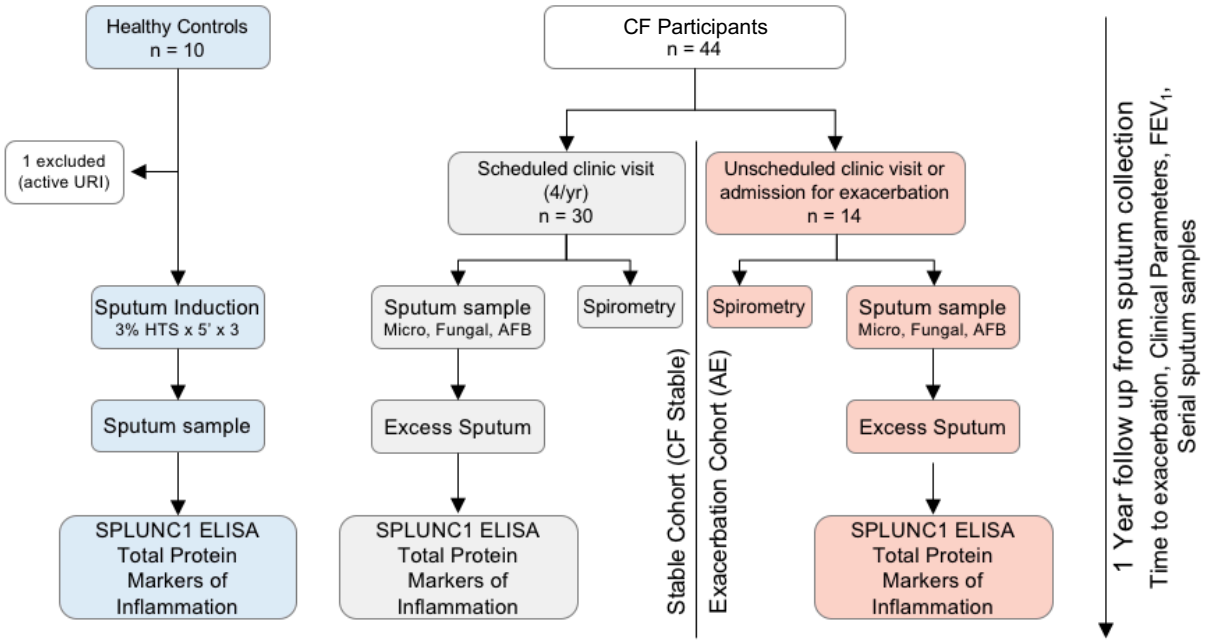
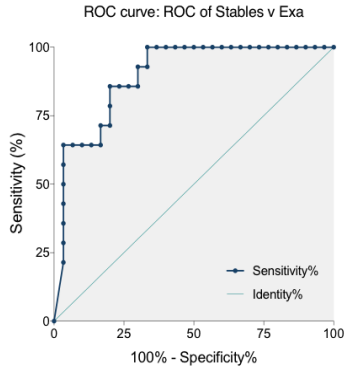


Supplemental Figure 1. Study design (Yale discovery cohort). Forty-four adult patients with a confirmed diagnosis of CF were identified from the Yale Adult CF Program to participate in this study. These patients were recruited during their scheduled routine visits, unscheduled "sick" visits in which they reported new respiratory symptoms, and on their first day of admission to the hospital for treatment of an acute exacerbation (AE). Our recruitment period extended from 2014-2016. We organized study participants into two groups: 1) Stable CF patients (CF Stable): Individuals without new respiratory symptoms, who presented to clinic for their scheduled quarterly follow up and, 2) Patients having an AE: Individuals with new respiratory symptoms, clinically diagnosed with AE that were prescribed treatment for AE during scheduled visit, unscheduled sick visit, or first day of hospital admission for AE. All CF patients provided spontaneously expectorated sputum samples, sputum microbiology samples, and pulmonary function tests. Healthy controls underwent sputum induction. All patients were followed for the development of AE for one year counted from the date of sputum collection.

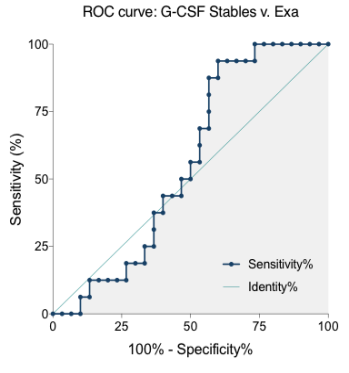
SPLUNC1

Cutoff	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
< 2063	92.86	66.13% to 99.82%	70	50.6% to 85.27%	3.095
< 2165	92.86	66.13% to 99.82%	66.67	47.19% to 82.71%	2.786
* < 2334	100	76.84% to 100%	66.67	47.19% to 82.71%	3
< 2613	100	76.84% to 100%	63.33	43.86% to 80.07%	2.727
< 2935	100	76.84% to 100%	60	40.6% to 77.34%	2.5



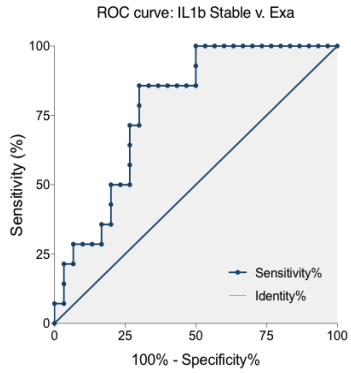
G-CSF

Cutoff	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
< 147.1	68.75	41.34% to 88.98%	46.67	28.34% to 65.67%	1.289
< 153.7	68.75	41.34% to 88.98%	43.33	25.46% to 62.57%	1.213
* < 171.6	75	47.62% to 92.73%	43.33	25.46% to 62.57%	1.324
< 191.2	81.25	54.35% to 95.95%	43.33	25.46% to 62.57%	1.434
< 198.5	87.5	61.65% to 98.45%	43.33	25.46% to 62.57%	1.544



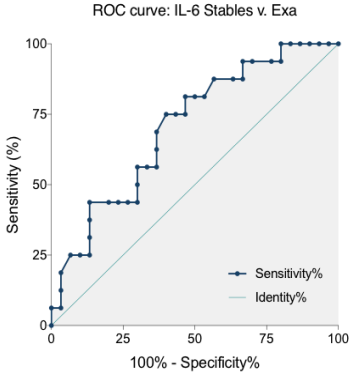
IL-1β

Cutoff	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 940.2	100	76.84% to 100%	43.33	25.46% to 62.57%	1.765
> 1135	100	76.84% to 100%	46.67	28.34% to 65.67%	1.875
* > 1574	100	76.84% to 100%	50	31.3% to 68.7%	2
> 1907	92.86	66.13% to 99.82%	50	31.3% to 68.7%	1.857
> 2053	85.71	57.19% to 98.22%	50	31.3% to 68.7%	1.714



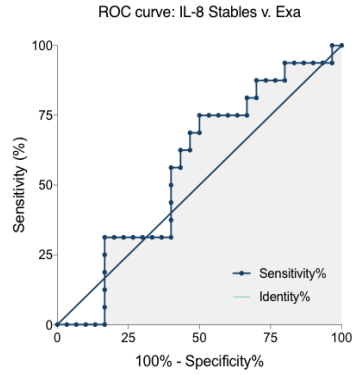
IL-6

Cutoff	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
< 9.65	62.5	35.43% to 84.8%	63.33	43.86% to 80.07%	1.705
< 10	68.75	41.34% to 88.98%	63.33	43.86% to 80.07%	1.875
* < 10.65	75	47.62% to 92.73%	60	40.6% to 77.34%	1.875
< 11.15	75	47.62% to 92.73%	56.67	37.43% to 74.54%	1.731
< 11.35	75	47.62% to 92.73%	53.33	34.33% to 71.66%	1.607



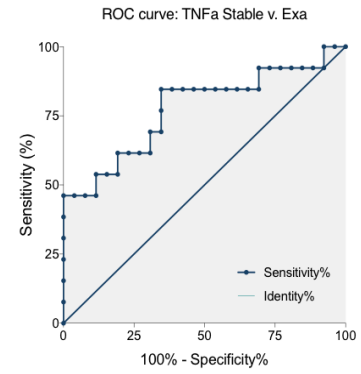
IL-8

Cutoff	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 27972	75	47.62% to 92.73%	50	31.3% to 68.7%	1.5
> 28408	68.75	41.34% to 88.98%	50	31.3% to 68.7%	1.375
* > 28647	68.75	41.34% to 88.98%	53.33	34.33% to 71.66%	1.473
> 29357	62.5	35.43% to 84.8%	53.33	34.33% to 71.66%	1.339
> 30084	62.5	35.43% to 84.8%	56.67	37.43% to 74.54%	1.442

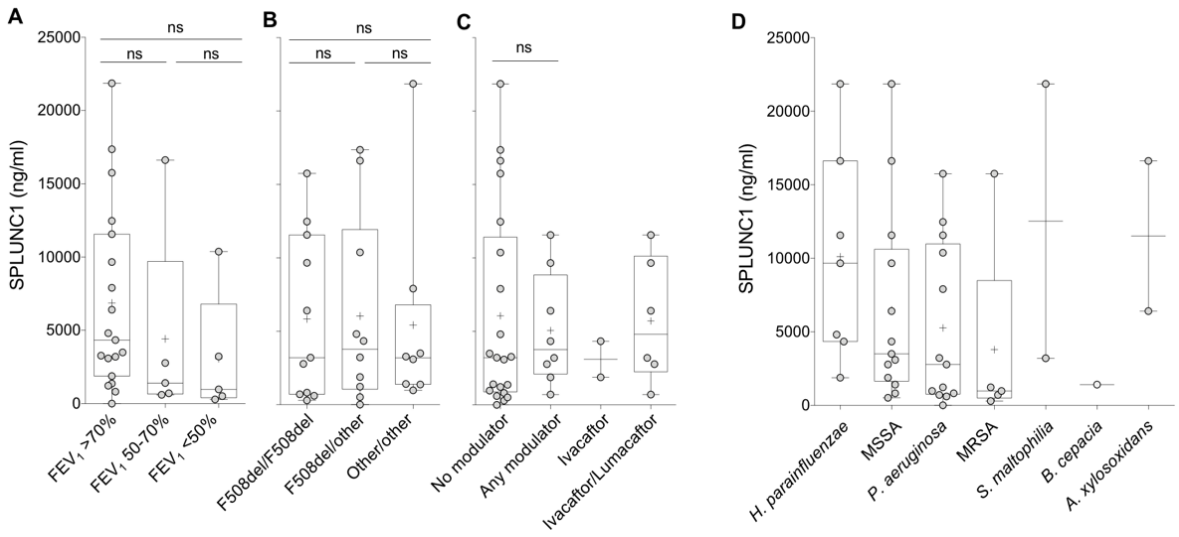


TNFα

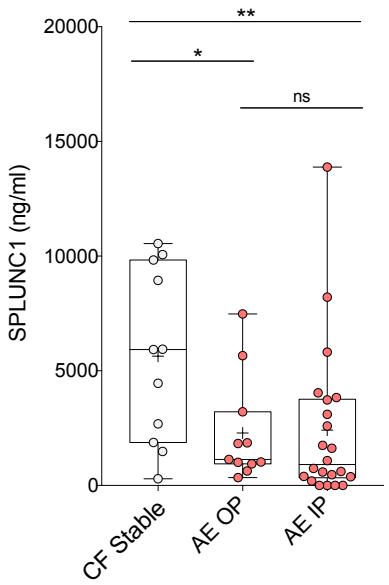
Cutoff	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
> 98.65	84.62	54.55% to 98.08%	57.69	36.92% to 76.65%	2
> 104.8	84.62	54.55% to 98.08%	61.54	40.57% to 79.77%	2.2
* > 111.2	84.62	54.55% to 98.08%	65.38	44.33% to 82.79%	2.444
> 116.2	76.92	46.19% to 94.96%	65.38	44.33% to 82.79%	2.222
> 124.4	69.23	38.57% to 90.91%	65.38	44.33% to 82.79%	2



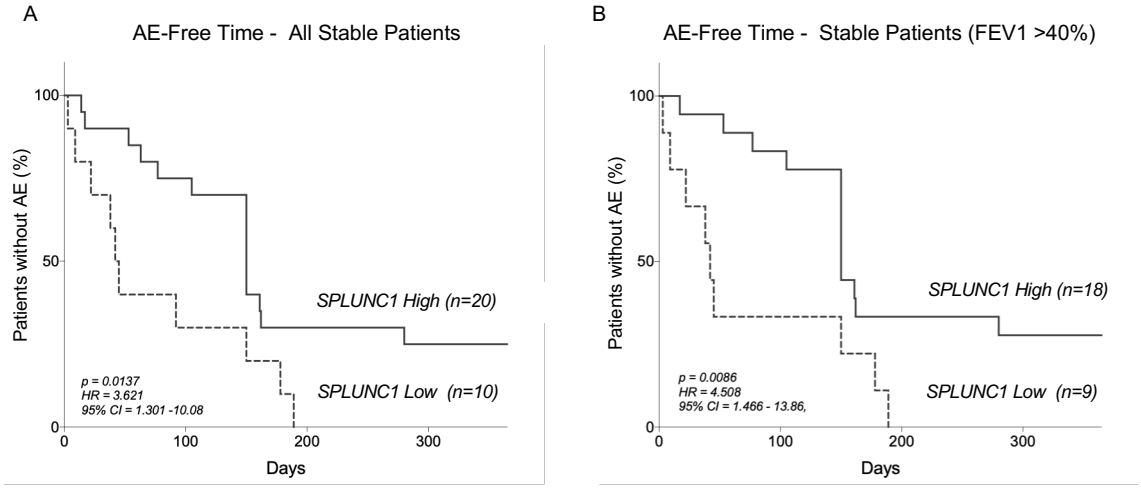
Supplemental Figure 2. Receiver Operator Curve Development for defining SPLUNC1, IL-1β, TNFα, GCSF, IL-6, and IL-8- High and Low categories. Cutoff values (*) were selected to provide maximum sensitivity with the highest specificity possible (highlighted in gray).



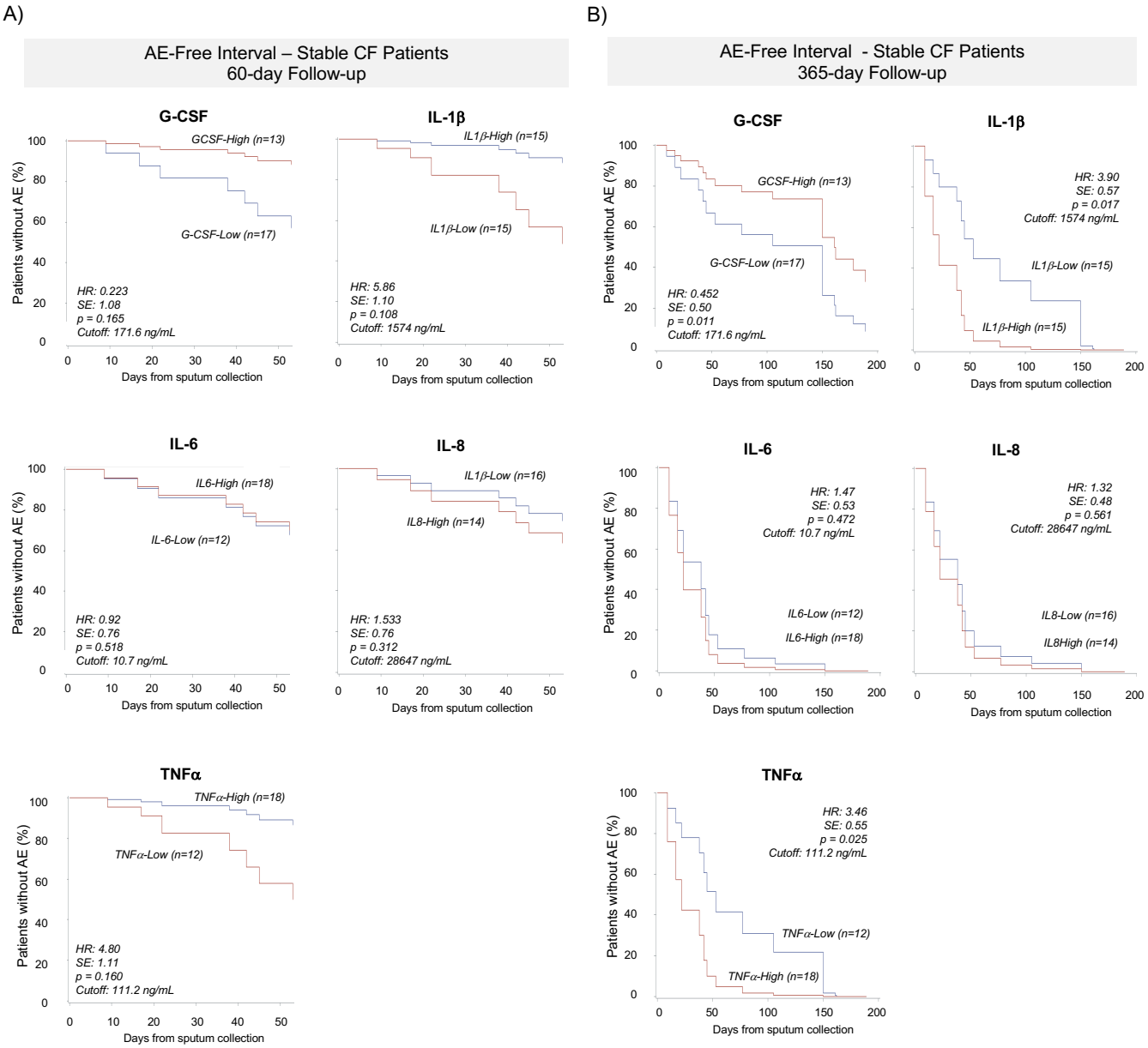
Supplemental Figure 3. CF Stable SPLUNC1 levels do not vary according to FEV_1, CF-causing mutation, modulator therapy, or sputum microbiology. Sputum SPLUNC1 levels in stable CF patients according to A) FEV_1, B) *F508del* genotype, C) CFTR-modulator therapy, and D) Sputum microbiology from the Yale discovery cohort including 30 adults with CF. Samples were obtained by voluntary expectoration during clinical assessment, SPLUNC1 quantified by ELISA. FEV_1 (Percent of Predicted, %) obtained by spirometry during clinical assessment; *FEV_1: Forced Expiratory Volume in the first second; + = Mean; Bar inside box: Median; Whiskers: Minimum/Maximum. Mann-Whitney test*; *ns = not statistically significant.*



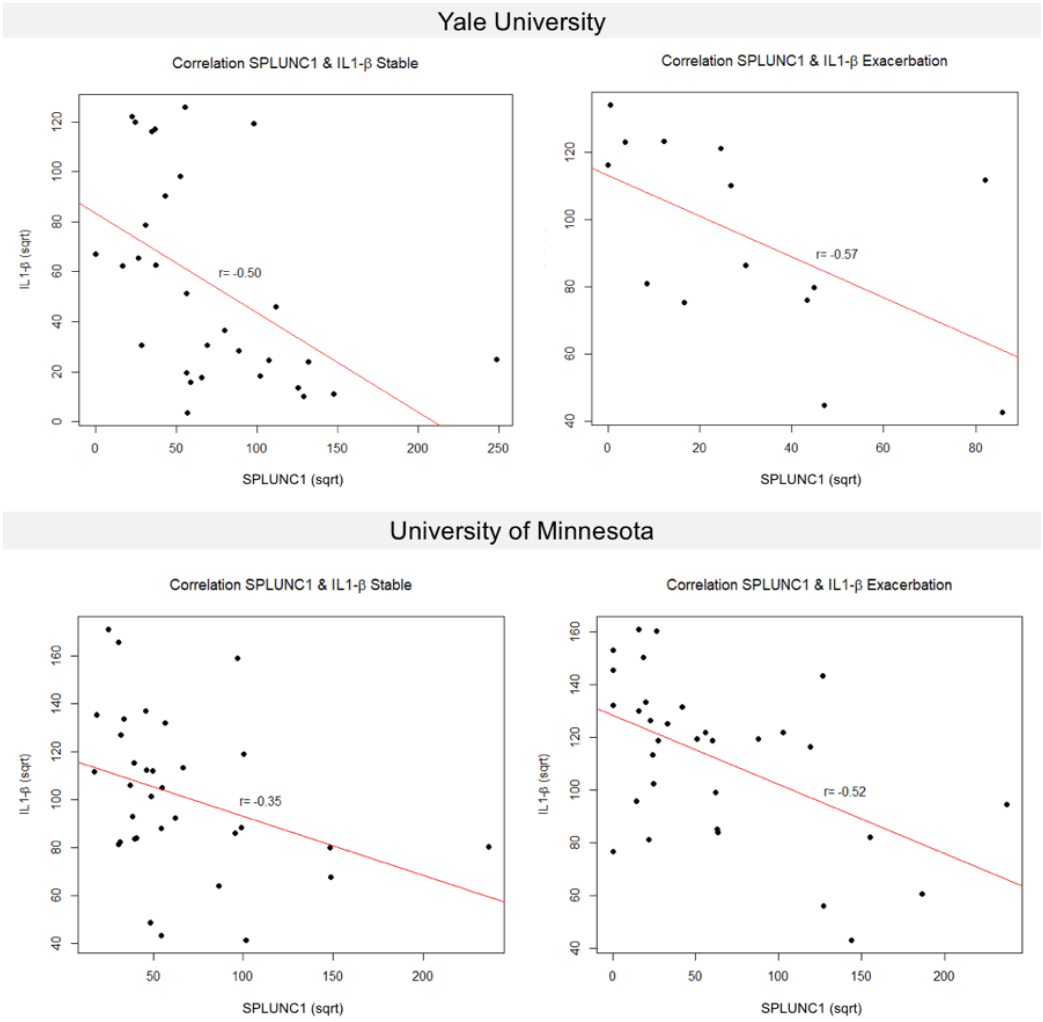
Supplemental Figure 4. SPLUNC1 is Decreased During CF Exacerbations Requiring Outpatient or Inpatient Antibiotic Treatment. SPLUNC1 levels in sputum samples from a clinical cohort including adult and pediatric CF patients (University of Minnesota). Samples were obtained by voluntary expectoration during clinical assessment, SPLUNC1 quantified by ELISA. *CF Stable:* No symptoms of AE, no antibiotic treatment. *AE Outpatient (AE OP):* Clinical symptoms consistent with exacerbation, treated with oral antibiotics at the time of sputum collection. *AE Inpatient (AE IP):* Admitted for inpatient antibiotic course, sample collected during first day of treatment. + = *Mean; Bar inside box: Median; Whiskers: Minimum/Maximum; Mann-Whitney test*; * = $p<0.05$, ** = $p<0.01$, *ns = not statistically significant.*



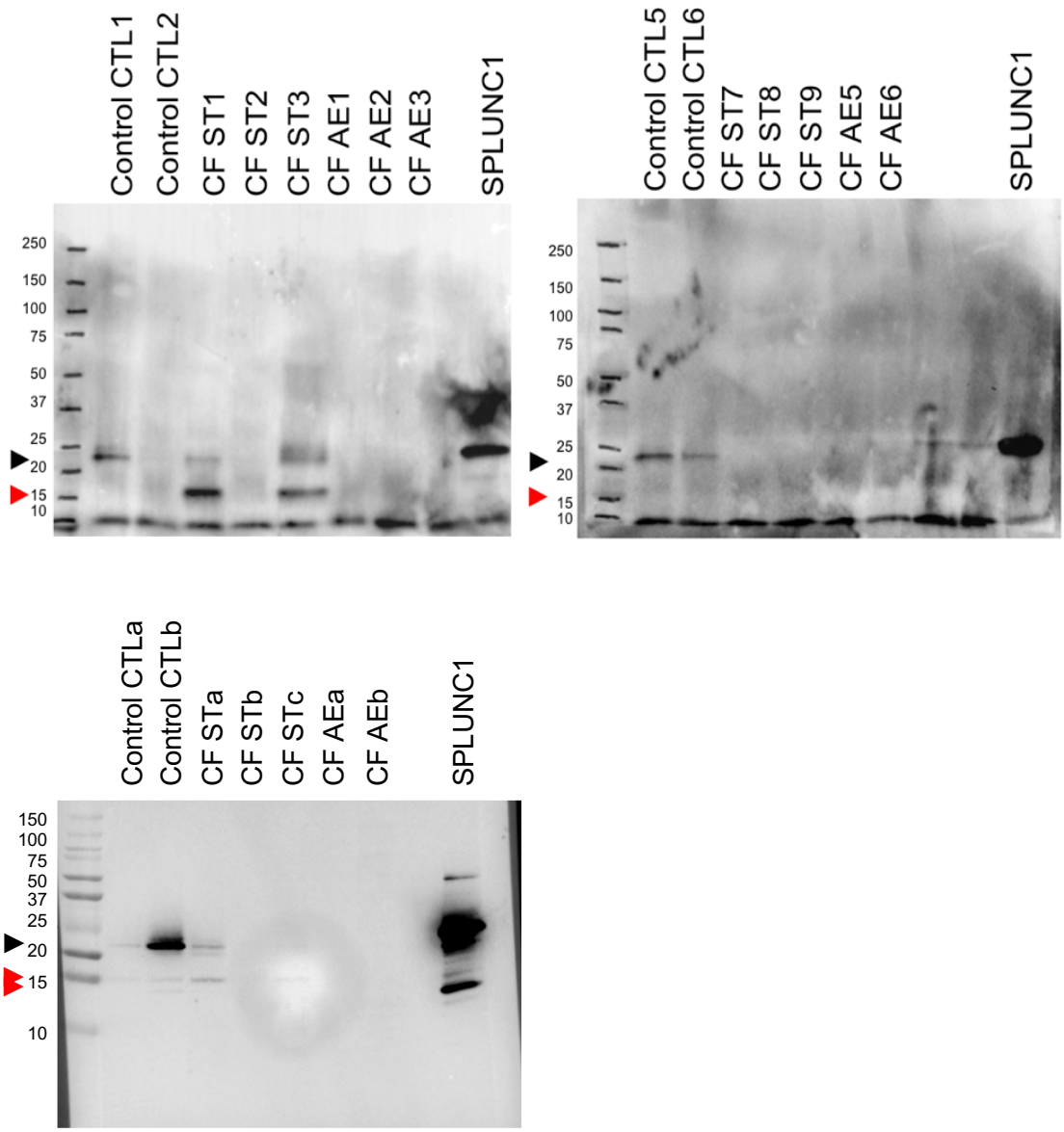
Supplemental Figure 5. SPLUNC1 Predicts AE-Free Time (unadjusted survival model). A) Stable CF patients were separated into SPLUNC1-high and SPLUNC1-Low groups according to a SPLUNC1 threshold of 2334 ng/mL. Time to AE was measured over 365 days from the date of sputum collection in all patients. **B)** Stable patients with an FEV_1 >40% of predicted were separated into SPLUNC1-high and SPLUNC1-low cohorts as above. Time to exacerbation was measured for up to one year from the date of sputum collection. Mantel-Haenszel estimator was used to calculate exacerbation-free interval in each group. Values are not adjusted for clinical variables. Adjusted values from Cox proportional hazards model are presented in Figure 4. *AE: CF exacerbation, HR: Hazard ratio, CI: Confidence interval.*



Supplemental Figure 6. Sputum Cytokines Do Not Predict Short Term AE-Free Time. (A) AE-Free time in Stable CF patients separated into IL-1β-, TNFα-, G-CSF-, IL-6-, and IL-8-High and -Low groups over a 60-day follow up period. Marker-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 IL-1β-, TNFα-, G-CSF-, IL-6-, and IL-8-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 correctors/modulators, and microbiology for *P. aeruginosa, A. xylosoxidans, H. parainfluenzae, Methicillin-sensitive S. aureus, and Methicillin-resistant S. aureus. HR: Hazard ration; SE: Standard error.*

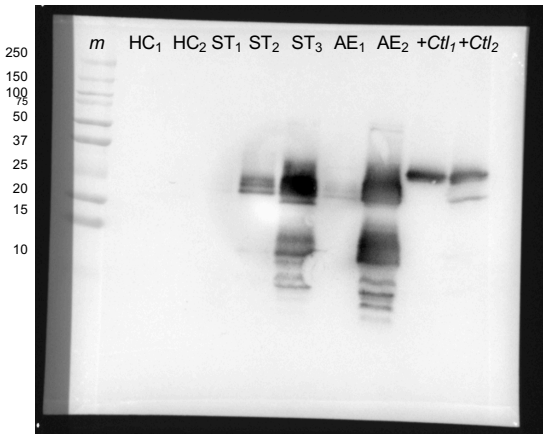


Supplemental Figure 7. SPLUNC1 Negatively Correlates with IL-1β in Stable and AE states. Pearson correlation demonstrates an inverse relationship between IL-1β and SPLUNC1 levels in Stable (left) and AE (right) states. Sputum samples from two cohorts including adult (Yale University, Stable n=30, AE n=14) and mixed adult/pediatric (University of Minnesota, Stable n=33, AE n=32) CF participants. SPLUNC1 and IL-1β quantified by ELISA, values are square-root-transformed for analysis. *r=correlation coefficient. All four correlations have a p <0.05.*

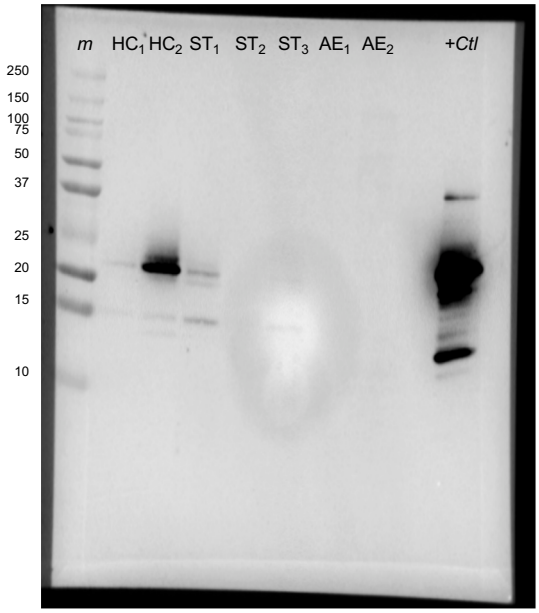


Supplemental Figure 8. SPLUNC1 Western Blot does not distinguish disease states but Identifies SPLUNC1 fragments. Full unedited WB showing SPLUNC1 in sputum from study participants. Sputum supernatants were loaded onto 4-15% Bis-Tris (top) or 16% Tricine gels (bottom, for improved SPLUNC1 fragment identification). Samples were electrophoresed at 130 volts. Membranes were initially probed for NE prior to stripping and re-probing for SPLUNC1. SPLUNC1 antibody: goat polyclonal hPLUNC1 antibody raised against residues Q20 - V256 of hPLUNC1 (1:3000, R&D systems), with a secondary anti-goat IgG-HRP. *ST: Stable CF; AE: CF exacerbation; Black arrowheads: SPLUNC1, Red arrowheads: SPLUNC1 fragments.*

A



B



Supplemental Figure 9. Full unedited Western Blots for figure 5D. Membranes were initially probed for NE prior to stripping and re-probing for SPLUNC1. A) NE antibody: Mouse monoclonal anti-hELA2 raised against residues M1–N252 (1:3000, R&D systems). B) SPLUNC1 antibody: goat polyclonal hPLUNC1 antibody raised against residues Q20 - V256 of hPLUNC1 (1:3000, R&D systems), a secondary anti-goat HRP. *HC: Healthy Control; ST: Stable CF; AE: CF exacerbation; m: marker; +ctl: recombinant protein positive control.*

SPLUNC1: A Novel Marker of Cystic Fibrosis Exacerbations

Supplementary Methods Section

Sara Khanal, Megan Webster, Naiqian Niu, Jana Zielonka, Myra Nunez, Geoffrey Chupp, Martin D. Slade, Lauren Cohn, Maor Sauler, Jose L. Gomez, Robert Tarran, Lokesh Sharma, Charles S. Dela Cruz, Marie Egan, Theresa Laguna, Clemente J. Britto

Definition of CF Exacerbation

A CF exacerbation was defined as the emergence of 4 of 12 signs or respiratory symptoms, prompting a change in therapy and initiation of antimicrobial treatment (modified from Fuchs' criteria[1]). These criteria included: change in sputum; change in hemoptysis; increased cough; increased dyspnea; malaise, fatigue or lethargy; fever; hyporexia or weight loss; sinus congestion; change in sinus discharge; change in chest physical exam; or FEV_1 decrease >10% from a previous value[1]. Individuals without new symptoms and those that did not meet AE criteria were characterized as "CF Stable".

Study Design

This was a two-center, prospective cohort study of Cystic Fibrosis (CF) participants during periods of clinical stability and acute exacerbation (AE). The primary objective of this study was to define an association between AE and sputum levels of SPLUNC1. All patients received standard-of-care therapies as recommended by CF Foundation guidelines, including airway clearance therapies, nutritional support, antibiotics when indicated, and cystic fibrosis transmembrane conductance regulator (CFTR) modulators when they became clinically available. Each subject provided a sputum sample and underwent 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, expectorated sputum samples, and pulmonary function testing data were collected at each clinic visit. Sputum was processed as previously described and frozen immediately after collection [2,3].

Cohort Characteristics

Yale Cohort (Discovery cohort): Forty-four adult individuals with a confirmed diagnosis of CF from the Yale Adult CF Program participated in this study. These participants were recruited during a) scheduled routine visits, b) unscheduled “sick” visits, in which they reported AE symptoms, and c) on the first day of admission to the hospital for AE treatment. Our recruitment period extended from 2014-2016. We organized study participants in two groups: 1) Stable CF participants (CF Stable): No new respiratory symptoms, presenting to clinic for scheduled quarterly follow up and, 2) AE participants (AE): Those diagnosed with AE that were prescribed treatment with a change in airway clearance and antibiotics (*Table 1*).

University of Minnesota (UMN, Validation cohort): Thirty-five adult and pediatric participants with a confirmed diagnosis of CF, previously enrolled in a sputum study of AE at UMN were included as a validation cohort. This was a prospective cohort study of patients hospitalized for AE treatment [4]. All patients received standard-of-care therapies. Each subject performed pulmonary function tests (PFT) within 72 hours of the initiation of intravenous antibiotics and provided sputum samples (*Table 2*)[5].

We also recruited ten healthy volunteers (Healthy Controls, HC) to undergo sputum induction according to previous protocols[6]. The study protocol was approved by the Yale University Institutional Review Board and informed consent was obtained from each subject.

Sputum Collection and Processing

CF participants with or without AE expectorated sputum spontaneously for routine airway cultures and provided an additional sample for our studies. Induced sputum samples were obtained from HC as previously described [6,7]. Briefly, participants inhaled nebulized 3% hypertonic saline for 5 minutes on three cycles. To reduce squamous cell contamination, participants were asked to rinse their mouth with water and clear their throat. Expectorated sputum samples were collected into specimen cups and placed on ice. Sputum plug material from HC and CF participants were selected and weighed prior to washing with 9x their volume of PBS. Samples were incubated in Dulbecco's Phosphate-Buffered Saline (DPBS) with agitation for 15 minutes and centrifugated at 5000 RCF for 5 minutes at room temperature. Supernatants were stored at -80°C.

SPLUNC1 ELISA

SPLUNC1 concentrations were measured in sputum supernatants using a direct human SPLUNC1 ELISA. Human SPLUNC1 recombinant protein was used as standard reference (Abnova, Taipei, Taiwan, cat# H00051297-P01). Detection antibody: polyclonal mouse anti-human SPLUNC1 IgG (1:000 dilution, cat# SAB1401687, MilliporeSigma, Burlington, MA). This antibody recognizes the following amino acid sequence within the SPLUNC1 protein: MFQTGGLIVFYGLLAQTMAQFGGLPVPLDQTLPLNVNPALPL SPTGLAGSLTNALSNGLLSGGLLGILENLPLLDILKPGGGTSGGLLGGLLGKVTSVIPGLNNIIDIKVTDP QLLELGLVQSPDGHRLYVTIPLGIKLQVNTPLVGASLLRLAVKLDITAEILAVRDKQERIHLVLGDCTHS PGSLQISLLDGLGPLPIQGLLDSLTGILNKVLPELVQGNVCPLVNEVLRGLDITLVHDIVNMLIHGLQFVI KV. Secondary antibody: Goat anti-mouse IgG HRP-conjugated (1:2500 dilution, Invitrogen, Carlsbad, CA cat# G21040). 3,39,5,59-tetramethylbenzidine (TMB) substrate was applied to develop the reaction (KPL, Gaithersburg, MD, cat# 5120-0047-50-76-00). Reactions were measured at optical densities of 450 and 550 nm using a SpectraMax Gemini XS Reader (Molecular Devices, San Jose, CA) . The limits of detection of this assay ranged 1 ng/mL - 20,000 ng/mL. The SPLUNC intra- and inter-assay variability was calculated using 5 randomly selected HC and 8 CF sputum samples. For inter-assay variability, SPLUNC1 was measured in duplicate in two separate plates on different days. Mean intra-assay variability was calculated at 5.18% (STDEV 1.28%) and inter-assay variability at 18.44% (STDEV 12.95%). Specimens were run in duplicate, using four serial dilutions to ensure reproducibility.

Western Blot

For Western blots, two HC, three stable CF, and three AE supernatants were thawed on ice for each assay. The samples were vortexed and centrifuged at 5000 RCF for five minutes at room temperature. The total protein concentrations in the supernatants were determined by BCA assay (Thermo Fisher Scientific, Waltham, MA, cat# 23225) and 20 μg of total protein was loaded for each sample onto 4-15% Bis-Tris gels (cat# 4568093, BioRad, Hercules, CA). Samples were electrophoresed at 130 volts. 1X Tris/Glycine/SDS Buffer was used as the running buffer (BioRad, Hercules, CA, cat# 1610732). Gels were transferred onto PDVF membranes (BioRad, Hercules, CA, cat# 1704156) and 3% nonfat milk and 3% Bovine serum albumin in 1X TBS and 1% Tween 20 (TBST) (AmericanBio, Canton, MA, cat# AB14330-01000) was used as blocking agent for 1 hour. After blocking, the membrane was probed for human neutrophil elastase (NE, hELA2) using mouse monoclonal anti-hELA2 (1:3000 dilution, R&D systems, Minneapolis, MN, cat# MAB-91671-100). The membrane was washed three times for 15 min with TBST and incubated with a 1:2500 dilution of horseradish peroxidase-conjugated anti-mouse IgG (Invitrogen, Carlsbad, CA, cat# G21040) for 40 min. Membranes were then "stripped" and re-probed using SPLUNC1 antibody (1:3000, R&D systems, Minneapolis, MN, cat#AF1897-SP). This antibody detects fragments of SPLUNC1 in sputum from HC and CF patients at ranges between 10 and 20 kD (Supplementary Figure 8), however, SPLUNC1 fragments are not detected in all samples from CF and controls. Membranes were developed using chemiluminescence. The loading control used for the densitometry was β-actin and similar protein abundance was confirmed by Ponceau Red staining. Protein band densitometry was determined using ImageJ software version 1.7 (https://imagej.nih.gov/ij/index.html).

Multiplexed ELISA Assay

Two custom-made multiplexed cytokine ELISA assays were used for sputum cytokine measurements (U-Plex Biomarker Kit, Mesoscale Diagnostics. Rockville, MD, cat# K15235N-1, coefficient of variability <25%). The assays were used to measure the cytokine levels of IFN-α2a, IFN-γ, IL-6, IL-8, CXCL10, TNF-α, G-CSF, IL-1β, IL-13, IL-29, MCP-1, and MIP-1α. Biotinylated capture antibody was combined with an assigned linker for each cytokine and incubated for 30 minutes. After 30 minutes, stop solution was added to stop the linker-antibody reaction to make a 10X solution and incubated for 30 minutes (U-Plex-coupled antibody solution). The U-Plex-coupled antibody solution was diluted in extra stop solution to bring the concentration to 1X. The 1X coating solution was added to each well of the U-Plex plate and incubated with shaking at 4C overnight. Next day, cytokine calibrator standards were prepared for each plate. Calibrator standard was reconstituted by adding a diluent to prepare a 1:4 dilution of each individual cytokine. We repeated 4-fold serial dilutions six additional times to generate a total of 7 calibrator standards. Sputum samples were diluted 1:2 in the provided diluent. The plates were washed with PBS plus 0.05% Tween-20 three times. The calibrator standards and sputum samples were diluted again in a 1:2 concentration in the provided diluent and added to the plates and incubated at room temperature while shaking for 1 hr. The detection antibody was diluted in the provided diluent to bring final concentration from 100X to 1X. The plates were washed 3 times and detection antibody was added to each well and incubated at room temperature while shaking for 1 hr. The plates were washed 3 times and 2X Read Buffer T was added to each well for reading. The plates were read on a Quickplex SQ 120 reader (MSD, Rockville, MD, cat# AI0AA-0) using MSD Discovery Workbench software (MSD, Rockville, MD, Version 4).

Sputum Neutrophil Elastase Activity

Neutrophil elastase (NE) activity was determined using the NE-specific substrate Suc-Ala-Ala-Ala-MCA (Peptides International, Louisville, KY, cat# MAA-3133;) at a final concentration of 100 μM, as described previously[8]. NE starting concentrations were based on previous observations that reported NE levels in CF sputum ranging from 0.1-1 μM[8,9]. There is limited data to inform the selection of a *P. aeruginosa*'s Elastase B (LasB) starting dose within a physiologic range, as this concentration would vary according to inoculum, bacterial growth rates, and stage of treatment or resolution. However, a LasB dose of 3.75 μM has been reported to induce cytokine expression and inhibit host defense protein expression[10]. As this dose was associated with cellular toxicity, we selected the lower starting dose of 1 μM to determine if minimal doses of LasB would be sufficient to regulate SPLUNC1 following *P. aeruginosa* infection. Following incubation with elastases, the fluorogenic substrate is cleaved to generate the reaction product 7-amino-4-methylcoumarin (AMC), whose fluorescence is measured by spectrophotometry. Briefly, sputum was diluted 1:1 with assay buffer (115 mM NaCl; 10 mM HEPES). 10 μl of sputum-buffer mix was added per well, and 10 μl of 100 μM MAA-3133 added to each well. Assays were performed at 37°C in a Tecan infinite M1000 plate-reader, with fluorescence readings taken every 15 minutes. Samples were excited at 380 ± 5 nm and emitted fluorescence collected at 460 ± 10 nm. Resultant AMC concentrations were determined from a standard curve with linear regression analysis.

Measurements of SPLUNC1 Degradation by Human and Bacterial Elastase

Recombinant human SPLUNC1(rhSPLUNC1, Abnova, Taipei, Taiwan cat# H00051297-P01) was incubated with recombinant human neutrophil elastase (R&D systems, Minneapolis, MN, cat#9167-SE-020) or *Pseudomonas aeruginosa* elastase (LasB, a gift from Dr. Karen Agaronyan, Yale) diluted to concentration ranges of 0.1, 0.25, 0.5, and 1.0 μM, which was determined based on NE ranges of concentration in CF sputum obtained from the NE-specific substrate Suc-Ala-Ala-Ala-MCA activity. rhSPLUNC1was diluted in NE and LasB solutions to get a final concentration of 0.1, 0.25, 0.5, and 1.0 μM. SPLUNC1 ELISA was performed. The samples were run in duplicate with four serial dilutions to ensure reproducibility.

Regulation of Epithelial SPLUNC1 Expression by Cytokines

Mouse tracheal epithelial cells (mTECs) were isolated from C57BL/6 mice and cultured on transwell plates (Costar, St. Louis, MO, cat#3460) with mTEC Basic Medium, containing DMEM with Hepes, 3.6 mM $NaHCo_3$, 4.5 mM L-Glutamine, 1X Pen-Strep, and 2.5 µg/ml Amphotericin, added to the basal and apical side of the transwell. After cells became confluent, they were transitioned to Air-Liquid Interface (ALI) and the following reagents were added to the culture medium (mTEC-plus ALI medium): 5 µg/ml Transferrin, 0.1 µg/ml Cholera Toxin, 2 mg/ml Bovine Pituitary Extract, 25 ng/ml Epidermal Growth Factor, 10 µg/ml Insulin, and 5% FBS. In this phase, the apical surface of the cells remained at air interface, and medium was administered only to the basal chamber of the transwell as previously described [11,12]. Retinoic acid (0.01 µM Retinoic acid) was added to the mTECs to promote differentiation for four weeks, after ALI. After two weeks of growing cells at ALI, mTECs were treated with recombinant murine IL-1β (Peprotech, Rocky Hill, NJ, cat# 211-11b) or TNF-α (Peprotech, Rocky Hill, NJ, cat# 315-01A), at 10 ng/ml on the apical surface for 24 hours. NCI-H292 human airway epithelial cells (shown to express high levels of SPLUNC1 and to be robustly regulated by inflammatory cytokines [12]), were cultured to confluence in RPMI medium 1640 (Gibco, Waltham, MA, cat# 11875-093) with 10% FBS and 1% combination of Penicillin/Streptomycin. Upon reaching confluence, submerged H292 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 added to the culture medium for 24 hours. Upon completion of the experiments the culture medium was discarded and the cells were collected for RNA isolation.

qPCR

Cellular mRNA was extracted from individual cell culture wells for qPCR using RNeasy plus lysis buffer (Qiagen, Hilden, Germany, cat# 74134). After measuring the RNA concentration of each sample, 1 µg of RNA was used to synthesize cDNA using the iScript reaction mix (Biorad, Hercules, CA cat# 170-8891). After cDNA synthesis, SYBR Fast qPCR Kit (MilliporeSigma, Burlington, MA, cat# kk4619) was used to run the qPCR assay. Murine PCR primers used for amplification included *splunc1* (5′-GTCCACCCTTGCCACTGAACCA-3′and 3′-CACCGCTGAGAGCATCTGTGAA-5′) and *β-actin* (5′-GTCCACACCCGCCACCAGTTCG and 3′-GACCCATTCCCACCATCACACCCT-5′). Human PCR primers used for amplification included *SPLUNC1* (5′-TGCTGGAACTTGGCCTTGTGCA-3′ and 3′-ACCAGGGGCGTATTCACTTGGA-5′) and glyceraldehyde 3–phosphate dehydrogenase (*GAPDH*; 5′-TGGAGAAGGCTGGGGGCTCATTT-3′ and 3′-TGGTGCAGGAGGCATTGCTGAT-5′). For each set of primers, validation experiments showed a linear dependency of threshold cycle values at different RNA concentrations. The data were analyzed after murine genes were normalized to housekeeping gene *β-actin* and human genes were normalized to housekeeping gene *GAPDH*. 384-well reaction plate with master mix, sample, and primers was prepared (Applied Biosystems, Foster City, CA, cat# 4309849) and the Sybr Green-based assay was run in a Viia7 Real Time PCR System (Applied Biosystems, Foster City, CA). Housekeeping genes were selected based on our previous experience in gene expression studies of human and mouse airway epithelial cells [12]. Data analysis was performed using GraphPad Prism 7 (GraphPad, San Diego, CA).

Statistical Analysis

Descriptive statistics were calculated for the entire subject population. Pearson correlations, or Spearman correlations for variables that were not normally distributed, were calculated between SPLUNC1 and clinical parameters. In order to select the optimal threshold of SPLUNC1 and cytokine concentrations that identify a subject group at higher AE risk, we calculated 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 this threshold, we applied statistical modeling (Mantel-Haenszel estimator) to predict AE-free intervals. AE interval was defined as the time in days from sputum sampling in a stable subject to the time of the first AE after that visit. Cox proportional hazards model was conducted with clinical parameters as covariates, these parameters included: 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*. A backward elimination strategy with a significance level to stay of 95% (a=0.05) was employed to achieve a parsimonious model. Note that the predictor of interest was forced to be included in the parsimonious model. All statistical analyses were conducted using SAS 9.4 with a level of significance of 95% (α=0.05).

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SPLUNC1: A Novel Marker of Cystic Fibrosis Exacerbations

Supplementary Tables Section

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Supplementary Table 1: SPLUNC1 levels sorted by FEV_1.

Yale Stable				
Age	**Sex**	**FEV1 (L)**	**FEV1 (%)**	**SPLUNC1**
51	M	NA	NA	1374.2
67	F	2.5	121.0	3498.7
70	M	3.3	113.0	61732.1
41	M	4.2	108.0	4810.9
39	F	3.1	103.0	7902.2
27	F	2.9	91.0	11563.0
65	F	2.3	90.5	17359.2
68	F	1.9	88.0	21862.6
54	F	2.0	86.5	1225.4
23	F	2.4	84.5	3279.3
30	F	2.2	82.0	816.3
29	F	2.4	80.0	0.0
20	F	2.6	79.0	3200.5
32	F	2.7	78.5	1880.6
32	M	3.3	78.0	9663.0
22	F	2.6	78.0	6412.3
43	F	2.1	77.0	15749.9
24	F	2.1	75.0	12469.5
54	M	2.9	73.5	4340.2
25	M	3.0	71.0	3094.8
29	F	1.7	63.0	703.0
31	F	1.9	62.0	609.8
34	M	2.3	55.0	2775.4
58	M	1.9	54.0	1408.3
79	M	1.6	52.0	16625.1
58	F	1.1	46.5	974.8
33	M	1.9	42.0	288.5
35	M	1.3	30.0	3218.7
22	M	0.7	17.0	10378.8
38	M	0.6	12.0	515.7

Yale AE				
Age	**Sex**	**FEV1 (L)**	**FEV1 (%)**	**SPLUNC1**
39	F	2.7	89.0	0.0
27	F	2.8	87.0	279.8
43	F	2.0	74.0	73.8
25	M	3.0	71.0	148.4

25	F	2.0	69.0	2012.5
23	F	2.2	66.5	7360.5
31	F	1.9	62.0	0.4
32	F	1.8	58.5	603.2
29	M	2.1	53.5	6749.8
41	M	1.4	33.0	2216.5
36	M	1.3	31.0	900.3
32	F	0.7	23.0	714.4
38	M	0.6	12.0	14.5
28	M	NA	NA	1886.5

Supplementary Table 2: SPLUNC1 levels sorted by F508del mutations genotype

Yale Stable			
Age	**Sex**	**mutation**	**SPLUNC1**
51	M	Other/other	1374.2
39	F	Other/other	7902.2
23	F	Other/other	3279.3
58	M	Other/other	1408.3
67	F	Other/other	3498.7
68	F	Other/other	21862.6
58	F	Other/other	974.8
25	M	Other/other	3094.8
29	F	F508del/other	0.0
38	M	F508del/other	515.7
54	F	F508del/other	1225.4
79	M	F508del/other	16625.1
32	F	F508del/other	1880.6
22	M	F508del/other	10378.8
54	M	F508del/other	4340.2
65	F	F508del/other	17359.2
35	M	F508del/other	3218.7
41	M	F508del/other	4810.9
70	M	F508del/other	61732.1
27	F	F508del/F508del	11563.0
30	F	F508del/F508del	816.3
20	F	F508del/F508del	3200.5
32	M	F508del/F508del	9663.0
24	F	F508del/F508del	12469.5
29	F	F508del/F508del	703.0
34	M	F508del/F508del	2775.4
33	M	F508del/F508del	288.5
22	F	F508del/F508del	6412.3
43	F	F508del/F508del	15749.9
31	F	F508del/F508del	609.8

Yale AE			
Age	**Sex**	**mutation**	**SPLUNC1**
25	M	Other/other	148.4
39	F	Other/other	0.0
29	M	F508del/other	6749.8
38	M	F508del/other	14.5
41	M	F508del/other	2216.5
28	M	F508del/other	1886.5
27	F	F508del/F508del	279.8
25	F	F508del/F508del	2012.5
32	F	F508del/F508del	603.2
36	M	F508del/F508del	900.3
32	F	F508del/F508del	714.4
43	F	F508del/F508del	73.8
23	F	F508del/F508del	7360.5
31	F	F508del/F508del	0.4

Supplementary Table 3: SPLUNC1 levels sorted by Sputum Microbiology shown by A) Complete microbiology in single individuals and B) Individual organism counts.

Yale Stable			
Age	**Sex**	**Complete Microbiology**	**SPLUNC1**
51	M	NA	1374.2
67	F	MSSA	3498.7
70	M	MSSA; *H. parainfluenzae*	61732.1
41	M	*H. parainfluenzae*	4810.9
39	F	PA	7902.2
27	F	PA; MSSA; *H. parainfluenzae*	11563.0
65	F	Normal flora	17359.2
68	F	MSSA; *S. maltophilia; H. parainfluenzae*	21862.6
54	F	PA; MRSA	1225.4
23	F	Normal flora	3279.3
30	F	PA; MSSA	816.3
29	F	PA; MRSA	0.0
20	F	*S. maltophilia*	3200.5
32	F	MSSA; *H. parainfluenzae*	1880.6
32	M	MSSA; H. parainfluenzae	9663.0
22	F	MSSA; *A. Xylosoxidans*	6412.3
43	F	PA; MRSA	15749.9
24	F	PA	12469.5
54	M	MSSA; *H. parainfluenzae*	4340.2
25	M	MSSA	3094.8

29	F	PA; MRSA; *M. catarrhalis*	703.0
31	F	PA	609.8
34	M	PA; MSSA	2775.4
58	M	MSSA; *B. cepacia*	1408.3
79	M	MSSA; *H. parainfluenzae, A. xylosoxidans*	16625.1
58	F	MRSA; PA	974.8
33	M	MRSA; *b-hemolytic streptococcus*	288.5
35	M	PA	3218.7
22	M	PA	10378.8
38	M	MSSA	515.7

Yale Stable			
Age	**Sex**	**Individual organism count**	**SPLUNC1**
70	M	*H. parainfluenzae*	61732.1
41	M	*H. parainfluenzae*	4810.9
27	F	*H. parainfluenzae*	11563.0
68	F	*H. parainfluenzae*	21862.6
32	F	*H. parainfluenzae*	1880.6
32	M	*H. parainfluenzae*	9663.0
54	M	*H. parainfluenzae*	4340.2
79	M	*H. parainfluenzae*	16625.1

67	F	MSSA	3498.7
70	M	MSSA	61732.1
27	F	MSSA	11563.0
68	F	MSSA	21862.6
30	F	MSSA	816.3
32	F	MSSA	1880.6
32	M	MSSA	9663.0
22	F	MSSA	6412.3
54	M	MSSA	4340.2
25	M	MSSA	3094.8
34	M	MSSA	2775.4
58	M	MSSA	1408.3
79	M	MSSA	16625.1
38	M	MSSA	515.7

39	F	PA	7902.2
27	F	PA	11563.0
54	F	PA	1225.4
30	F	PA	816.3
29	F	PA	0.0
43	F	PA	15749.9

24	F	PA	12469.5
29	F	PA	703.0
31	F	PA	609.8
34	M	PA	2775.4
58	F	PA	974.8
35	M	PA	3218.7
22	M	PA	10378.8

54	F	MRSA	1225.4
43	F	MRSA	15749.9
29	F	MRSA	703.0
58	F	MRSA	974.8
33	M	MRSA	288.5

68	F	*S. maltophilia*	21862.6
20	F	*S. maltophilia*	3200.5

22	F	*A. Xylosoxidans*	6412.3
79	M	*A. Xylosoxidans*	16625.1

58	M	*B. cepacia*	1408.3

Yale AE			
Age	**Sex**	**Complete Microbiology**	**SPLUNC1**
39	F	PA; *b-hemolytic streptococcus*	0.0
27	F	PA; MSSA; *H. parainfluenzae*	279.8
43	F	PA; MRSA	73.8
25	M	MSSA; *H. parainfluenzae*	148.4
25	F	PA	2012.5
23	F	*A. Xylosoxidans*	7360.5
31	F	PA	0.4
32	F	PA; MSSA	603.2
29	M	PA; MRSA; *H. parainfluenzae*	6749.8
41	M	MSSA	2216.5
36	M	MSSA	900.3
32	F	PA	714.4
38	M	MSSA	14.5
28	M	PA; MRSA	1886.5

Yale AE			
Age	**Sex**	**Individual organism count**	**SPLUNC1**

25	M	*H. parainfluenzae*	148.4
29	M	*H. parainfluenzae*	6749.8

27	F	MSSA	279.8
25	M	MSSA	148.4
32	F	MSSA	603.2
41	M	MSSA	2216.5
36	M	MSSA	900.3
38	M	MSSA	14.5

39	F	PA	0.0
27	F	PA	279.8
43	F	PA	73.8
25	F	PA	2012.5
31	F	PA	0.4
32	F	PA	603.2
29	M	PA	6749.8
32	F	PA	714.4
28	M	PA	1886.5

43	F	MRSA	73.8
29	M	MRSA	6749.8
28	M	MRSA	1886.5

23	F	*A. Xylosoxidans*	7360.5

Supplementary Table 4: SPLUNC1 levels sorted by active modulator use.

Yale Stable				
Age	**Sex**	**Ivacaftor**	**Ivacaftor/Lumacaftor**	**SPLUNC1**
51	M	no	no	1374.2
67	F	no	no	3498.7
70	M	no	no	61732.1
41	M	no	no	4810.9
39	F	no	no	7902.2
65	F	no	no	17359.2
68	F	no	no	21862.6
54	F	no	no	1225.4
23	F	no	no	3279.3
30	F	no	no	816.3
29	F	no	no	0.0
43	F	no	no	15749.9
24	F	no	no	12469.5
25	M	no	no	3094.8

31	F	no	no	609.8
58	M	no	no	1408.3
79	M	no	no	16625.1
58	F	no	no	974.8
33	M	no	no	288.5
35	M	no	no	3218.7
22	M	no	no	10378.8
38	M	no	no	515.7
54	M	yes	no	4340.2
32	F	yes	no	1880.6
27	F	no	yes	11563.0
20	F	no	yes	3200.5
32	M	no	yes	9663.0
22	F	no	yes	6412.3
29	F	no	yes	703.0
34	M	no	yes	2775.4

Yale AE				
Age	**Sex**	**Ivacaftor**	**Ivacaftor/Lumacaftor**	**SPLUNC1**
39	F	no	no	0.0
25	M	no	no	148.4
31	F	no	no	0.4
29	M	no	no	6749.8
41	M	no	no	2216.5
38	M	no	no	14.5
28	M	no	no	1886.5
27	F	no	yes	279.8
43	F	no	yes	73.8
25	F	no	yes	2012.5
23	F	no	yes	7360.5
32	F	no	yes	603.2
36	M	no	yes	900.3
32	F	no	yes	714.4