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SPLUNC1: A Novel Marker of Cystic Fibrosis Exacerbations

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Abstract

Acute pulmonary Exacerbations (AE) are episodes of clinical worsening in cystic fibrosis (CF), often precipitated by infection. Timely detection is critical to minimize morbidity and lung function declines associated with acute inflammation during AE. Based on our previous observations that airway protein Short Palate Lung Nasal epithelium Clone 1 (SPLUNC1) is regulated by inflammatory signals, we investigated the use of SPLUNC1 fluctuations to diagnose and predict AE in CF.

We enrolled CF participants from two independent cohorts to measure AE markers of inflammation in sputum and recorded clinical outcomes for a 1-year follow-up period.

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AUTHOR CONTRIBUTIONS

CB, NN, SK, JZ, LS, and CDC planned the project, designed and performed experiments, analyzed the data, and wrote the manuscript. TL and MN, contributed to the design and analysis of clinical data, and provided access to the UMN cohort samples. MW, and RT performed, and analyzed experiments related to NE densitometry and activity in sputum. GC generated the sputum collection protocol for the Yale cohort and facilitated access to banked human samples. LC, MS, JLG, ME, and GC contributed to the design and analysis of all experiments, and the final manuscript. MDS provided biostatistics support and analyzed all experiments. All authors reviewed, revised, and approved the manuscript for submission.

DISCLOSURES

Dr. Tarran reports the following financial and intellectual property disclosures: Eldec Pharmaceuticals, outside the submitted work; In addition, Dr. Tarran has a patent on Peptide inhibitors of Ca2+ channels pending, a patent on PEPTIDE INHIBITORS OF SODIUM CHANNELS with royalties paid, and a patent on Regulation of sodium channels by PLUNC proteins with royalties paid. Dr. Cohn reports the following financial disclosures: Genentech, Novartis, Astra-Zeneca, GlaxoSmithKline, Regeneron, Pieris, Sanofi, all outside the submitted work. The other authors of this manuscript do not have any conflicts of interest that could be perceived to bias their work.

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SPLUNC1 levels were high in healthy controls *(n=9, 10.7μg/mL),* and significantly decreased in CF participants without AE *(n=30, 5.7μg/mL, p=0.016).* SPLUNC1 levels were 71.9% lower during AE *(n=14, 1.6μg/mL, p=0.0034)* regardless of age, sex, CF-causing mutation, or microbiology findings. Cytokines Il-1β and TNFα were also increased in AE, whereas lung function did not consistently decrease. Stable CF participants with lower SPLUNC1 levels were much more likely to have an AE at 60 days *(HR: 11.49, Standard Error: 0.83, p=0.0033).* Low-SPLUNC1 stable participants remained at higher AE risk even one year after sputum collection *(HR: 3.21, Standard Error: 0.47, p=0.0125).* SPLUNC1 was downregulated by inflammatory cytokines and proteases increased in sputum during AE.

In acute CF care, low SPLUNC1 levels could support a decision to increase airway clearance or to initiate pharmacological interventions. In asymptomatic, stable patients, low SPLUNC1 levels could inform changes in clinical management to improve long-term disease control and clinical outcomes in CF.

PLAIN LANGUAGE SUMMARY

SPLUNC1 is an abundant host defense protein found in the respiratory tract that decreases with inflammation. Individuals with cystic fibrosis experiencing clinical worsening (exacerbation) have lower levels of SPLUNC1 in their sputum. In stable cystic fibrosis patients, lower levels of SPLUNC1 may predict an upcoming respiratory illness. Therefore, SPLUNC1 may serve as a tool for early diagnosis and treatment of cystic fibrosis exacerbations.

BACKGROUND

Cystic fibrosis (CF) is a multi-system, autosomal recessive disease caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene[1–4]. CF acute pulmonary exacerbations (AE) are generally reversible episodes of acute deterioration, associated with increased morbidity and worsening quality of life[5–9]. AE are frequently triggered by respiratory viruses, but also by oropharyngeal flora and bacterial respiratory pathogens such as *P. aeruginosa* and *S. aureus*[10–12]. Increased lung inflammation during AE, manifested as higher immune cell counts and rising concentrations of airway cytokines and proteases, contribute to tissue injury and disease progression[7,13]. Increased AE frequency decreases CF survivorship and accelerates lung function decline[14,15]. Importantly, delays in AE detection and treatment may have long-term effects on lung function recovery and response to antibiotic treatment[16,17]. These observations suggest that early AE detection could help improve clinical outcomes in CF.

Early or mild AE presentations can go undetected during routine visits[7,18,19], exacerbating an already variable AE diagnostic approach among healthcare providers[20] and impacting treatment outcomes[21]. Biomarkers of airway inflammation or lung function like the Forced Expiratory Volume in the first second (FEV_1), are routinely used to support AE diagnosis and management[18,19]. However, FEV_1 changes often occur as a late consequence of AE, limiting its clinical use in early detection[22]. Inflammatory cytokines (e.g. IL-6, IL-8, and TNFα) have also been linked to AE, but there is limited data on their ability to predict these events[23–25].

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Our group and others previously demonstrated that airway concentrations of host defense protein Short Palate Lung Nasal epithelium Clone 1 (SPLUNC1), are closely regulated by inflammatory signals and proteases[26–29]. SPLUNC1 is primarily expressed by non-ciliated epithelial and mucus cells of the upper and proximal lower respiratory tract[30–32]. SPLUNC1 is also present at low levels in extrapulmonary tissues, and myeloid cells[30,32]. SPLUNC1 has antimicrobial, immunomodulatory, and ion transport properties that are highly relevant to CF health, which may be disrupted at baseline and during AE[28,33,34].

SPLUNC1 decreases within hours of exposure to inflammation, irritants, or pathogens[33], and it is differentially regulated in lung disease[27,28,33,35–39]. In CF, previous studies have shown low levels in respiratory secretions[28], but increased bronchial SPLUNC1 staining in advanced disease[40]. Recently, genome-wide association studies (GWAS) showed that *SPLUNC1* expression was higher in stable CF patients compared to healthy controls, but lower in CF patients with more severe disease[41,42]. In asthma and active smokers, lower SPLUNC1 levels correlate with increased inflammation[35,37,38], however, studies of its regulation in COPD have been inconclusive[27,39]. Beyond airway disease, SPLUNC1 dysregulation has also been reported in idiopathic pulmonary fibrosis and respiratory malignancies[33,36,43]. The variable relationship between SPLUNC1 regulation, inflammation, and underlying lung disease suggests that SPLUNC1 has a role as rheostat of respiratory health, whose function and regulation are context-specific.

Based on the known downregulation of SPLUNC1 by pathogens and inflammatory signals, we hypothesized that its sputum concentrations would decrease during AE, and that lower levels of SPLUNC1 would impair its host defense functions, leading to adverse clinical outcomes. Here, we show that SPLUNC1 decreases sharply as inflammation increases in AE, and that in stable patients, lower SPLUNC1 levels portend an increased AE risk. SPLUNC1 downregulation occurs shortly after exposure to cytokines and proteases, suggesting that it could detect AE at early stages, reducing diagnostic uncertainty and informing proactive interventions to decrease AE impact on CF health[44–46].

MATERIALS AND METHODS

Definition of CF Exacerbation

AE were defined as the emergence of 4 of 12 signs or symptoms, prompting changes in therapy and initiation of antibiotics (modified from Fuchs' criteria[18]). These criteria included: change in sinus congestion, sputum, or hemoptysis; increased cough, dyspnea, malaise, fatigue or lethargy; fever; hyporexia or weight loss; change in chest physical exam; or FEV_1 decrease >10% from a previous value[18]. Individuals not meeting AE criteria were characterized as "CF Stable".

Study Design

This was a two-center, prospective study of CF participants during periods of clinical stability and AE. All patients received standard-of-care therapy and CFTR modulators when they became available. Our primary objective was to define an association between AE and sputum levels of SPLUNC1. Each participant provided a sputum sample and underwent

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spirometry within 24 hours of sample collection. Participants were followed at quarterly outpatient clinic visits, or sooner when indicated, for up to one year (Supplemental Figure 1). Clinical information, sputum, and spirometry data were collected at each visit.

Cohort Characteristics

Discovery cohort: 44 adults with confirmed CF diagnosis from the Yale Adult CF Program were recruited from 2014 to 2016 during a) scheduled routine visits, b) unscheduled visits in which they reported AE symptoms, and c) on the first day of admission to the hospital for AE treatment. We organized study participants in two groups: 1) Stable CF participants (CF Stable): No new respiratory symptoms, presenting to clinic for scheduled follow up and, 2) AE participants (AE): Diagnosed with AE (Table 1). We also recruited 10 healthy controls (HC) to undergo sputum induction according to published protocols[47]. The study was approved by the Yale University Institutional Review Board and informed consent was obtained from each participant.

Validation cohort: 35 adult and pediatric participants with confirmed CF, previously enrolled in a prospective study of patients hospitalized for AE treatment at the University of Minnesota (UMN) were included[23]. All patients received standard-of-care therapy and each participant provided sputum samples and performed pulmonary function tests within 72 hours of antibiotic initiation (Table 2)[48].

Sputum Collection and Processing

CF participants expectorated sputum spontaneously for cultures and provided an additional study sample. Induced sputum samples were obtained from HC by induction as previously reported[47,49]. Sputum was diluted, filtered, centrifuged, and processed as previously reported[50].

SPLUNC1 and Cytokine ELISA

A direct SPLUNC1 ELISA was developed in our laboratory to measure SPLUNC1 in sputum (details in supplemental methods). Briefly, high-binding polystyrene ELISA plates (Corning, NY, cat# 9018) were coated with sputum supernatants or recombinant human SPLUNC1 protein (rhSPLUNC1) as reference (Abnova, Taipei, Taiwan, cat# H00051297-P01). A polyclonal mouse anti-human SPLUNC1 IgG (MilliporeSigma, Burlington, MA, cat# SAB1401687) was used as detection antibody and HRP-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA, cat# G21040,) as secondary. Chromogenic tetramethylbenzidine substrate was applied (KPL, Gaithersburg, MD, cat# 5120-0047-50-76-00) and reactions were measured at optical densities of 450 and 550 nm. The assay limits of detection were 1–20,000 ng/mL. Mean intra-assay variability: 5.18% (STDEV 1.28%), Inter-assay variability: 18.44% (STDEV 12.95%).

Custom-made multiplexed cytokine ELISA assays were used to measure cytokine levels in sputum. Briefly, biotinylated capture antibodies for CXCL10, G-CSF, IFN-α2a, IFN-γ, IL-1β, IL-13, IL-29, IL-6, IL-8, MCP-1, MIP-1α, and TNF-α, were combined with an assigned “linker” for each cytokine. The linker-antibody mix was then coated onto U-plex plates and incubated overnight according to manufacturer’s specifications (U-Plex

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Biomarker Kit, Mesoscale Diagnostics (MSD). Rockville, MD, cat# K15235N-1). The following day, recombinant human cytokines and sputum samples were loaded onto U-Plex plates. Finally, detection antibodies for each cytokine were applied and Read Buffer T was added to each well to quantify the reaction. Plates were read on a Quickplex SQ 120 reader (MSD, cat# AI0AA-0) using MSD Discovery Workbench software version 4.0.

Western Blot

Western blots were performed as previously reported, using human neutrophil elastase (hELA2), mouse monoclonal anti-hELA2 IgG (R&D systems, cat# MAB-91671-100) and mouse polyclonal anti-human SPLUNC1 IgG[28]. Horseradish peroxidase-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA, cat# G21040) was used as secondary antibody. Membranes were developed using chemiluminescence and protein band densitometry was determined using ImageJ software version 1.7 (https://imagej.nih.gov/ij/index.html).

Sputum Neutrophil Elastase (NE) Activity and SPLUNC1 Degradation Assays

NE activity was determined using the 7-amino-4-methylcoumarin (MCA) assay (Peptides International, Louisville, KY, #MAA-3133), as described[28]. RhSPLUNC1 was incubated with recombinant human NE (rhNE, R&D systems, cat#9167-SE-020) or *Pseudomonas aeruginosa* elastase (LasB, a gift from Dr. Karen Agaronyan, Yale) at decreasing concentrations for 3 and 8 hours. SPLUNC1 concentrations were measured by ELISA. Starting NE concentrations (1 μM) were selected based on previous sputum NE level measurements by our group[28]. There were no published data on airway levels of LasB during AE to inform dose selection. However, a dose capable of inhibiting host defense peptide expression and inducing cytokine expression had been previously reported (3.75 μM)[51]. Based on this, we chose a starting dose of 1 μM to define minimal LasB doses capable of regulating SPLUNC1.

Regulation of Epithelial Cytokine Expression

Mouse tracheal epithelial cells (mTECs) were isolated from C57BL/6 mice and cultured at air–liquid interface (ALI) as described[26]. mTECs were treated with recombinant murine IL-1β (Peprotech, Rocky Hill, NJ, cat# 211-11b) or TNF-α (Peprotech, cat# 315–01A) at 10 ng/mL for 24 hours. NCI-H292 human airway epithelial cells, were treated with recombinant human IL-1β (Gibco, Gaithersburg, MD, cat# PHC0811,) or TNF-α (R&D, Minneapolis, MN, cat# 210-TA-005) at 10 ng/ml. Cellular mRNA was extracted for qPCR, and qPCR assays were performed to quantify *SPLUNC1* transcriptional regulation as described[26].

Statistical Analysis

Descriptive statistics were calculated for the entire participant population. Pearson, or Spearman correlations for variables that were not normally distributed, were calculated between SPLUNC1 and clinical parameters. In order to select optimal thresholds to separate groups at higher AE risk, we developed receiver-operator curves (ROC) based on the distribution of SPLUNC1, IL-1β, TNFα, G-CSF, IL-6, and IL-8 levels in the discovery cohort (Supplemental Figure 2). Using these thresholds, we applied statistical modeling

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(Mantel-Haenszel estimator) to predict AE-free intervals. AE-free intervals were defined as the time in days from sputum sampling in a stable patient to the time of the first AE after that visit. Finally, a Cox proportional hazards model was conducted with clinical parameters as covariates. A backward elimination strategy with a significance level to stay of 95% (a=0.05) was employed to achieve a parsimonious model. All statistical analyses were conducted using SAS 9.4 with a level of significance of 95% (α=0.05).

Please see details in Supplementary Methods.

RESULTS

SPLUNC1 is Decreased in the Sputum of Stable CF Participants

SPLUNC1 levels ranged from 4.41 to 22.24μg/mL in the sputum of healthy controls (HC). In stable CF participants, SPLUNC1 was significantly decreased, whereas total sputum protein was increased (Figure 1A, 1B). To further define the inflammatory profile of stable CF participants, we measured sputum concentrations of cytokines previously reported to be increased in CF. Of these, IFNα, IFNγ, IL1β, IL-8, IL-13, and TNFα were significantly increased in CF compared to HC (Figure 1C). There were no differences in SPLUNC1 levels of stable participants according to severity of lung function impairment, *F508del* genotype, use of CFTR modulators, or microbiology findings (Supplemental Tables 1–4, Supplemental Figure 3A–D). These findings indicate that SPLUNC1 is abundant in sputum and decreased in stable CF participants.

SPLUNC1 Decreases Further During AE

We measured SPLUNC1 levels in sputum from stable and AE participants to determine if SPLUNC1 is a marker of AE. SPLUNC1 decreased sharply during AE in the discovery cohort (71.9% decrease) and in the validation cohort (38.6% decrease) (Figure 2A). In contrast, FEV_1 did not decrease in the discovery cohort's AE group, but was significantly lower in the validation cohort (Figure 2B).

Mean SPLUNC1 levels of AE participants treated with oral antibiotics (AEO) and IV antibiotics (AEIV) in the UMN cohort were lower than stable CF levels. However, there was no difference in SPLUNC1 levels between AEO and AEIV (Supplemental Figure 4). The lack of difference between these treatment groups suggests that acute drops in SPLUNC1 occur during AE regardless of its severity.

Next, we sought to define SPLUNC1 fluctuations during AE within the same individuals, relative to their stable-state reference value (individual-specific fluctuations). We compared SPLUNC1 and FEV_1 (%) in paired samples from the same participants, collected during stable and AE periods. SPLUNC1 decreased during AE in the majority of paired samples from both the Yale and UMN cohorts (Figure 3A). In contrast, FEV_1 decreased during AE in the in the majority UMN samples, but not in those form the Yale cohort (Figure 3B). These findings indicate that while SPLUNC1 is consistently decreased during AE, FEV_1 changes during AE vary across cohorts.

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Low SPLUNC1 Levels Predict AE Risk in Stable CF Participants

To determine if SPLUNC1 is a predictor of AE risk, we first performed a Mantel-Haenszel survival estimator analysis for AE-free time. We separated the cohorts into high/low-SPLUNC1 groups based on a concentration threshold defined by ROC analysis comparing AE and Stable patients (Supplemental methods, Supplemental Figure 2). In stable CF participants, the SPLUNC1-low group had a median AE-free time of 43.5 days, compared to 150 days in the SPLUNC1-high group, this relationship was preserved in a subgroup analysis of patients with FEV_1 >40% of predicted (Supplemental Figure 5). This suggests that higher SPLUNC1 levels are associated with longer AE-free intervals independently of stable-state FEV_1.

Next, we performed Cox-proportional Hazards modeling to assess the likelihood of AE while adjusting for demographics, CFTR genotype, CF-related comorbidities, microbiology, and lung function. In the short term (60 days), participants in the SPLUNC1-low group had a significantly increased risk of AE (*Hazard ratio: 11.49, $p=0.003$,* Figure 4A), which persisted upon long-term follow up at 1 year (*Hazard ratio: 3.21, $p=0.013$,* Figure 4B).

In order to compare SPLUNC1 to previously reported biomarkers as predictors of AE, we defined ROC thresholds and AE-free time for G-CSF, IL1-β, IL-6, IL-8, and TNFα (Supplemental Figure 2). In a similar multivariate proportional hazards model, cytokine high/low groups based on these markers did not show an increased hazard ratio of AE at 60 days, and only high IL-1β, and TNFα were associated with an increased AE risk at 1 year of follow-up (*Hazard ratios: 3.90, and 3.46 respectively, $p<0.05$,* Supplemental Figure 6). These findings suggest that SPLUNC1 is a better predictor of AE risk in the short and long term than previously reported sputum AE markers.

Human and Bacterial Elastases Found in CF Sputum Degrade SPLUNC1

Our group and others have shown that NE degrades SPLUNC1, and that NE inhibitor Sivelestat only partially prevents SPLUNC1 degradation by CF sputum[27,28]. In order to understand the role of NE and bacterial elastase in decreasing SPLUNC1 during AE, we incubated rhSPLUNC1 with recombinant human neutrophil elastase (NE) or *Pseudomonas aeruginosa*'s Elastase B (LasB) at increasing concentrations for 3 and 8 hours. Both elastases induced a concentration-dependent decrease in full-length SPLUNC1 (Figures 5A, B). Next, we quantified NE concentrations in HC and CF sputum during stable and AE periods. NE was increased overall in CF, but it did not increase significantly from stable levels during AE (Figure 5C). Finally, to define individual-specific NE and SPLUNC1 changes, we performed Western blots of HC and CF sputum, probing for NE, followed by re-probing for SPLUNC1. Although NE was increased in CF relative to HC, NE levels were not different between Stable and AE states (Figure 5D).

To determine if NE activity, rather than concentration, increased during AE we measured NE-specific fluorescent cleavage products. When incubated with NE, CF sputum had much higher NE activity than HC sputum; however, there was no difference between stable and AE participants (*Figure 5E).*

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Sputum Cytokines IL-1β and TNFα are Increased During AE

To further define the AE inflammatory profile of CF participants, we measured concentrations of thirteen cytokines in stable and AE samples. Only IL-1β and TNFα were significantly increased during AE (Figure 6A). We also sought to define a relationship between SPLUNC1 and cytokine levels in sputum during stable and AE states using Pearson's correlation. In stable and AE groups from both cohorts, IL-1β levels inversely correlated with SPLUNC1 (Supplementary Figure 7), while CXCL10, G -CSF, IFNγ, IL-6, MCP1, MIP-1α, and TNFα did not consistently correlate with SPLUNC1 across cohorts (not shown).

In order to determine if increased IL-1β and TNFα contributed to decreased SPLUNC1 during AE, we treated mTEC and a human airway epithelial cell line with these cytokines and measured *SPLUNC1* mRNA expression. At concentrations encountered in AE sputum, both IL-1β and TNFα decreased *SPLUNC1* expression by airway epithelial cells (Figure 6B). Together with our observations from NE and LasB experiments, these findings suggest that during AE, SPLUNC1 is decreased through protein degradation and cytokine-driven transcriptional downregulation.

DISCUSSION

AEs contribute to increased morbidity in CF and treatment delays are associated with poor FEV_1 recovery and impaired treatment responses[5–9,15–17,52,53]. Yet, few biomarkers are clinically available to guide early AE interventions in order to minimize hospitalizations and improve quality of life[44–46]. Here, we describe a novel role for SPLUNC1 as an AE biomarker and predictor that could support clinical decision-making to improve AE outcomes.

The key finding of our study is that among CF participants, SPLUNC1 levels were considerably lower during AE compared to stable state. We propose that the mechanism for SPLUNC1 decreases in CF is multifactorial, with contributions from protease degradation and gene expression downregulation by inflammatory cytokines. Prospectively, stable patients with low SPLUNC1 had an increased likelihood of AE at 60 days and one year. These findings suggest that SPLUNC1 levels could inform the diagnosis and clinical management of AE in the short and long term.

By the time symptoms develop or FEV_1 declines, airway inflammation and damage may already be underway[14,15]. In the face of a suspected AE with incomplete clinical criteria and normal spirometry, a low SPLUNC1 level would support a decision to increase airway clearance, adjust monitoring, or initiate pharmacological interventions when appropriate. In asymptomatic stable patients, low SPLUNC1 levels could also prompt immediate or long-term changes in clinical management.

Although symptom and spirometry monitoring at home increase AE detection, they do not prevent FEV_1 decline, possibly because of the delayed nature of FEV_1 changes in response to airway inflammation[54]. The application of clinical biomarkers is even more challenging in children, where FEV_1 and cytokine abnormalities are inconsistently detected

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until adolescence, despite early evidence of structural lung disease[44,55–57]. SPLUNC1 measurements at home could detect subtle inflammatory changes that complement symptom and spirometry monitoring.

Previous studies examined the correlation between sputum biomarkers, infection, inflammation, and lung function decline in CF[25,58–67]. While some defined novel AE biomarkers[25,68–72], to our knowledge, ours is the first study to compare a broad panel of markers in AE and stable state that includes adults and children.

Daily changes in inflammatory signals or pathogen exposures may cause small fluctuations, however, SPLUNC1 levels drop sharply during AE. We previously showed that LPS and IFN-γ have tonic suppressive effects on SPLUNC1 at baseline[26]. However, high-dose LPS and IFN-γ exposures decrease epithelial *SPLUNC1* expression drastically, indicating a dose-dependent response. SPLUNC1's tight regulation suggests that it is an ideal biomarker to detect early and subtle changes in lung homeostasis[26].

SPLUNC1 has host protective functions relevant to CF, including regulation of airway surface liquid, antimicrobial properties, and immunomodulatory effects[73–79]. Therefore, SPLUNC1 decreases in CF may not only be a marker, but also a contributor to pathogenesis during AE and disease progression. Decreased SPLUNC1 in CF may impair mucociliary clearance and facilitate bacterial colonization, leading to tissue injury and exacerbated inflammation. In fact, some SPLUNC1-deficient animal models have shown increased susceptibility to infectious and non-infectious inflammation[80,81]. In our study, we observed increased IL-1β and TNFα during AE, but only IL-1β inversely correlated with SPLUNC1. This correlation may reflect the transcriptional effects of higher relative concentrations of IL-1β during AE when compared to TNFα (Figure 6A). Furthermore, others have shown that IL-1β may have a more potent neutrophil recruitment effect than TNFα[82]. Thus, the increased concentrations of IL-1β may enhance neutrophil recruitment that in turn increases SPLUNC1 degradation, strengthening the inverse correlation between SPLUNC1 and IL-1β. Our data showing increased NE levels and activity, although not different between AE and stable state, suggest that SPLUNC1 cleavage at functional sites by NE (and other proteases during AE) may disrupt its host defense functions, worsening inflammation and accelerating lung disease[34,83].

Our study has some limitations. First, our study has a small sample size and predicts AE risk based on cross-sectional data. However, our findings show it was adequately powered to demonstrate differences in key observations, confirmed on a validation cohort. In the future, we would favor serial SPLUNC1 measurements to establish stable-state baselines that reflect day-to-day fluctuations. Second, there is no distinct SPLUNC1 level that separates HC from CF, or stable- from AE-state in all participants. The overlap of SPLUNC1 levels among some HC and CF patients, may be in part explained by changes in inflammation and environmental exposures between sputum samplings. However, our study highlights the value of individual biomarker variability in characterizing disease states. Third, we used IV or oral therapy as surrogates for disease severity. We appreciate that choice of therapy route is based on many factors, including access to care, AE complications, and severity of FEV_1 decline[84]. We decided to present these data to show that SPLUNC1 can

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be used as a marker in AE of any severity and regardless of factors driving therapeutic decisions. Finally, our cohorts had a higher prevalence of advanced lung disease and *CFTR* genotypes linked to severe disease. We addressed this in our multivariate models by using a backwards elimination strategy to confirm that the predictive ability of SPLUNC1 was not affected by *CFTR* genotype. Larger prospective studies are needed to replicate our findings in sub-cohorts that reflect specific *CFTR* genotypes, comorbidities, and the impact of novel CFTR modulator combinations.

In the age of highly-effective CFTR modulator therapy, we look forward to rising life expectancy and quality of life[4]. We hope that measurements of non-invasive, airway-relevant biomarkers such as SPLUNC1 will become a resource to guide acute management of respiratory complications and inform our partnership with CF patients for the betterment of their long-term health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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TAKE-HOME MESSAGE

Sputum concentrations of the secreted airway protein SPLUNC1 decrease during CF exacerbations. Lower SPLUNC1 levels in stable participants portend a significantly increased risk of exacerbation that could inform therapeutic interventions.

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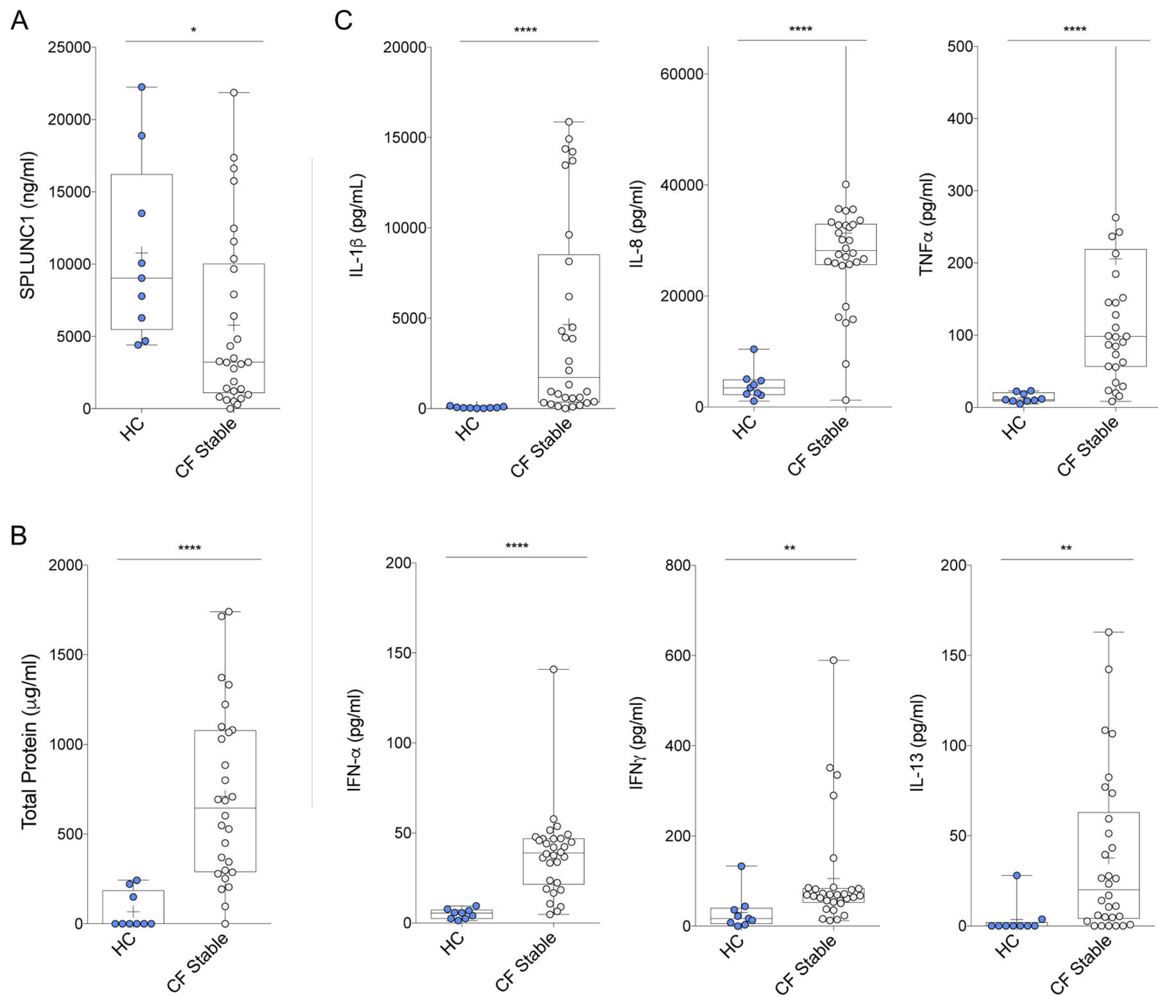

Figure 1. SPLUNC1 is Decreased in the Sputum of Stable CF Patients.

A) SPLUNC1 levels (ELISA) in sputum samples from the Yale cohort of adult CF patients without respiratory symptoms (*CF Stable*) and healthy controls (HC). B) Total protein in sputum (BCA assay) from the same patients. C) Inflammatory cytokine levels (ELISA) in sputum from the same patients. Additional cytokines tested without significant difference: CXCL10, G-CSF, IFNλ, IL-6, IL-13, MCP1, MIP1α. CF Samples were obtained by voluntary expectoration during clinical assessment, HC samples obtained by sputum induction with nebulized normal saline solution. *+ = Mean; Bar inside box: Median; Whiskers: Minimum/Maximum. Mann-Whitney Test with Bonferroni correction*, *$*$ = $p<0.05$; $**$ = $p<0.01$; $****$ = $p<0.0001$.*

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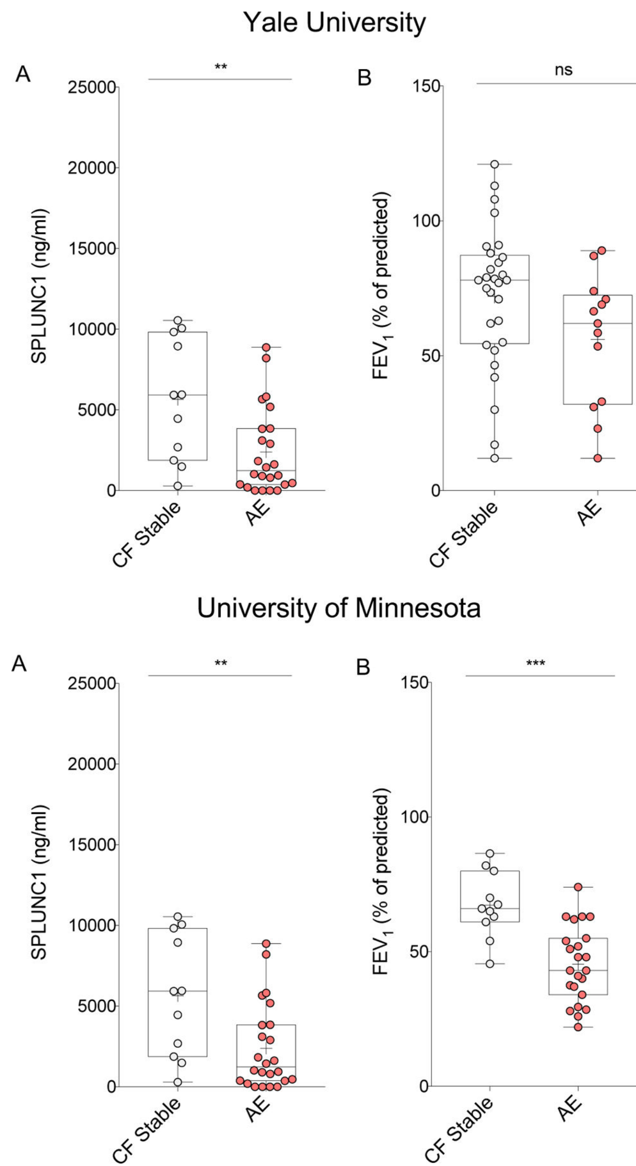

Figure 2. SPLUNC1 is Decreased During Acute CF Exacerbations (AE).

Sputum SPLUNC1 and FEV_1 from two clinical cohorts including adult (Yale University, n=43) and mixed adult/pediatric (University of Minnesota, n=35) CF patients. Samples were obtained by voluntary expectoration during clinical assessment, A) SPLUNC1 quantified by ELISA, B) FEV_1 (Percent of Predicted, %) obtained by spirometry during clinical assessment; *CF Stable: No symptoms of AE, no antibiotic treatment. AE: Acute CF exacerbation, symptoms of AE and ongoing antibiotic therapy;* FEV_1*: Forced Expiratory Volume in the first second; + = Mean; Bar inside box: Median; Whiskers: Minimum/ Maximum. Mann-Whitney test, ** = p<0.005; *** = p<0.001; ns = not statistically significant.*

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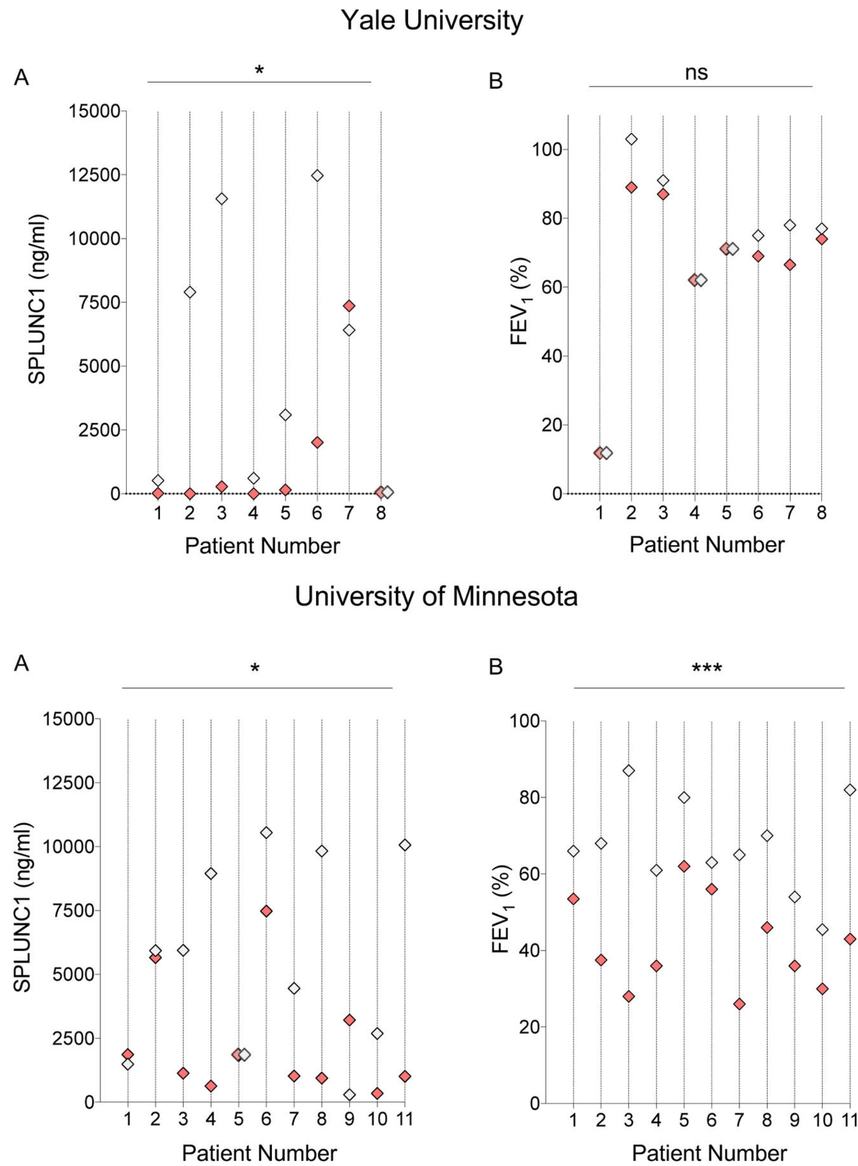

Figure 3. Individual-Specific SPLUNC1 and FEV_1 Decreases During AE.

A) Paired SPLUNC1 levels in sputum samples from the same individual with and without AE (ELISA); B) Paired FEV_1 measurements from the same individual with and without AE (Percent of Predicted, %) obtained by spirometry during clinical assessment; Samples from two clinical cohorts including adult (Yale University, n=8) and mixed adult/pediatric CF patients (University of Minnesota, n=11). Each vertical line and number represent a single patient that provided one Stable and one AE sample. *CF Stable (Gray markers): No symptoms of AE, no antibiotic treatment. AE (Red markers): Acute CF Exacerbation, symptoms of AE and ongoing antibiotic therapy; When values were the same, these were represented by two overlapping diamonds along the patient's line. Wilcoxon matched-pairs signed rank test; * = $p<0.05$, *** = $p = 0.0001$, ns=not statistically significant.*

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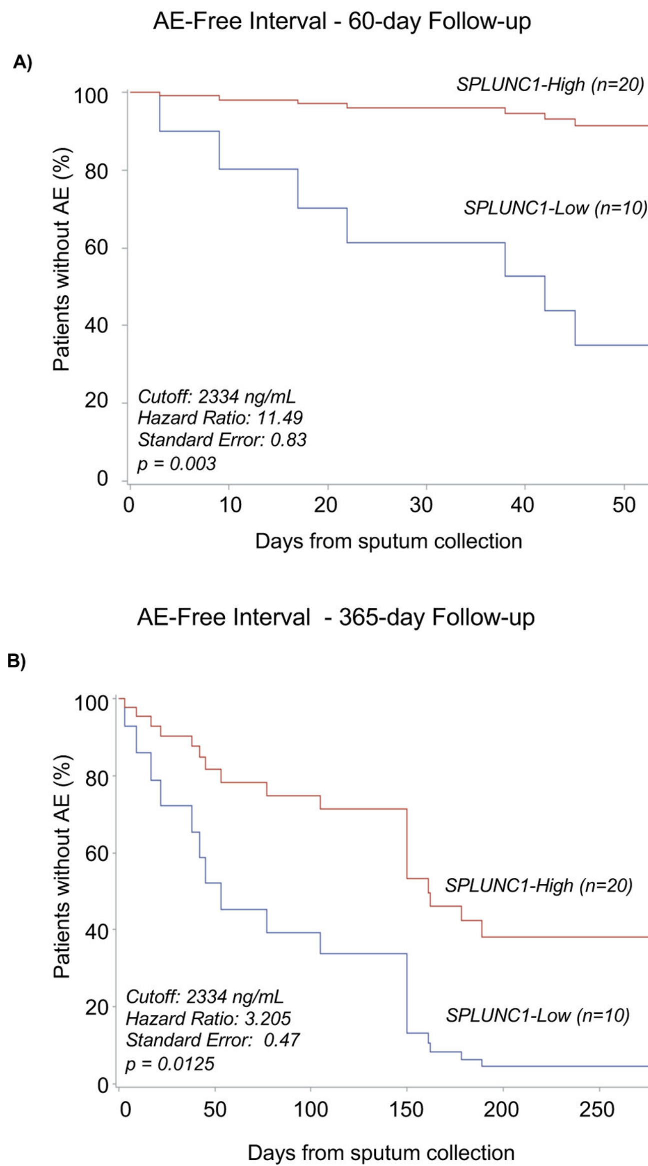

Figure 4. SPLUNC1 Predicts AE-Free Time.

A) AE-Free time in Stable CF patients separated into SPLUNC1-High and SPLUNC1-Low groups over a 60-day follow-up period. SPLUNC1-high and –low groups were defined according to sputum concentration thresholds obtained from receiver-operator curves separating CF Stable and AE levels (Supplemental Figure 4). AE-Free time was defined as the number of days from sputum collection in Stable patients until the date of their next AE. B) AE-Free time in Stable CF patients separated into SPLUNC1-High and -Low groups over a 365-day follow up period. Cox Proportional Hazards model used to calculate AE-free intervals and adjust for age, sex, BMI, FEV_1, number of *F508del* mutations, presence of CF-related diabetes or pancreatic insufficiency, use of CFTR modulators, and microbiology for *P. aeruginosa, A. xylosoxidans, H. parainfluenzae, Methicillin-sensitive S. aureus, and Methicillin-resistant S. aureus.*

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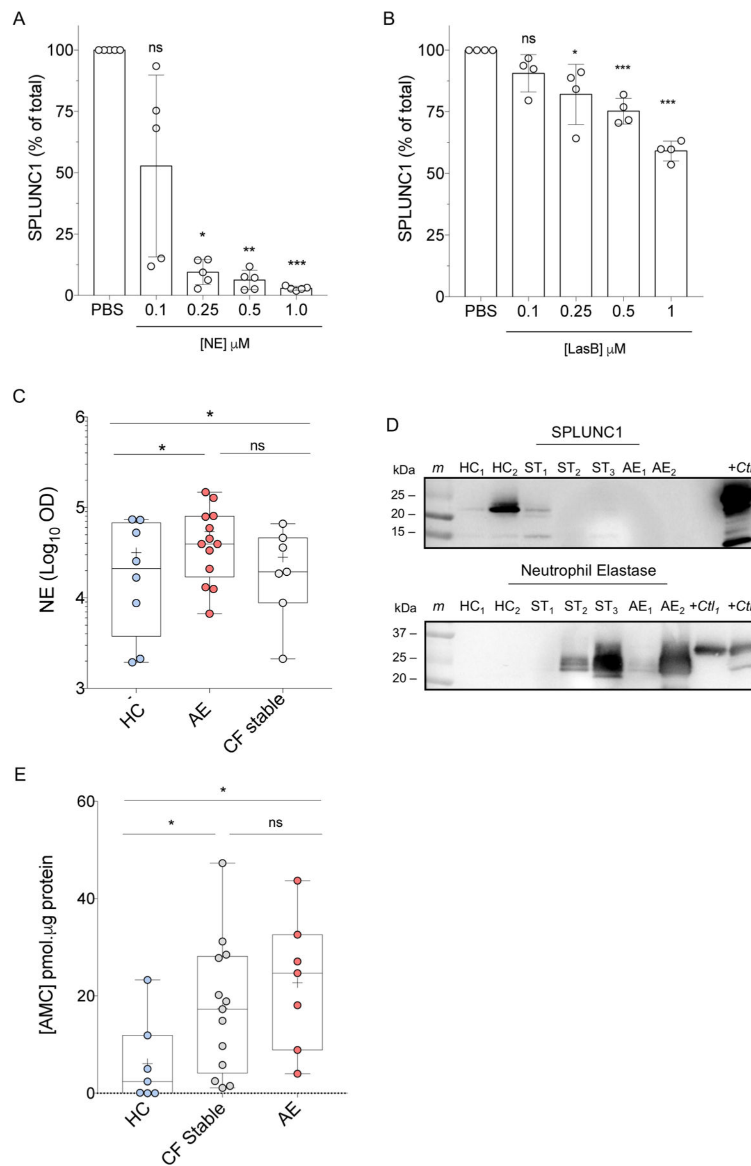

Figure 5. Elastase Concentration and Activity are Increased in CF.

A) SPLUNC1 densitometry showing degradation by human neutrophil elastase (NE) relative to PBS control, at specified concentrations over 3 hours at 37°C. **B)** SPLUNC1 densitometry showing degradation by Elastase B (LasB) from *P. aeruginosa* relative to PBS control at specified concentrations over 8 hours at 37°C. **C)** NE densitometry in sputum from healthy controls (HC), Stable CF patients (CF Stable), and AE patients (AE) assessed by WB. **D)** Representative WB showing endogenous expression of SPLUNC1 (20–25 kD) and NE (25–30 kD) in HC and CF sputum samples from the same individuals. Membranes were probed for NE prior to stripping and re-probing for SPLUNC1. **E)** NE Activity in CF sputum: AMC formation from florigenic NE substrate MAA-3133 following 6 h incubation with HC, CF stable, and AE sputum at 37°C. *For experiments in A and B: n = 4–5, 2 individual experiments*; *Mann-Whitney Test; + = Mean; Bar inside box: Median; Whiskers: Minimum/ Maximum; * p < 0.05; ** = p < 0.01; *** = p < 0.005; ns: not statistically significant; HC:*

Healthy Control; ST: Stable CF; AE: CF exacerbation; m: marker; +ctl: recombinant protein positive control; OD: optic density.

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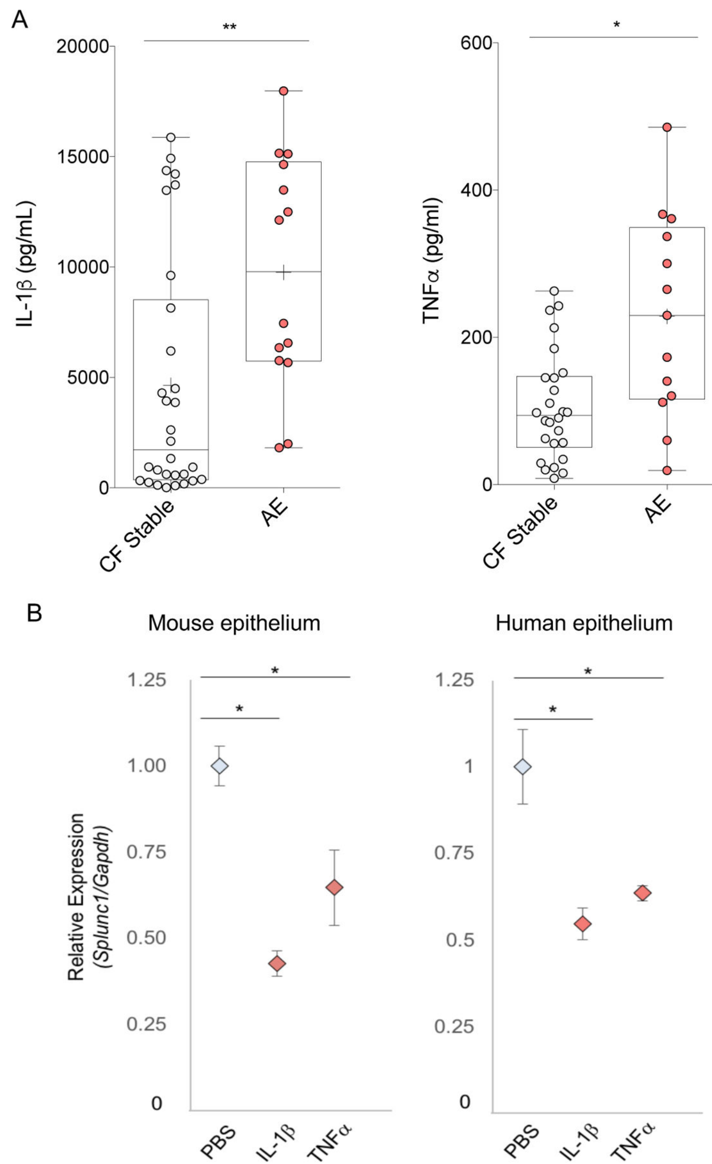

Figure 6. Cytokines IL-1β and TNF-α Increase During AE & Downregulate SPLUNC1 Expression.

A) Inflammatory cytokine levels in sputum from adult CF patients without respiratory symptoms (CF Stable) and with acute CF Exacerbation (AE). *Additional cytokines tested without significant difference: CXCL10, G-CSF, IFNα2, IFNγ, IFNλ, IL-6, IL-8, IL-13, MCP1, MIP1α. Mann-Whitney Test with Bonferroni correction.* B) Relative *SPLUNC1* mRNA expression in mouse tracheal epithelial cells (Mouse epithelium) grown at air-liquid interface and in the NCI-H292 human airway epithelial cell line (Human epithelium) treated with recombinant TNFα and IL-1β (10 ng/mL) for 24 hours *(2-way ANOVA); + = Mean; Bar inside box: Median; Whiskers: Minimum/Maximum;* mRNA expression quantified by qPCR. *Represents 2 experiments; * = p<0.05; ** = p<0.01.*

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Table 1.

Yale Cohort – Demographics

Demographic characteristics of the Yale Adult CF Program cohort (Discovery Cohort).

Number of Patients (n)	**HC (9)**	**CF Stable (30)**	**AE (14)**
Age			
Age (Mean)	*33.5*	41.1	32.1
Age (STD)	*10.7*	17.0	6.4
Age (Range)	*27–45*	20–79	23–43
Sex			
Female (n)	*1*	17	8
Female (%)	*11.1*	56.7	57.1
Male (n)	*8*	13	6
Male (%)	*88.9*	43.3	42.9
Mutation Background			
F508del/F508del (n)	*NA*	11	8
F508del/F508del (%)	*NA*	36.7	57.1
F508del/other (n)	*NA*	11	4
F508del/other (%)	*NA*	36.7	28.6
Other mutations (n)	*NA*	8	2
Other mutations (%)	*NA*	26.7	14.3
FEV_1 (L)			
FEV_1 (Mean)	*NA*	2.2	1.9
FEV_1 (STD)	*NA*	0.7	0.7
FEV_1 (Range)	*NA*	0.57–3.3	0.55–2.98
FEV_1 (%)			
FEV_1 (Mean)	*NA*	67.5	56.1
FEV_1 (STD)	*NA*	24.3	24.3
FEV_1 (Range)	*NA*	12–121	12–89
BMI (Kg/m^2)			
BMI (Mean)	*NA*	24.4	22.7
BMI (STD)	*NA*	4.1	4
BMI (Range)	*NA*	17.6–35.8	17.6–35.8
Exacerbations per year (AE/y)			
AE/y (Mean)	*NA*	1.9	3.7
AE/y (Range)	*NA*	0–10	1–10
CF Comorbidities			
PI (n)	*NA*	25	14
PI (%)	*NA*	83.3	100
CFRD (n)	*NA*	11	9
CFRD (%)	*NA*	36.7	64.3
Microbiology			
PA Colonization (n)	*NA*	12	9

Number of Patients (n)	HC (9)	CF Stable (30)	AE (14)
PA Colonization (%)	*NA*	40	64.3
CFTR Modulators			
Ivacaftor (n)	*NA*	2	0
Ivacaftor (%)	*NA*	6.7	0
Ivacaftor/Lumacaftor (n)	*NA*	6	7
Ivacaftor/Lumacaftor (%)	*NA*	20	50

HC: Healthy controls; CF Stable: CF participants without exacerbation; AE: Participants with active exacerbation.

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Table 2.
University of Minnesota Cohort – Demographics

Demographic characteristics of the University of Minnesota CF Center cohort (Validation Cohort).

Age	CF Stable (11)	AE (24)
Age (Mean)	27.1	30.0
Age (STD)	7.7	10.5
Age (Range)	14–40	13–57
Sex		
Female (n)	7	12
Female (%)	63.6	50
Male (n)	4	12
Male (%)	36.4	50
Mutation Background		
F508del/F508del (n)	6	14
F508del/F508del (%)	54.5	58.3
F508del/other (n)	5	9
F508del/other (%)	45.5	37.5
Other mutations (n)	1	0
Other mutations (%)	9.1	0
FEV_1 (L)		
FEV_1 (Mean)	2.4	1.7
FEV_1 (STD)	0.6	0.6
FEV_1 (Range)	1.39–3.85	0.70–2.80
FEV_1 (%)		
FEV_1 (Mean)	67.3	45.3
FEV_1 (STD)	12.1	14.1
FEV_1 (Range)	45.5–86.5	26–74
BMI		
BMI (Mean)	21.8	21.3
BMI (STD)	2.1	3.1
BMI (Range)	18.1–24.6	13.1–25.8
Exacerbations per year (AE/y)		
AE/y (Mean)	4.1	4.3
AE/y (Range)	1–12	1–10
CF Comorbidities		
PI (n)	11	23
PI (%)	100	95.8
CFRD (n)	6	9
CFRD (%)	54.5	37.5
Microbiology		
PA Colonization (n)	5	9
PA Colonization (%)	45.5	37.5

Age	CF Stable (11)	AE (24)
CFTR Modulators		
Ivacaftor (n)	0	0
Ivacaftor (%)	0	0
Ivacaftor/Lumacaftor (n)	0	0
Ivacaftor/Lumacaftor (%)	0	0

CF Stable: CF participants without exacerbation; AE: Participants with active exacerbation.

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