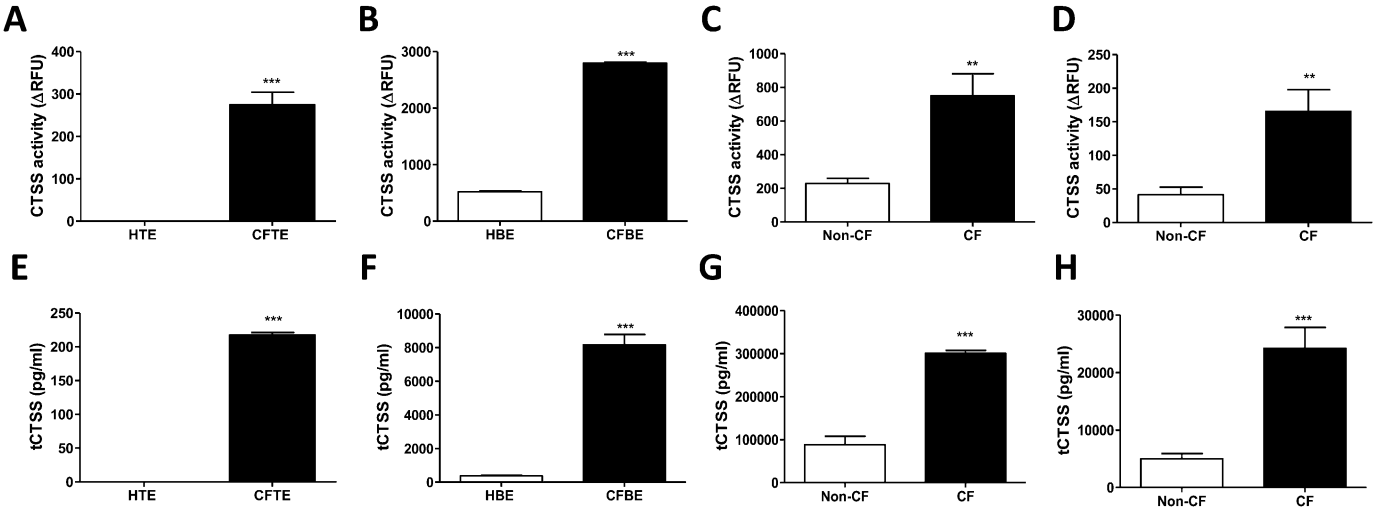

Figure 3. Basal cathepsin S (CTSS) secretion from unstimulated human cystic fibrosis (CF) and non-CF pulmonary epithelial cells. CTSS activity in cell-free supernatants of non-CF and CF (*A*) tracheal (human tracheal epithelial cell line 9HTEo− [HTE], CF tracheal epithelial cell line CFTE29o− [CFTE]) and (*B*) bronchial epithelial (human bronchial epithelial cell line 16HBE14o− [HBE], CF bronchial epithelial cell line CFBE41o− [CFBE]) cell lines, (*C*) primary human bronchial epithelial cell (PBEC, n = 4) apical washes, and (*D*) PBEC basolateral media was analyzed using FR-AMC (pH 7.5). Results were expressed as the change (Δ) in relative fluorescence units (ΔRFU) over time. (*E–H*) Total CTSS (tCTSS) levels in cell line supernatants (*E* and *F*), PBEC apical washes (*G*), and PBEC basolateral media (*H*) were quantified by ELISA. $^{**}P < 0.01$, $^{***}P < 0.001$.

$r = -0.58$; $P = 0.0048$) correlated negatively with FEV_1 (% predicted) suggesting that increased CTSS is associated with a decline in lung function in CF. In addition, CF BAL fluid CTSS activity was found to correlate significantly with BAL neutrophil counts (Figure 1E; $r = 0.6875$; $P = 0.0001$) but not with macrophage counts (Figure 1F; $r = 0.2759$; $P = 0.1725$).

CTSS Is Readily Detectable in BAL Fluid from Preschool Patients with CF

Our recent findings demonstrate that in CF lung secretions, the level of active NE is significantly increased in Ps+ compared with Ps− CF BAL fluid and sputum (9, 10). In contrast, CTSS activity and levels were found to be significantly elevated in CF BAL fluid regardless of the status of chronic *Pseudomonas* infection (Figures 1A and 1B). Therefore, to extend our analysis of CTSS and NE activities in young patients with CF without chronic *P. aeruginosa* infection, BAL fluid samples were obtained from a cohort of preschool children with CF (n = 43) who tested negative for *P. aeruginosa* infection. In addition, BAL fluid was obtained from *Pseudomonas*-negative non-CF children (n = 9). Details of pathogens detected in this cohort of CF and nonpatients with CF are presented in Table E2. As illustrated in Figure 2A, the levels of CTSS activity were significantly increased in CF BAL compared with BAL samples from children without CF ($P < 0.05$). In contrast, no significant difference in NE activity (Figure 2B) was detectable between the non-CF and CF BAL samples ($P = 0.562$). Interestingly, although CTSS activity was detectable in all CF and non-CF BAL samples, NE activity was undetectable in most samples from both groups (66.7% non-CF; 60.5% CF). This level of detection of NE is in agreement with previous reports in the literature (3, 7, 31). In agreement with our findings above, CF BAL fluid CTSS activity was found to correlate significantly with BAL neutrophil counts (Figure 2C; $r = 0.4806$; $P = 0.0015$) but not with macrophages (Figure 2D; $r = 0.2522$; $P = 0.1165$).

Expression and Secretion of CTSS from Unstimulated CF and Non-CF Epithelial Cells

Evidence to date suggests that CTSS has a restricted tissue distribution with

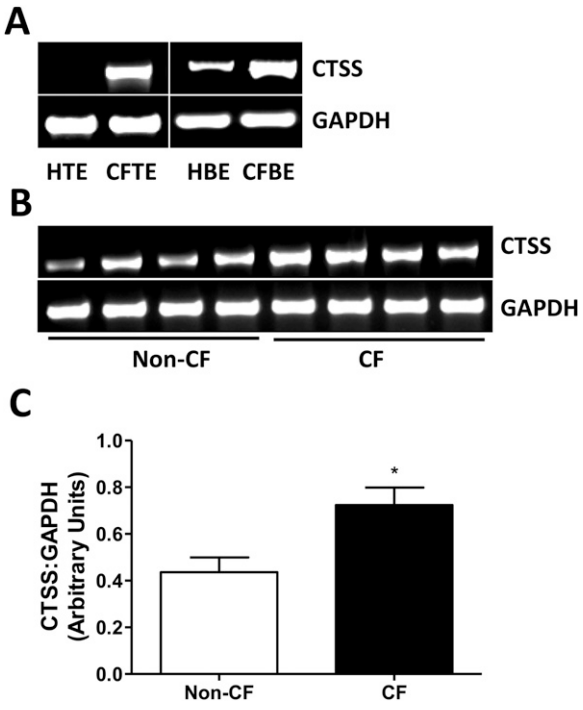

Figure 4. Increased basal cathepsin S (CTSS) mRNA expression in cystic fibrosis (CF) epithelial cells. (*A*) CTSS and glyceraldehyde phosphate dehydrogenase (GAPDH) expression were detected in tracheal (human tracheal epithelial cell line 9HTEo− [HTE], CF tracheal epithelial cell line CFTE29o− [CFTE]) and bronchial epithelial (human bronchial epithelial cell line 16HBE14o− [HBE], CF bronchial epithelial cell line CFBE41o− [CFBE]) cell lines and (*B*) primary human bronchial epithelial cells (PBEC, n = 4) by reverse-transcriptase polymerase chain reaction. (*C*) Densitometry of PBEC CTSS relative to GAPDH. *$P < 0.05$.

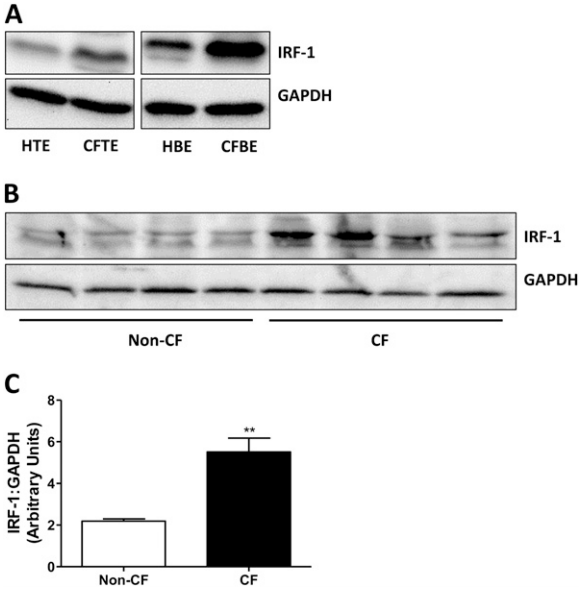

Figure 5. IRF-1 levels in non–cystic fibrosis (CF) and CF epithelial cells. (*A*) Western blotting of IRF-1 and glyceraldehyde phosphate dehydrogenase (GAPDH) in tracheal (human tracheal epithelial cell line 9HTEo− [HTE], CF tracheal epithelial cell line CFTE29o− [CFTE]) and bronchial (human bronchial epithelial cell line 16HBE14o− [HBE], CF bronchial epithelial cell line CFBE41o− [CFBE]) epithelial cell lines and (*B*) primary cultures of well-differentiated human bronchial epithelial cells (PBECs) (n = 4) whole-cell lysates. (*C*) Densitometry of PBEC IRF-1 relative to GAPDH. **$P < 0.01$.

predominant expression in antigen-presenting cells; however, more recent work has demonstrated expression of CTSS in airway epithelial cells (24, 25, 32). Given the prevalence of CTSS in the lungs of children with CF, we investigated whether pulmonary epithelial cells represent a possible source of CTSS in the CF lung. As illustrated in Figure 3, unstimulated CF tracheal (Figure 3A) and bronchial (Figure 3B) epithelial cell lines secreted significantly elevated levels of active CTSS compared with non-CF cells. These findings were consistent in well-differentiated PBECs because increased levels of extracellular CTSS activity were detected in both CF apical washes and basolateral media compared with non-CF (Figures 3C and 3D). These findings were consistent with findings by ELISA (Figures 3E and 3H) and Western blotting (*see* Figure E1) demonstrating that CF airway epithelial cells secrete more CTSS compared with non-CF control cells. We next examined the levels of CTSS mRNA in these cells by reverse-transcriptase polymerase chain reaction. In concurrence with the protein data, CTSS expression was found to be elevated in CF tracheal and bronchial epithelial cell lines (CFTE, CFBE) compared with non-CF control cells (HTE, HBE) (Figure 4A). These findings were confirmed in PBECs, with significantly increased CTSS mRNA levels detectable in CF PBECs compared with non-CF (Figures 4B and 4C). In contrast to CTSS, no changes in cathepsin B expression were found between CF and non-CF epithelial cells (*see* Figure E2).

Increased Levels of IRF-1 as a Result of miR-31 Deregulation Result in Increased Expression and Secretion of CTSS by CF Epithelial Cells

Several transcription factors are reported to regulate the expression of CTSS. Of particular interest is IRF-1, which has been shown to regulate CTSS expression in airway epithelial cells (25). Therefore, we investigated the status of IRF-1 in whole-cell lysates of CF and non-CF cells by Western blotting. Levels of IRF-1 were found to be elevated in CF compared with non-CF epithelial cell lines and consistent between tracheal and bronchial cell types (Figure 5A). Furthermore, levels of IRF-1 were significantly increased in CF PBECs compared with non-CF (Figures 5B and 5C). To establish a functional relationship between IRF-1 and CTSS in CF epithelial cells, we used short interfering RNA (siRNA) to knock down IRF-1 levels in CFBEs and examined the effects on CTSS levels. As illustrated in Figure 6A, transfection of CFBEs with IRF-1 siRNA effectively decreased IRF-1 protein levels compared with mock transfected (Con) and the scrambled control (Scr). This decrease in IRF-1 protein was associated with a marked reduction in CTSS expression (Figure 6B) but not cathepsin B (*see* Figure E3). Consistent with the reduced expression of CTSS, both extracellular CTSS activity (Figure 6C) and levels (Figure 6D) were significantly decreased in CFBEs transfected with IRF-1 siRNA compared with Scr control cells, demonstrating the functional importance of IRF-1 in CFBE CTSS expression.

To elucidate the mechanism responsible for increased levels of IRF-1 protein in CF epithelial cells, we considered the role of microRNAs (miRNAs). miR-31 is one of the most highly expressed miRNAs present

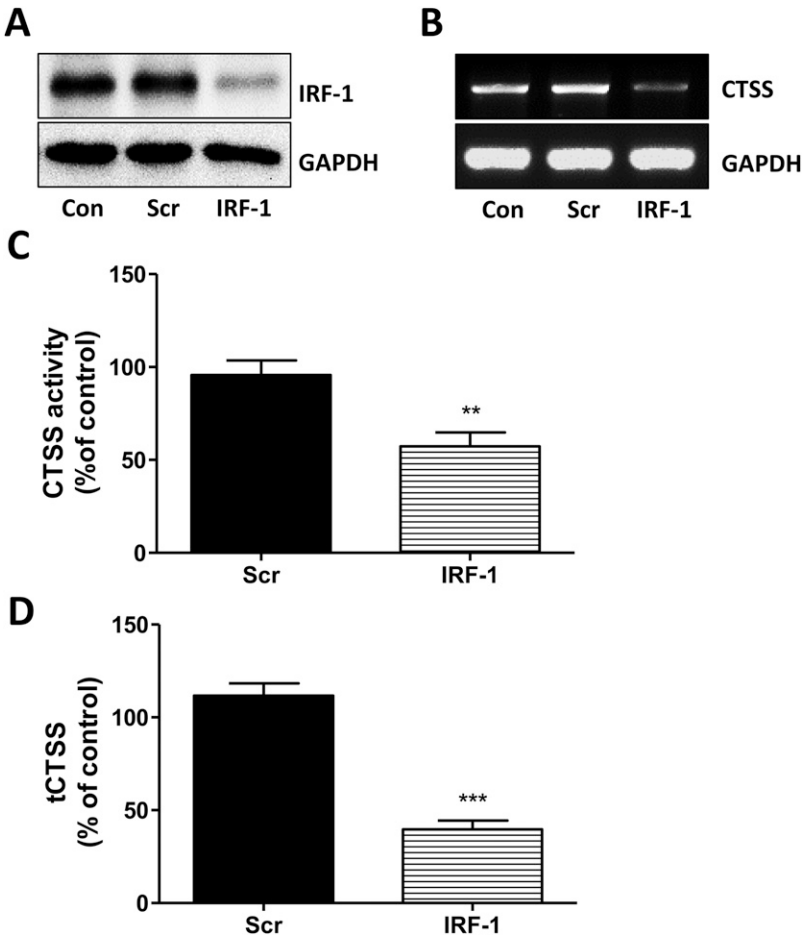

Figure 6. IRF-1 knockdown decreases cathepsin S (CTSS) expression and secretion from cystic fibrosis epithelial cells. The effects of IRF-1 knockdown on CTSS levels in cystic fibrosis bronchial epithelial cells (CFBEs) were investigated using IRF-1 siRNA. CFBEs were transfected with 100 nM IRF-1 siRNA (IRF-1), scrambled control (Scr), or mock transfected (Con) for 48 hours. After a further 24-hour incubation in fresh media, (*A*) knockdown of IRF-1 levels was confirmed by Western blotting and (*B*) CTSS mRNA levels were assessed by reverse-transcriptase polymerase chain reaction. (*C*) Extracellular CTSS activity was determined using the FR-AMC (pH 7.5) substrate and (*D*) extracellular tCTSS levels were quantified by ELISA. The data are the mean ± SEM of n = 3 and are expressed as % of mock transfected control cells (*C* and *D*). $^{**}P < 0.01$, $^{***}P < 0.001$. GAPDH = glyceraldehyde phosphate dehydrogenase; tCTSS = total CTSS levels.

in human non-CF primary well-differentiated airway epithelial cultures (33) and *in silico* analysis predicted that IRF-1 was a target of miR-31. Both microRNA.org and TargetScan 6.2 predicted two binding sites for miR-31 located at positions 606–613 (8mer, mirSVR score −0.8544) and 2182–2187 (7mer-1A, mirSVR score −0.001) of the IRF-1 3′ UTR (*see* Figure E4). miR-31 is not predicted to target CTSS mRNA. miR-31 was found to be significantly decreased in CF bronchial brushings compared with non-CF using Taqman MicroRNA Arrays v2.0 (34). Relative expression of miR-31 was validated independently by quantitative reverse-transcriptase polymerase chain reaction in separate cohorts of CF and non-CF individuals (n = 5) and, in agreement with previous data (34), miR-31 expression was significantly decreased in CF bronchial brushings compared with non-CF (Figure 7A).

Similarly, a consistent decrease in miR-31 expression was observed in CF PBECs (n = 4; Figure 7B) and both tracheal (Figure 7C) and bronchial (Figure 7D) epithelial cell lines compared with non-CF control cells. We next assessed the effect of miR-31 overexpression on CFBE IRF-1 and CTSS levels. Transfection of CFBEs with pre–miR-31 (PM-31) led to a significant increase in mature miR-31 compared with mock or negative control (PM-Neg) transfected cells (data not shown). As illustrated in Figure 8A, transfection of PM-31 into CFBE cells resulted in a decrease in the levels of IRF-1 detectable by Western blot compared with mock transfected (Con) cells or cells transfected with PM-Neg. This decrease in IRF-1 was associated with a significant decrease in both CTSS expression (Figure 8B) and secretion (Figure 8C) in CFBEs overexpressing miR-31 compared with control cells.

Discussion

In this report, our findings show that CTSS is a highly abundant protease found in the CF lung, even in young children with CF in the absence of chronic *Pseudomonas* infection. We have demonstrated that CF pulmonary epithelial cells may contribute to the protease burden of the CF lung by expressing and releasing increased levels of active CTSS. Although we have described epithelial cells as a potential source of CTSS in the CF airway, other cell types, such as neutrophils and macrophages, may also contribute to the CTSS levels found in CF BAL fluid. In this study, we found that CF BAL fluid CTSS activity correlated significantly with BAL neutrophil counts, but not macrophage counts. However, further work is required to delineate the contribution of various cell types to pulmonary CTSS load. In addition, the significant correlation observed also supports the hypothesis that proteases, such as CTSS, may also play a role in neutrophil recruitment as proposed previously (35).

Further investigation identified miR-31 as a potentially important regulator of

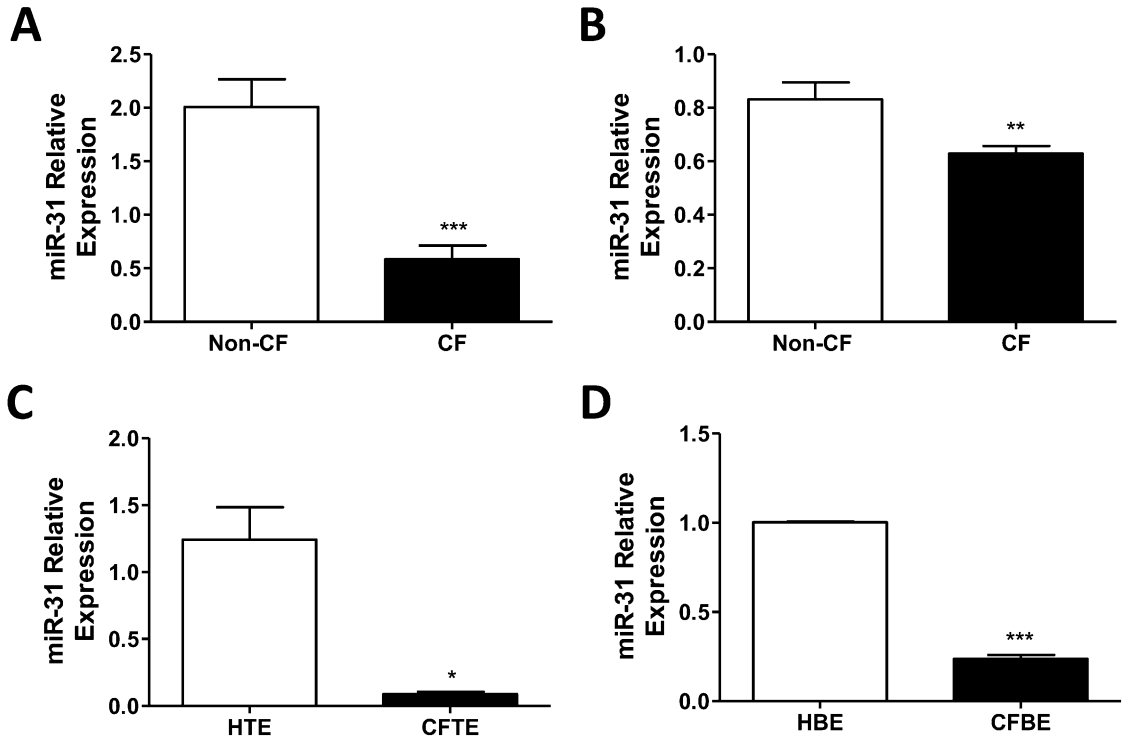

Figure 7. miR-31 expression is decreased in cystic fibrosis (CF) epithelial cells. miR-31 levels in (*A*) bronchial brushings (n = 5), (*B*) primary cultures of well-differentiated human bronchial epithelial cells (n = 4) and (*C*) tracheal and (*D*) bronchial epithelial cell lines were quantified by quantitative reverse-transcriptase polymerase chain reaction and normalized to miR-16. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. CFBE = CF bronchial epithelial cell line CFBE41o−; CFTE = CF tracheal epithelial cell line CFTE29o−; HBE = human bronchial epithelial cell line 16HBE14o−; HTE = human tracheal epithelial cell line 9HTEo−.

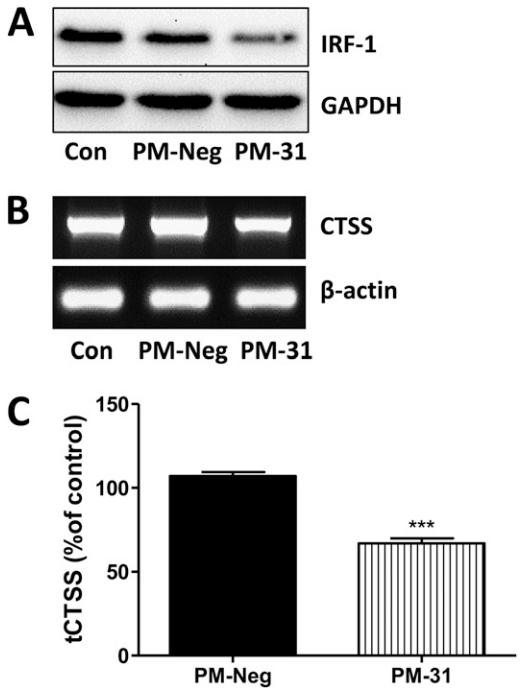

Figure 8. miR-31 dysregulation in cystic fibrosis epithelial cells leads to increased cathepsin S (CTSS) expression via IRF-1. The effects of pre–miR-31 overexpression on IRF-1 and CTSS levels were determined by transfecting cystic fibrosis bronchial epithelial cells (CFBEs) with 30 nM pre–miR-31 (PM-31), negative control (PM-Neg), or mock transfected (Con) for 48 hours. (*A*) IRF-1 levels in whole-cell lysates were analyzed by Western blotting. (*B*) CTSS mRNA levels were assessed by reverse-transcriptase polymerase chain reaction. (*C*) Extracellular CTSS was quantified by ELISA. The data are the mean ± SEM of n = 3 and are expressed as % of mock transfected control cells. ***$P < 0.001$. GAPDH = glyceraldehyde phosphate dehydrogenase; tCTSS = total CTSS levels.

CTSS expression via the transcription factor IRF-1 in CF epithelial cells. miRNAs are short noncoding RNAs that modulate target gene expression via translational repression or degradation of their target RNAs (36). miRNAs may regulate up to one-third of all protein coding genes in human genome (37) and it is believed that dysregulation of miRNAs can contribute to disease pathology (38). Recent work has identified unique miRNA profiles in the CF lung and analysis of target genes has revealed important roles for miRNAs in the regulation of CFTR expression and function and inflammation (34, 39, 40). miR-31 is one of the most highly expressed miRNAs present in human non-CF airway epithelial cells (33) and our data demonstrate that miR-31 expression was significantly decreased in CF airway epithelial cell lines and primary cells. Altered miR-31 expression has been reported in such diseases as cancer, psoriasis, coronary artery disease, and lupus (41–44); however, the mechanisms of this dysregulation are poorly understood.

Similar to other human genes, miRNA expression can be altered by several mechanisms, such as chromosomal abnormalities, mutations, defects in their biogenesis machinery, epigenetic silencing (DNA methylation, histone modification), or by transcription factors. Work to date suggests that miR-31 expression is regulated by several distinct signaling networks in an intricate lineage- and cell type–dependent manner (41). miR-31 expression levels can be enhanced by tumor necrosis factor, bone morphogenetic protein-2, and transforming growth factor-β1 and by the transcription factor C/EBP-β (42, 45–47). Other work has found that miR-31 is subject to epigenetic and nonepigenetic silencing in different human breast cancer cell lines (41, 48). In addition, reduced mature miR-31 levels may arise because of the defective post-transcriptional processing of the miR-31 RNA precursor rather than transcriptional repression of the miR-31 gene itself (49). Further work is required to elucidate the cause of decreased miR-31 in the CF lung and how this relates to CFTR dysfunction.

Altered miR-31 expression has been reported in such diseases as cancer and psoriasis, and a number of target genes have been identified including RhoA, PP2A regulatory subunit B α isoform, and serine/threonine kinase 40 (41, 42, 50). In this study, we implicate the transcription factor IRF-1 as a novel target of miR-31. IRF-1 plays an important role in the regulation of cellular responses in host defense, such as the innate and adaptive immune responses, antigen presentation, and cellular apoptosis (51). Of interest, increased IRF-1 expression has also been documented in CF blood polymorphonuclear leukocytes compared with non-CF polymorphonuclear leukocytes (52) but the role of miRNAs in this up-regulation and the downstream effects of increased IRF-1 on neutrophil function are currently unknown.

Neutrophil-dominated inflammation has been shown to play a major role in the pathogenesis of CF lung disease. Work to date has highlighted an association between NE and bronchiectasis (3) and disease severity (8) in young children with CF. Although we found that CTSS was active in a greater number of BAL fluid samples compared with NE, our NE findings correlate closely with those of Sly and colleagues who demonstrated that approximately 18–41% of CF BAL fluid samples from similar age groups contained active NE (3, 31). Our recent findings demonstrate that in CF lung secretions, the level of active NE is significantly increased in patients with chronic *Pseudomonas* infection compared with *Pseudomonas*-negative patients with CF (9, 10). These findings are in contrast to the profile of CTSS observed in these patients where BAL fluid CTSS was found to be significantly elevated regardless of the status of chronic *Pseudomonas* infection compared with healthy control BAL fluid. The findings from this cross-sectional investigation also demonstrate the presence of active CTSS in BAL fluid from young patients with CF and raise the important question of the potential role of CTSS in CF lung disease.

Previous work indicates that CTSS may also play a role in the diminution of the lung's antimicrobial defenses thereby favoring conditions for bacterial infection (13, 14, 23). Furthermore, given its potent elastinolytic activity (17–20, 53), it is possible that CTSS may play a role in tissue damage and remodeling present in early CF (2, 3, 6, 7), an increasingly recognized clinical finding over the last 5 years. In addition, the presence of elevated CTSS in the CF airways may explain, in part, why previous therapeutic approaches to protease neutralization in the CF lung, which have not taken elevated CTSS activity into account, have not been entirely successful to date. At present, there is no available therapy to prevent, limit, or reverse airway remodeling. Further work, including longitudinal analysis of patients with CF, is essential to elucidate the role of CTSS in the pathogenesis and progression of CF lung disease.

In conclusion, these novel data not only identify pulmonary epithelial cells as a source of CTSS in the CF lung, but also highlight a mechanism for the increased CTSS via dysregulated miRNA expression, which may represent a link between epithelial CFTR dysfunction and CF lung disease. Because of its multiple biologic functions, CTSS may play an important role in the pathogenesis of lung disease in CF. These findings may have important implications with regard to the pathogenesis of CF lung disease and the development of therapeutics in the future aimed at limiting the progression of structural lung disease or preserving lung function in individuals with CF. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank all patients who participated in this study.

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