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SPLUNC1 Degradation by the Cystic Fibrosis Mucosal Environment Drives Airway Surface Liquid Dehydration

Megan J. Webster[1], **Boris Reidel**[1], **Chong D. Tan**[1], **Neil E. Alexis**[2], **Scott H. Donaldson**[1,4], **Mehmet Kesimer**[1], **Carla M.P. Ribeiro**[1,3], and **Robert Tarran**[1,3]

[1]Marsico Lung Institute, The University of North Carolina, Chapel Hill, NC, 27599,

[2]Center for Asthma and Lung Biology, The University of North Carolina, Chapel Hill, NC, 27599,

[3]Department of Cell Biology & Physiology, The University of North Carolina, Chapel Hill, NC, 27599,

[4]Division of Pulmonary and Critical Care Medicine, The University of North Carolina, Chapel Hill, NC, 27599,

Abstract

Cystic fibrosis (CF) is a multi-organ disease caused by mutations in the cystic fibrosis transmembrane regulator (*CFTR)* gene that leads to diminished transepithelial anion transport. CF lungs are characterised by airway surface liquid (ASL) dehydration, chronic infection/inflammation and neutrophilia. Dysfunctional CFTR may up-regulate the epithelial Na^+ channel (ENaC), further exacerbating dehydration. We previously demonstrated that the short palate lung and nasal epithelial clone 1 (SPLUNC1) negatively regulates ENaC in normal airway epithelia. Here, we used pulmonary tissue samples, sputum and human bronchial epithelial cultures (HBECs) to determine whether SPLUNC1 could regulate ENaC in a CF-like environment. We found that SPLUNC1 was reduced in CF secretions, and that CF secretions rapidly degraded recombinant SPLUNC1 (rSPLUNC1). Interestingly, normal sputum, which contained SPLUNC1 and SPLUNC1-derived peptides, inhibited ENaC in both normal and CF HBECs. Conversely, CF sputum activated ENaC and rSPLUNC1 was unable to reverse this phenomenon. Additionally, we observed upregulation of ENaC protein levels in human CF bronchi. SPX-101 is a novel, SPLUNC1-derived peptide. Unlike SPLUNC1, SPX-101 resisted protease degradation, bound to the mucosal surface of HBECs, inhibited ENaC and prevented ASL dehydration following extended pre-incubation with CF sputum. Our data indicate CF mucosal secretions drive ASL hyperabsorption and protease-resistant peptides, like SPX-101, can reverse this effect to re-hydrate CF ASL.

Correspondence to: Robert Tarran, 7102 Marsico Hall, 125 Mason Farm Road, University of North Carolina, Chapel Hill, NC, 27599, robert_tarran@med.unc.edu, Contact: 919-966-7052.

Author Contributions

M. J. Webster, B. Reidel, C. D. Tan, R. Tarran designed and performed research and analyzed data. N. E. Alexis, S. H. Donaldson and C. M. P. Ribeiro provided sputum and SMM samples, designed experiments and interpreted data. M. Kesimer designed experiments and analyzed data. M. J. Webster, B. Reidel and R. Tarran wrote the manuscript. All other authors edited and approved the manuscript.

Conflicts of Interest

RT is founder of and has equity in Spyryx Biosciences. CMPR is founder of and has equity in Irex Pharma. No other conflicts exist.

Introduction

Cystic fibrosis (CF) is a genetic, multi-organ disease caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene that result in defective transepithelial anion transport (1, 2). In the airways, ENaC is the rate-limiting step that governs transepithelial Na^+ absorption (3). The absence of CFTR leads to increased ENaC activity and Na^+ hyperabsorption, which in combination with anion hyposecretion causes ASL dehydration reducing mucociliary clearance (4–6) Together, these changes are predicted to cause the accumulation of dehydrated intraluminal mucus plugs that serve as a nidus for subsequent bacterial infections. A failure to resolve these infections causes chronic inflammation/neutrophilia and elevated lung luminal protease levels. These proteases, including neutrophil elastase (NE) and cathepsins B, S, L, then cause bronchiectasis and ultimately destroy the lung (7, 8). Indeed, a correlation between NE activity and CF lung disease severity has recently been reported (9).

Short palate lung and nasal epithelial clone 1 (SPLUNC1) is a multi-functional 25 kDa innate defence protein that is secreted into the airway surface liquid (ASL) (10–13). SPLUNC1 is an allosteric regulator that binds extracellularly causing a conformational change to ENaC. This binding event is followed by NEDD4.2-dependent ubiquitination of αENaC and subsequent internalisation of the channel, which serves to reduce Na^+ and ASL absorption (12–14). Resolution of the SPLUNC1's crystal structure has allowed assignment of its functions to distinct regions. For example, the N-terminal 'S18' region inhibits the epithelial Na^+ channel (ENaC) to modulate ASL volume (12, 13).

Interactions between CFTR and ENaC have been reported in heterologous expression systems (5, 15, 16). However, the nature of these interactions and the underlying mechanism of Na^+ hyperabsorption are unknown. As such, the role of ENaC in CF lung pathophysiology remains controversial (17, 18). SPLUNC1 inhibits ENaC in normal ASL. However, due to a series of pH-sensitive salt bridges, SPLUNC1 fails to function in the moderately acidic CF ASL (~pH 6.5)(12). Thus, under thin film conditions where native ASL has accumulated, SPLUNC1 can spontaneously inhibit ENaC in normal but not CF epithelia. Since they lack the pH-sensitive salt bridges, peptides of SPLUNC1's ENaC inhibitory domain are pH-independent and inhibit ENaC in CF-affected epithelia (13). For example, a SPLUNC1-derived peptide, SPX-101, is currently undergoing clinical trials for the treatment of CF lung disease (19). We have previously focused on the initiating events in CF (6, 12). However, given the high levels of neutrophilia and protease activity within CF airways, we postulated that endogenous SPLUNC1 was susceptible to a non-pH related degradation in CF lungs, which if it occurred within the S18 region, would prevent ENaC inhibition. Thus, using patient-derived samples, we tested the hypotheses that (i) SPLUNC1 is degraded in CF airways, leading to increased ENaC activity and (ii) that SPX-101 could overcome this deficiency to induce ENaC inhibition and rehydrate CF ASL.

Methods

A detailed description of Methods used is provided in the online supplement.

Normal/CF sputum and supernatant of mucopurulent material (SMM).

Induced and spontaneous sputum samples were obtained as described (20). SMM was harvested from the airways of excised human CF lungs as described (21). Donor demographics are shown in supplementary tables 1, 2 and 3.

Proteins and peptides.

SPLUNC1 was expressed and purified as described previously (12). SPX-101 (aaLPIPLDQTaa) was prepared by solid-state FMOC synthesis as described (19).

Western blot.

Endogenous NE and SPLUNC1 protein levels were determined using neat sputum samples from 18 donors. For degradation experiments, PBS, NE, normal or CF sputa (pooled from N = 6 donors/group) and SMM (pooled from N = 3 donors) were co-incubated with 10 μM rSPLUNC1 at 37°C. Where applicable, inhibitors were pre-incubated with sputa for one hour prior to rSPLUNC1 incubation.

Neutrophil elastase activity assay.

NE activity levels were determined using the NE sensitive peptide Suc-Ala-Ala-Ala-MCA as described in the supplementary material.

ENaC and Na^+/K^+-ATPase expression in human bronchi.

Human lungs were obtained as described above, donor demographics are shown in supplementary table 3. Bronchi were dissected from the underlying tissue; CF bronchi were selected from relatively disease-free regions. Tissues were rinsed using a lactated Ringers solution and proteins extracted using lysis buffer containing NP40 (22).

Determination of rSPLUNC1 and SPX-101 cleavage by mass spectrometry.

10 μM rSPLUNC1 and SPX-101 were incubated with pooled normal or CF sputa (N = 6 donors/group). Samples were snap frozen at −80°C and prepared by filter-aided sample preparation for proteomics (23). Solubilized peptide material was injected for label-free quantitative proteomic analysis utilizing a Q Exactive (Thermo Scientific) mass spectrometer coupled to an UltiMate 3000 (Thermo Scientific) nanoHPLC system, and data acquisition was performed as described (24).

Human bronchial epithelial cell culture.

Cells were harvested from human lungs deemed unsuitable for transplantation (non-CF donors) or post-transplantation (CF donors) as per UNC protocol #03–1396 and cultured as described on permeable supports and maintained at air-liquid interface in a modified bronchial epithelial growth medium (22). Cells were studied 21–28 days after seeding.

ASL height measurements.

30 μM rSPLUNC1 or SPX-101 were co-incubated with PBS or pooled normal/CF sputa (N = 6 donors/group) overnight at 37°C ± sivelestat before addition to HBEC mucosal surfaces and ASL heights were measured as described previously (12).

Binding of SPLUNC1 and SPX-101 to HBECs.

Cells were exposed apically to either amine-reactive Dylight-633 rSPLUNC1 or 5-TAMRA - SPX-101 pre-incubated with PBS, normal or CF pooled sputa. Cells were counterstained using Calcein-AM and imaged using a Leica SP8 confocal microscope.

Transepithelial Potential Difference.

A single-barreled transepithelial potential difference (V_t)-sensing microelectrode was positioned in the ASL by a micromanipulator and used in conjunction with a macroelectrode in the serosal solution to measure V_t using a voltmeter (World Precision Instruments) as described (12, 25)

Statistics.

Normally distributed data were analysed using ANOVA followed by the Tukey Test or Student's t-test. Non-parametric equivalents (Mann-Whitney U-test; Kruskal Wallis Test with Dunn's Multiple Comparisons Test) were used when data were not normally distributed. For SPLUNC1 degradation over time, curves were fit with single exponentials and analyzed using the Extra Sum of Squares F Test. Data analysis was performed using GraphPad Prism 7.0. Significance values are denoted as follows; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

Results

SPLUNC1 protein levels are decreased in CF sputum.

Consistent with previous studies, we detected SPLUNC1 levels by Western blot analysis in normal sputum samples (10, 26). However, we detected a significant reduction in endogenous SPLUNC1 protein levels in CF sputum (figure 1). Since NE can degrade SPLUNC1 (26), we next determined NE protein levels. In agreement with previous studies (27), we found increased NE levels in CF samples (figure1a and b). We next co-incubated recombinant SPLUNC1 (rSPLUNC1) with pooled sputa from normal and CF donors. Full-length rSPLUNC1 was exponentially degraded by CF sputum ($t_{1/2}$ = 26 min), but remained stable following incubation with normal sputum (figure 2a and b). Since CF lung disease increases the ASL protein content, we co-incubated rSPLUNC1 with volume-adjusted normal and CF sputa to test for rSPLUNC1 degradation independently of protein concentrations. rSPLUNC1 levels were significantly reduced in the presence of CF sputum only (figure 2c and d). To confirm that SPLUNC1 degradation was protein-mediated, samples were heat denatured in the presence of β-mercaptoethanol. rSPLUNC1 degradation was attenuated in heat denatured CF sputum (supplementary figure S1a and b). Since collection methods differed for normal and CF sputa (i.e. induced vs. spontaneous collection respectively), we confirmed that rSPLUNC1 stability was unaffected by the collection method and we found no difference in SPLUNC1 stability in spontaneous vs. induced sputum (supplementary figure S2).

NE in CF sputum is partially responsible for SPLUNC1 degradation.

We co-incubated rSPLUNC1 and CF sputum with inhibitors of NE (sivelestat) and trypsin/chymotrypsin like proteases (aprotinin) (21). An EDTA free protease inhibitor cocktail (PIC) was used to inhibit multiple proteases. Sivelestat and PIC significantly attenuated rSPLUNC1 degradation (figure 3a and b). Aprotinin failed to attenuate rSPLUNC1 degradation. Since NE was elevated in CF sputum, and SPLUNC1's degradation was sivelestat-sensitive, we co-incubated rSPLUNC1 with NE, which degraded rSPLUNC1 in a concentration dependent manner (figure 3c and d). Due to the potential importance of these findings, we exposed rSPLUNC1 to normal/CF sputa in the presence of protease inhibitors for extended times. Surprisingly, sivelestat was unable to prevent rSPLUNC1 degradation after extended incubation periods (figure 4a anb).

To confirm the translation of high NE protein levels into elevated NE activity, we added a NE-sensitive substrate to sputum ± sivelestat, and measured formation of the fluorescent product, AMC, which was significantly elevated after exposure to CF but not normal sputum (figure 4c). Sivelestat was unable to reduce AMC formation following 12 hours of exposure to CF sputum (figure 4d) and failed to recover SPLUNC1's ability to regulate ASL height (supplementary figure 3a and b).

Up-regulation of α-, β-, and γ- ENaC and Na^+/K^+ ATPase α1 protein expression in CF bronchi indicates increased ENaC activity.

SPLUNC1 was undetectable in SMM collected from excised CF lungs (figure 5a). Similarly, co-incubation of SMM and rSPLUNC1 resulted in a rapid diminution of rSPLUNC1 (figure 5b and c). Since SPLUNC1 affects ENaC proteostasis (14), we measured ENaC levels. Bronchi were collected from non-CF and relatively disease-free areas of CF lungs and we measured protein levels by Western blot (12). We observed a significant increase all ENaC subunits in CF bronchi (figure 5d and e). α and γ ENaC must be proteolytically cleaved before ENaC can conduct Na^+ (28). Interestingly, we detected significant increases in cleaved ENaC subunits in CF bronchi, suggesting that ENaC activity is upregulated (figure 5d and e). Consistent with increased ENaC levels, the Na^+/K^+ ATPase α1 subunit was also increased in CF bronchi (figure 5d and e).

CF sputum cleaves the ENaC inhibitory domain of SPLUNC1.

NE predominantly cleaves valine and alanine residues. Indeed, co-incubation of rSPLUNC1 with CF but not normal sputum generated elastase-specific peptides As a control, we performed a tryptic digest and here, peptides were detected regardless of genotype (figure 6a). Coverage analysis revealed valine-specific cleavage of SPLUNC1's S18 region (GGLPVPLDQTLPLNVPA) and the formation of PLDQTLPLNV, which is likely unable to regulate ENaC (figure 6b). We recently developed a novel, NE-resistant, SPLUNC1 derived peptide called SPX-101 (19). The ENaC interacting region of the SPX-101 peptide remained intact following exposure to CF sputum (figure 6b). To determine if rSPLUNC1 and SPX-101 remained active in the presence of CF sputum, we co-incubated PBS or sputum with rSPLUNC1 or SPX-101 overnight and added this apically to HBECs established from normal donors. rSPLUNC1 and SPX-101 increased ASL height in the presence of PBS and

normal sputum (figure 6c and d). Consistent with mass spectrometry data, only SPX-101, was capable of regulating ASL height in the presence of CF sputum (figure 6c and d).

CF sputum prevents SPLUNC1 binding and elevates ENaC activity.

Since ENaC is apically expressed, SPLUNC1 must also bind apically as part of the inhibition process (14). We therefore co-incubated fluorescently labelled rSPLUNC1 and SPX-101 with PBS or sputum, before apical addition to HBECs. We observed apical binding of Dylight633-rSPLUNC1 and 5-TAMRA-SPX-101 in both PBS and normal sputa-treated HBECs (figure 7a). However, after exposure to CF sputum, SPX-101, but not SPLUNC1 was able to bind apically (figure 7a). Furthermore, SPX-101 but not SPLUNC1, remained functionally active and internalised αENaC-GFP following exposure to NE, (supplementary figure S4).

We next determined whether SPLUNC1 degradation by CF secretions contributed to ENaC hyperactivity. We have previously reported increases in ENaC activity in primary CF HBECs (25). However, passaged HBECs do not display this phenomenon. So to start with similar transepithelial voltages (V_ts), we exposed passaged normal and CF HBECs to normal or CF sputum, or PBS (control) and measured the resultant amiloride-sensitive V_t as a marker of ENaC activity. Baseline amiloride-sensitive V_t's were not significantly different between normal and CF HBECs (figure 7b and c). However, addition of normal sputum reduced V_t whilst CF sputum significantly elevated V_t, independently of HBEC genotype. Addition of rSPLUNC1 in PBS significantly reduced V_t in normal but not CF HBECs, consistent with our previous findings that SPLUNC1 cannot function in CF HBECs (12). rSPLUNC1 was without further effect on the already reduce V_t in HBECs exposed to normal sputum, suggesting that endogenous S18-like peptides were present in sufficient quantities to inhibit ENaC as described (13). rSPLUNC1 failed to reduced amiloride-sensitive V_t in the presence of CF sputum. SPX-101 was able to significantly inhibit the amiloride-sensitive V_t in both normal and CF HBECs after exposure to either PBS or CF sputum (figure 7b and c).

Discussion

The lung mucosal environment contains ~1000 proteins, of which ~5% are proteases and their inhibitors (30). Under normal conditions, anti-proteases prevent excessive NE activity in the airways (31). However, chronic neutrophilia in the CF lung lumen significantly increases free NE levels (9, 31, 32). Similarly, we found significantly higher NE protein levels and activity in CF sputum compared with sputum from normal healthy donors. We also observed that SPLUNC1 protein levels were significantly reduced in CF sputum and that rSPLUNC1 was rapidly degraded in CF. To confirm that SPLUNC1 was degraded in CF sputum, we also performed proteomic analysis. Indeed, NE-specific SPLUNC1 coverage was markedly increased in CF sputum, indicating inflammation-induced degradation. Crucially, intact S18-like peptides that can inhibit ENaC, were absent in CF sputum. Despite being cleaved by NE, SPLUNC1 remains aggregated for several hours before dissociating (33). However, the lack of SPLUNC1 coverage in CF sputum, and the relatively short half-

life of rSPLUNC1 in CF sputum, suggest that at steady state, SPLUNC1 does not exist as a functional entity in CF airway secretions.

NE inhibitors have previously been tested in CF patients, with little effect. For example, AZD9668 was tested in CF patients and had no effect on inflammation or lung function (34). Similarly, recombinant α1 anti-trypsin also failed to affect lung inflammation (7). Similarly, our data indicated that sivelestat was only partially effective at preventing SPLUNC1 degradation in CF sputum and this effect was lost over time. ENaC is highly promiscuous and is cleaved by multiple proteases. Thus, possible contributions to the failure of NE inhibitors in the clinic are (i) SPLUNC1 and other proteins are still degraded by CF secretions in the absence of NE and (ii) ENaC can still be activated by other proteases in the CF lung leading to persistent ASL dehydration. Thus, whilst newer broad-spectrum anti-proteases such as QUB-TL1 may be more effective at preventing ENaC cleavage (36), whether or not they are fully effective in the CF lung remains to be determined.

Our working hypothesis is that SPLUNC1 causes removal of ENaC from the plasma membrane, preventing its cleavage and activation. We have recently developed SPX-101, a size-optimized SPLUNC1 derivative that can inhibit ENaC in CF HBECs and CF-like animal models (19). Since SPX-101 is currently undergoing clinical trials for the treatment of CF, we tested whether SPX-101 was stable and efficacious in CF sputum samples. Both SPLUNC1 and SPX-101 increased ASL height after an overnight incubation in normal sputum. However, after exposure to CF sputum, SPLUNC1 was degraded and failed to regulate ASL height, while SPX-101 remained intact and active. As a control, we also incubated SPLUNC1 and SPX-101 overnight with NE and probed for αENaC-GFP diminution, as an indicator of internalisation. While SPLUNC1 lost the ability to inhibit ENaC, SPX-101 again remained active even after prolonged exposure to NE (see online supplement).

SPLUNC1 was degraded in CF sputum from the central airways. However, by utilizing SMM, we also observed SPLUNC1 degradation in more distal regions of CF lungs. Consistent with the lack of SPLUNC1, we observed a significant increase in full-length α, β and γ ENaC subunits, suggesting that SPLUNC1 had failed to internalise ENaC. Crucially, α and/or γ ENaC must be proteolytically cleaved to be active (28, 37, 38) and we also detected significant increases in cleaved α and γ ENaC subunits in CF airways. The basolateral Na^+/K^+-ATPase serves to pump out Na^+ that enters the cell via ENaC in order to keep intracellular Na^+ at ~23 mM (39). This pump has previously been shown to be functionally upregulated in CF airways (29). Similarly, we also observed a significant increase in Na^+/K^+-ATPase protein levels in CF airways, consistent with the increase in cleaved α and γ ENaC subunits. Welsh and colleagues have proposed that the increase in ENaC activity detected electrophysiologically is an artefact caused by amiloride-induced apical membrane hyperpolarisation (40, 41). However, our biochemical data, which demonstrate that cleaved (i.e. active) ENaC, as well as the necessary Na^+/K^+-ATPase are upregulated in CF epithelia suggest that this is not the case.

Whilst defective ion transport is widely accepted to be the initial event in the pathogenesis of CF lung disease, the role of ENaC remains highly controversial (18). A number of studies

have demonstrated increased electrical Na^+ flux and fluid changes in CF airway epithelia that were attributable to increased ENaC activity (4, 42). In contrast, others failed to find differences in CF piglets and in cultured airway epithelia (40, 41, 43). Our data revealed that rSPLUNC1 failed to bind to HBEC apical membranes after pre-incubation with CF sputum whilst N-terminally labelled SPX-101 bound equally well after exposure to normal or CF sputum. rSPLUNC1 was amine-labelled with DyLight633, suggesting that multiple residues were labelled. Thus, while it is possible that CF proteases may have removed some dye-labeled residues, we limited our SPLUNC1/CF sputum pre-incubation to 2 hours, at which point, ~20% of the protein should have remained.

In our microelectrode studies, we first added bumetanide in order to avoid any confounding effects of amiloride-induced Cl^- secretion in non-CF HBECs (25). SPLUNC1 lowered the amiloride-sensitive V_t in non-CF but not CF HBECs whilst SPX-101 lowed V_t regardless of genotype. Here, the reduced bicarbonate secretion in CF HBECs contributes to the moderate acidification needed to prevent SPLUNC1 function. The requirement of functional CFTR for SPLUNC1 to inhibit ENaC is likely due to the presence of pH-sensitive salt bridges in the tertiary structure of SPLUNC1, which render it inactive below pH 7. Conversely, isolated ENaC-inhibitory peptides, such as SPX-101, lack the salt bridges and are pH-independent (12). Surprisingly, both non-CF and CF HBECs showed a significant decrease in the amiloride-sensitive V_t after being exposed to normal sputum for ~2 hours. This is likely due to the presence of endogenous S18-like ENaC inhibitory peptides (13). In contrast, addition of CF sputum significantly elevated the amiloride-sensitive V_t in both non-CF and CF HBECs relative to normal sputum. As with the ASL height and binding experiments, SPLUNC1 was ineffective at ameliorating these effects, due to excessive degradation. However, SPX-101 remained active and could significantly reduce the amiloride-sensitive V_t. These changes are likely due to an abundance of proteases, many of which can cleave and activate ENaC, as well as a lack of functional SPLUNC1, or SPLUNC1-derived peptides in CF sputum. Taken together, these findings demonstrate that the CF mucosal environment (i.e. the ASL secretions) influences ion transport in established CF lung disease. Previous studies have demonstrated cellular interactions between CFTR and ENaC (5, 15, 16). However, our study is the first to test the effects of CF airways secretions on ENaC activity and our data strongly indicate that the extracellular environment drives ENaC hyperactivity in CF airways.

In conclusion, we have shown that SPLUNC1 is absent or markedly reduced in CF airway secretions, which contributes to increased ENaC activity and ASL dehydration in CF HBECs. These data suggest that studying CF HBECs in the presence of CF sputum will serve as a more realistic model of CF airways and also indicate that novel therapies should be tested for efficacy in the presence of CF secretions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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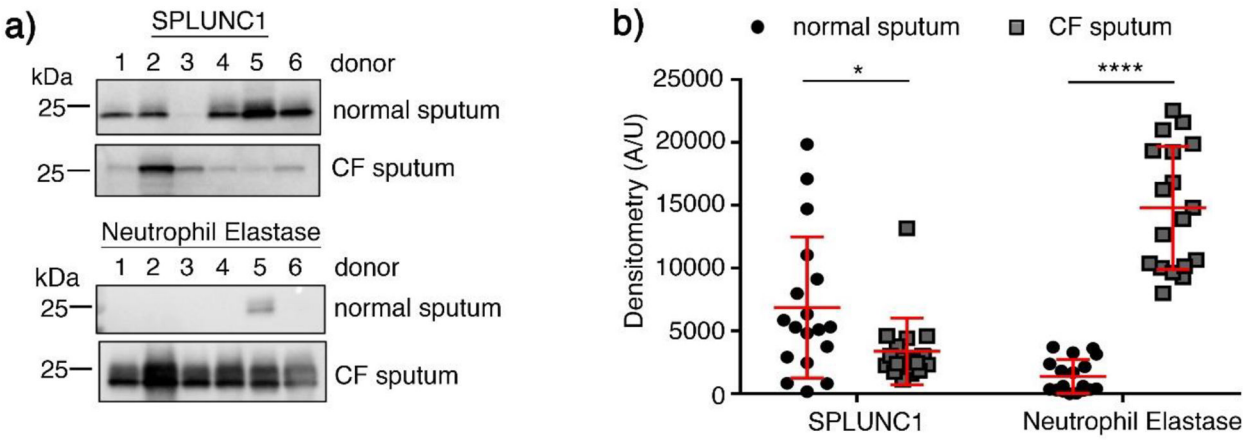

Figure 1. SPLUNC1 protein levels are significantly reduced in CF sputum.

a) Representative western blots showing endogenous expression of SPLUNC1 and NE in normal and CF sputum samples from N = 6 donors per group. Membranes were probed for NE prior to stripping and re-probing for SPLUNC1. **b)** Densitometrical analysis of SPLUNC1 and NE protein abundance in normal and CF sputum from N = 18 donors per group. Data shown as mean ± SD, t-test with Welche's correction, SPLUNC1 normal vs. CF sputum, * $P < 0.05$ and NE normal vs. CF sputum, **** $P < 0.0001$.

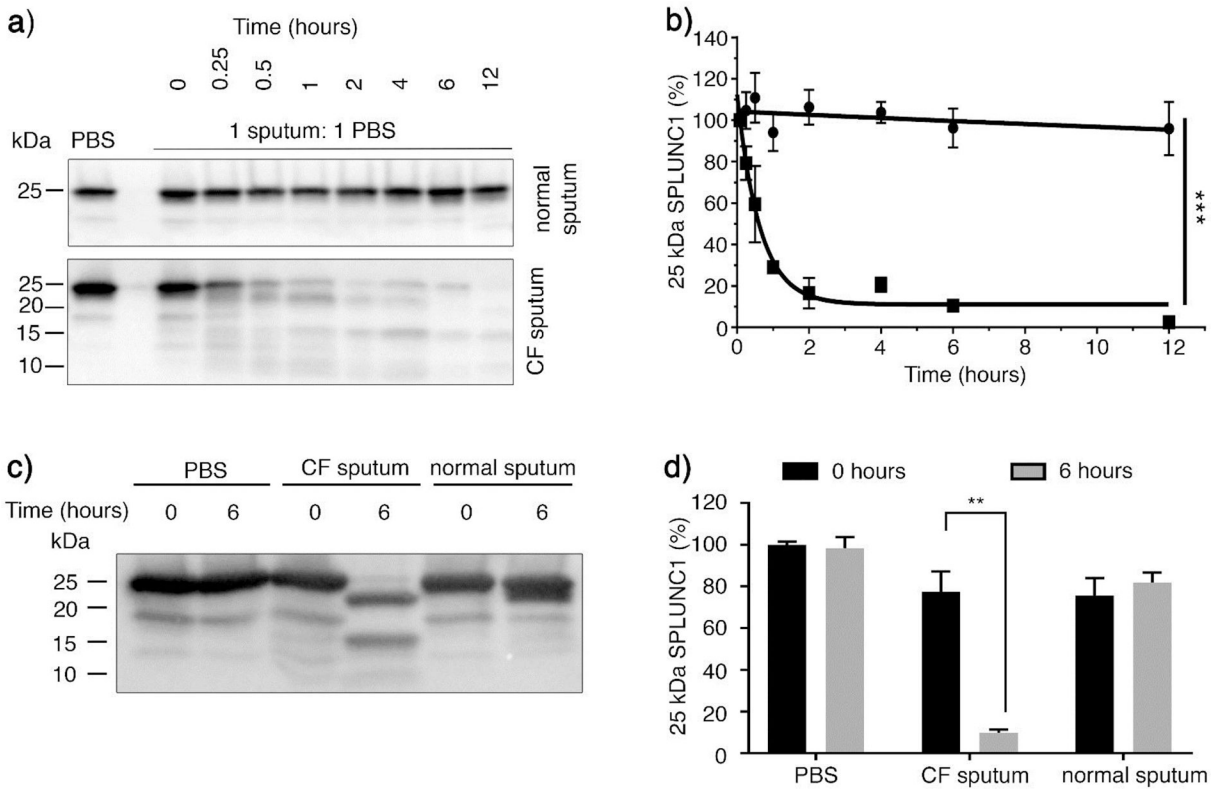

Figure 2.

rSPLUNC1 is rapidly degraded in CF sputum. a) Representative western blots showing SPLUNC1 abundance after incubation with CF and normal sputum (pooled from N = 6 donors) at 37°C. **b)** Densitometrical analyses showing rapid degradation of rSPLUNC1. Extra sum of squares F-test, *** $P < 0.001$, rate constant (k) PBS vs. SMM rSPLUNC1 degradation. CF sputum; $t_{1/2}$ = ~ 26 min, k = 1.57, normal sputum; $t_{1/2}$ = ~ 4 hours, k = 0.02. Data shown mean N = 3 – 4 individual experiments ± SD. **c)** Representative western blot showing significant degradation of 10 μM rSPLUNC1 by CF sputum following a 6 h incubation at 37°C, with 15 μg of total protein and **d)** associated densitometry. Data show mean N = 3–5 individual experiments ± SEM, ** $P < 0.01$ Mann-Whitney U test.

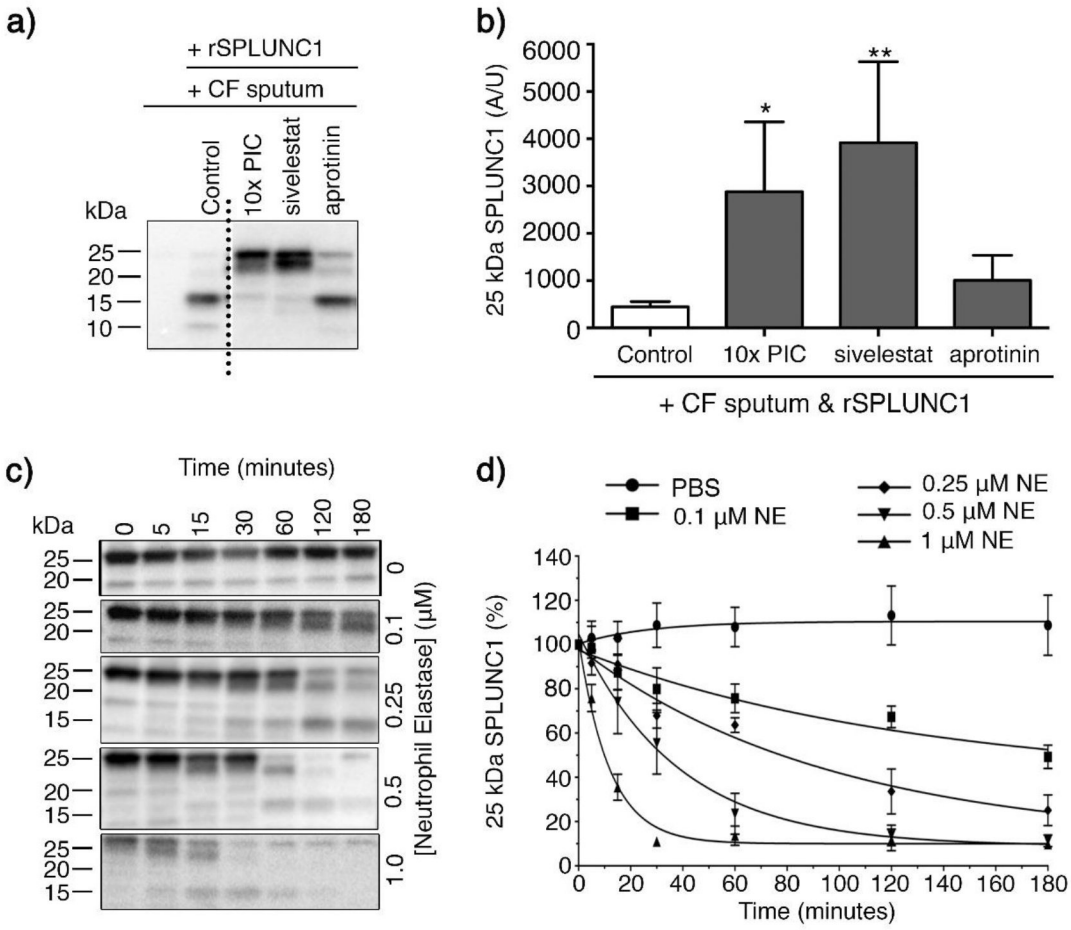

Figure 3. Sivelestat partially attenuates rSPLUNC1 degradation.

a) Representative western blot showing SPLUNC1 abundance following co-incubation with 1×/ 10× protease inhibitor cocktail (PIC) or 10 μM sivelestat/aprotinin and CF sputum (pooled from N = 6 donors) for 6 hours. **b)** Densitometry from N = 5 individual experiments ± SEM, % 25 kDa SPLUNC1 expression compared with CF sputum alone. Data show significant inhibition of rSPLUNC1 degradation by 10× PIC and 10 μM sivelestat only, * $P < 0.05$, ** $P < 0.01$ by Kruskal–Wallis test. **c)** Representative western blots following exposure of rSPLUNC1 to NE over time and **d)** associated densitometry from N = 4 – 6 individual experiments ± SEM. Extra sum of squares F-test, **** $P < 0.0001$, rate constant (k) different between each [NE] data set.

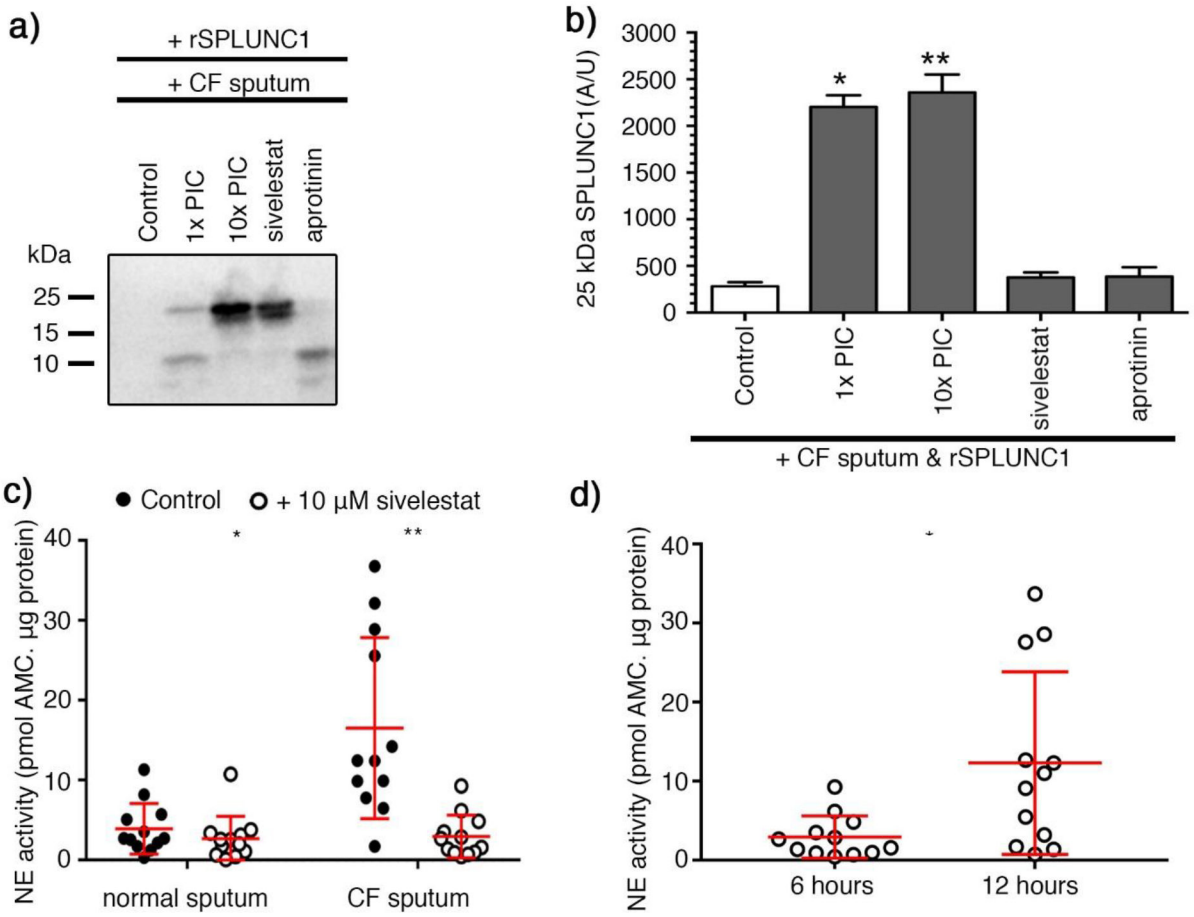

Figure 4. Sivelestat fails to prevent rSPLUNC1 degradation by CF sputum over extended periods.

a) Representative western blot showing 25 kDa rSPLUNC1 abundance following co-incubation of 1×/ 10× protease inhibitor cocktail (PIC), 10 μM sivelestat or 10 μM aprotinin and CF sputum (pooled from N = 6 donors) **b)** associated densitometry from N = 3 – 4 individual experiments ± SEM. % 25 kDa SPLUNC1 compared with CF sputum only. * $P < 0.05$, ** $P < 0.01$ by Kruskal–Wallis test. **c)** AMC formation following cleavage of 100 μM fluorogenic substrate Suc-Ala-Ala-Ala-MCA following 6 h incubation with normal and CF sputum at 37°C ± 10 μM sivelestat. Data shown as mean ± SD; normal, N = 12; CF, N =12 individual donors, * $P < 0.05$ normal vs. CF, CF vs. CF + sivelestat ** $P < 0.01$, by Kruskal-Wallis test with Dunn's multiple comparison. **d)** Change in fluorescence after cleavage of 100 μM fluorogenic substrate Suc-Ala-Ala-Ala-MCA by CF sputum after 6 or 12 hours + 10 μM sivelestat. Data shown as mean ± SD; normal, N = 12; CF, N =12 individual donors. * $P < 0.05$, Mann-Whitney U test.

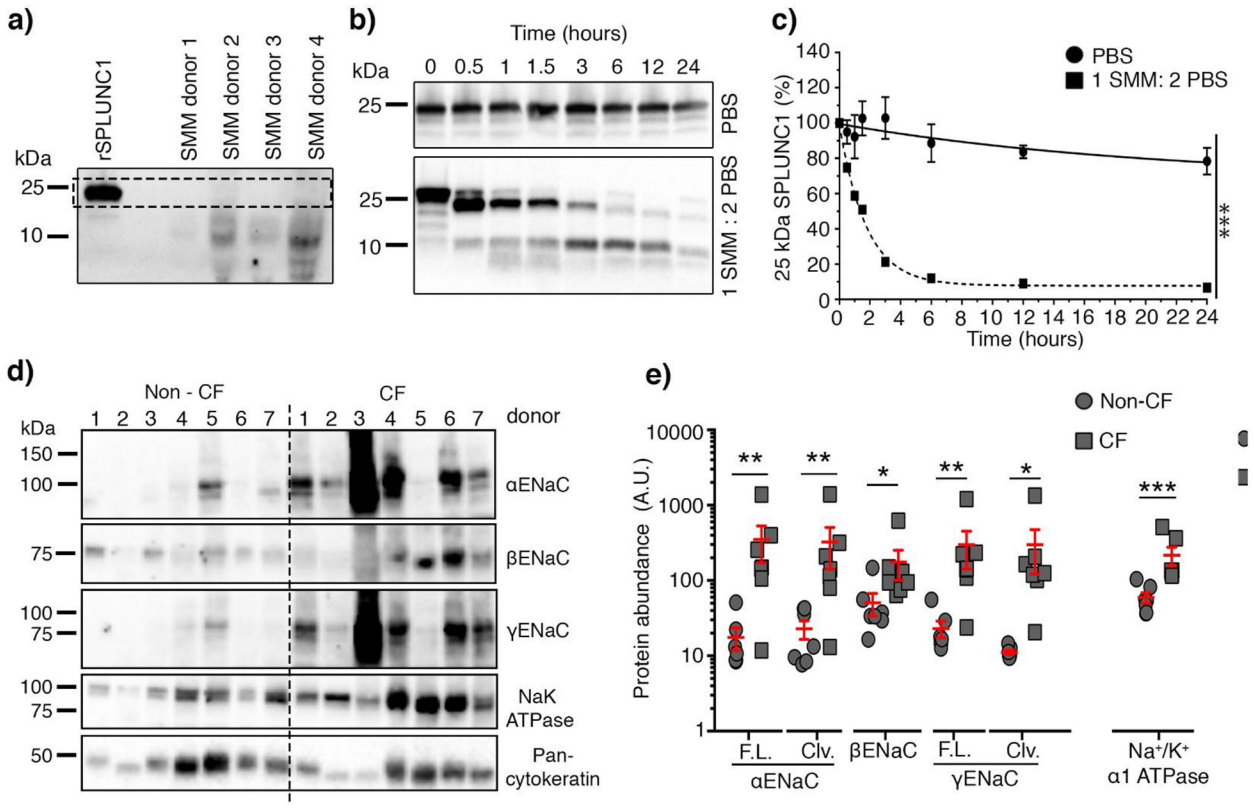

Figure 5. SPLUNC1 is degraded and α-, β-, and γ- ENaC are upregulated in CF bronchi.

a) Western blot showing abundance of full length (F. L.) and cleaved (Clv.) α, β, and γENaC, as well as the Na^+/K^+ ATPase and pan-cytokeratin in bronchi dissected from 7 non-CF and 7 CF lungs post-mortem or post-transplant. **b)** Associated densitometry, data shown are mean ± SD, $\log_{10}$ transformed. Protein abundance was normalised to pan-cytokeratin expression for each individual donor. Normal vs. CF expression; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, Mann Whitney U test. **c)** Western blot showing endogenous SPLUNC1 levels in SMM extracted from 4 individual CF donor lungs obtained post-transplant. d**)** Representative western blots showing 10 μM rSPLUNC1 levels over time in PBS or SMM diluted 1:2 in PBS (SMM pooled N = 3 – 4 donors). **e)** Associated densitometry of 25 kDa SPLUNC1. Data are from N = 3 independent experiments ± SD. Extra sum of squares F-test, *** $P < 0.001$, rate constant (k) PBS vs. SMM rSPLUNC1 degradation. PBS; $t_{1/2}$ = 14.6 hours, k = 0.05, SMM; $t_{1/2}$ = 1.2 hours, k = 0.60.

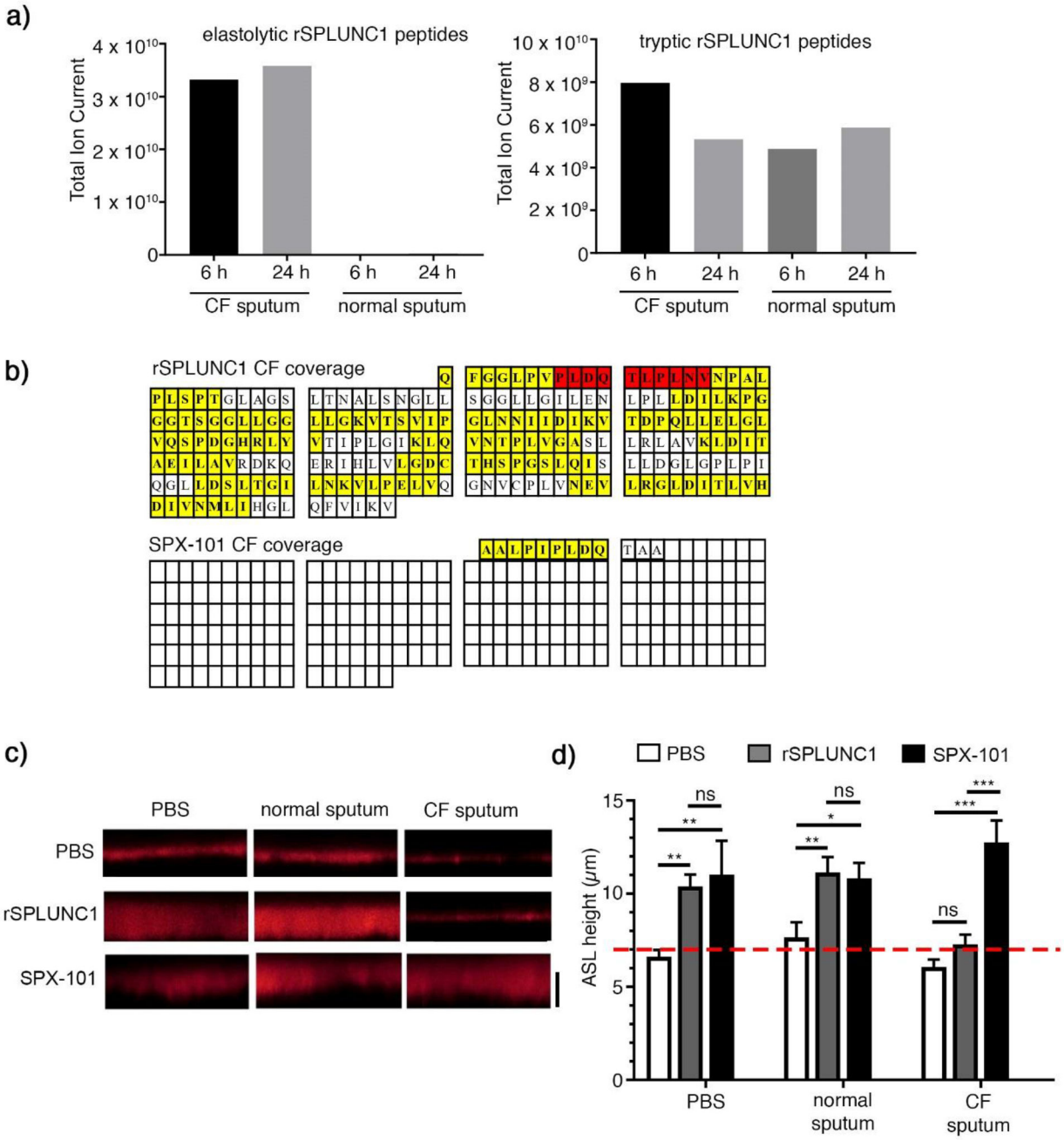

Figure 6. CF sputum cleaves SPLUNC1's ENaC inhibitory region.

a) Total ion chromatogram intensities for elastic and tryptic-specific SPLUNC1 peptides following incubation with CF and normal sputum at 37°C. **b)** SPLUNC1 coverage after proteomic analysis of elastic specific peptides. The peptide identified as PLDQTLPLNV indicating disruption of SPLUNC1's S18 region is highlighted in red. **c)** Representative ASL heights following a 2 hour incubation of HBECs with PBS, normal sputum or CF sputum (pooled N = 6 donors) ± 30 μM rSPLUNC1 or 30 μM SPX-101. **d)** Mean ± SEM ASL height measurements from n = 12 – 15 transwells cultured from N = 4 – 5 individual donors.

* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, Two-way ANOVA analysis with Tukey's post-test analyses.

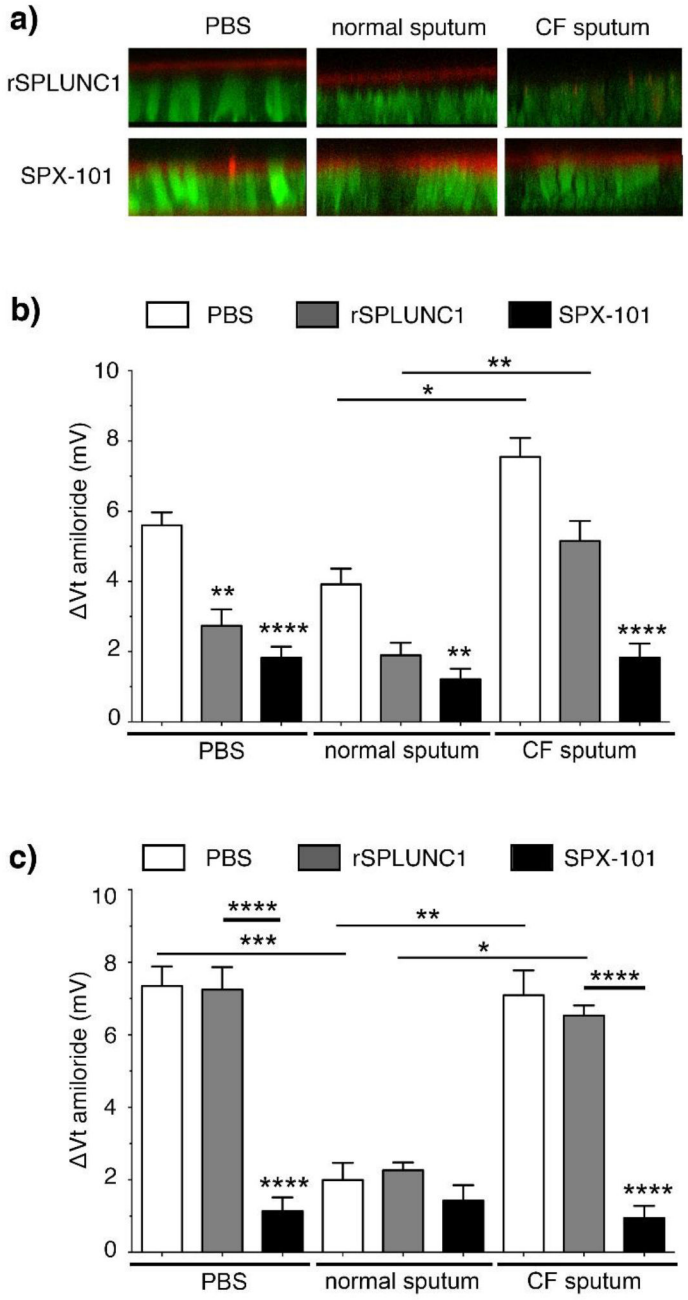

Figure 7. CF sputum prevents SPLUNC1 binding to HBEC mucosal surfaces and elevates the amiloride-sensitive V_t.

a) Dylight633-rSPLUNC1 and TAMRA-SPX-101 were incubated with PBS, normal and CF sputum for 2 hours at 37°C and added mucosally to HBECs for an additional 2 h before imaging. XZ confocal micrographs showing apical binding of Dylight633-rSPLUNC1 and TAMRA-SPX-101 (red) to normal HBECs. HBECs were stained with calcein-AM (green). **b and c)** 30 μM rSPLUNC1 and SPX-101 were incubated with PBS, normal or CF sputum (pooled from N = 6 donors) for 12 hours at 37°C and was added mucosally to HBECs for an additional 2 – 3 h. Voltage-sensing microelectrodes were then positioned in the ASL by micromanipulator and the amiloride-sensitive Vt was measured. N.B., all V_t measurements

were performed in the presence of basolateral 10 μM bumetanide to inhibit Cl^- secretion. Delta $V_{t\ amiloride}$ was then measured in normal **(B)** and CF **(C)** HBECs as indicated. Data shown as mean ± SEM from n = 16 – 24 HBECs cultured from N = 5 – 8 individual donors. Data were analyzed by ANOVA with Tukey's post-test analyses, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.