SPLUNC1 Loses Its Antimicrobial Activity in Acidic Cystic Fibrosis Airway Secretions

To the Editor:

Cystic fibrosis (CF) is a genetic disease caused by dysfunctional CF transmembrane conductance regulator (CFTR) anion secretion that leads to airway dehydration and poor pathogen clearance. These defects result in bacterial infection in the lower airways, persistent inflammation, tissue damage, and a progressive decline in lung function. Gram-negative *Pseudomonas aeruginosa* chronically infects patients with CF, and the *Burkholderia cepacia* complex is associated with severe but less common CF lung infections (1, 2). The antimicrobial compound SPLUNC1 (short palate, lung, and nasal epithelial clone; gene name *BPIFA1*) is found in airway secretions and targets gram-negative bacteria (3), but its relevance to CF pathogenesis remains unclear. CF airways are characterized by a mildly acidic airway surface liquid (ASL) pH, about 0.5 units lower than normal ASL pH of ~7.1, as a result of diminished bicarbonate transport through mutant CFTR (4, 5). Some antimicrobial peptides are pH sensitive, and it has been hypothesized that the acidic CF ASL leads to loss of antimicrobial function (6). Here, we hypothesized that SPLUNC1 is inactivated by acidic CF ASL leading to reduced antimicrobial activity in CF airways. Some of the results of these studies have been previously reported in the form of an abstract (7).

Although not all studies report that CF ASL has reduced pH (8), we and others have found CF ASL to be acidic (4, 5, 9), including CF human bronchial epithelial cells (HBECs) ASL and CF patient nasal secretions (4). Normal and CF human bronchial epithelial cells, obtained from main stem bronchi, were infected or not with 10^3 cfu/ml *B. cenocepacia* strain J2315 and were measured for ASL pH, using fluorescent dyes as described (4). Normal ASL pH was 7.2 and was unaffected by a 2-hour J2315 infection. CF ASL

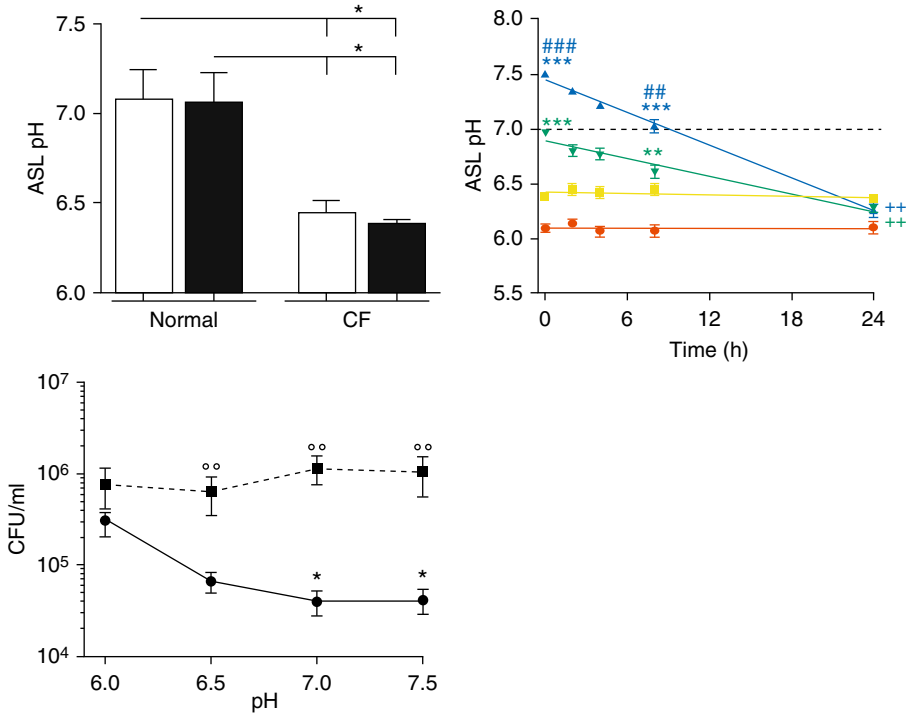

Figure 1. Alkalinized cystic fibrosis (CF) airway surface liquid (ASL) transiently reduces *Burkholderia cenocepacia* J2315 growth. Normal and CF ASL pH was measured immediately and 2 hours after phosphate-buffered saline addition without (open bars) and with (solid bars) 10^3 cfu/ml J2315 infection. All $n = 4$ donors per group (top left). Next, 100 μl modified Ringer's solution buffered at pH 6.0 (red), 6.5 (yellow), 7.0 (green), and 7.5 (blue) and labeled with 10 μM pHRodo Red dextran and 10 μM Alexa-Fluor 647 dextran dyes was added apically to CF human bronchial epithelial cells at $T = 0$ hours and left undisturbed for 24 hours in an environmental chamber. ASL pH was measured over time, using the pHrodo dye. $n = 11$ donors per group (right). Aliquots of ASL at 8 hours (circles) and 24 hours (squares), initially buffered at the indicated pH, were collected, and 100 μl was incubated with 10^3 cfu/ml J2315 for 2 hours at 37°C, 5% CO_2. Aliquots were serially diluted onto agar plates to determine bacterial counts. $n = 5$ donors per group (bottom left). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ comparing pH at the respective times with ASL buffered at pH 6. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ comparing pH at the respective times with ASL buffered at pH 6.5. $^{++}P < 0.01$ comparing $t = 24$ hours with $t = 0$ hours of the respective pH-buffered ASL. $^{\circ\circ}P < 0.01$ comparing 8–24 hours at each buffered pH.

This study was funded by NIH R01HL108927, P30DK065988, the Environmental Protection Agency Cooperative Agreement CR 83578501, the UK Cystic Fibrosis Trust (INOVCF, SRC003), and Cystic Fibrosis Foundation R026-CR11.

Originally Published in Press as DOI: 10.1164/rccm.201812-2303LE on April 23, 2019

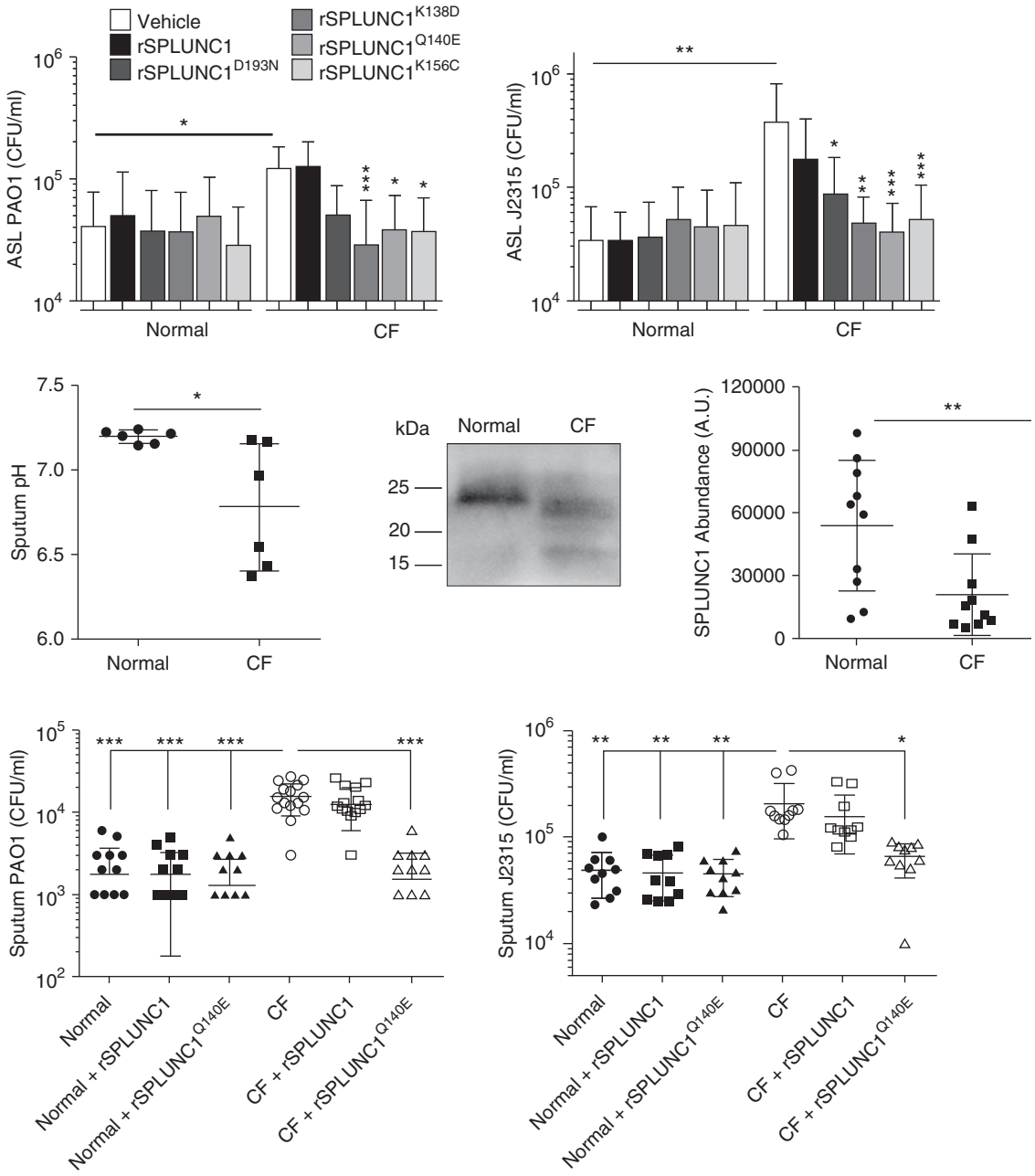

Figure 2. pH-independent SPLUNC1 mutants reduce bacterial burden in cystic fibrosis (CF) airway surface liquid (ASL) and sputum. Normal and CF human bronchial epithelial cells were pretreated with 10 μM rSPLUNC1 and rSPLUNC1 mutants for 1 hour and then infected with 10^3 cfu/ml PAO1 and J2315 apically for 2 hours, and bacterial burden in the ASL was determined. $n = 6$ donors per group (top). Normal and CF sputa were collected and measured for pH and for Western blot of SPLUNC1 densitometry of full-length (25 kD) SPLUNC1 (middle). Next, 100 μl normal and CF sputa were coincubated with or without 10 μM rSPLUNC1 or rSPLUNC1^{Q140E} with 10^3 cfu/ml PAO1 and J2315 for 2 hours at 37°C, 5% CO_2. Aliquots were serially diluted and plated on agar to determine bacterial counts (bottom). $n = 9$–10 donors per group. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the respective vehicle or as indicated with line.

pH was significantly lower (pH 6.4) and also unaffected by J2315 (Figure 1, left). To determine whether CF ASL pH could be normalized, and whether this could restore antimicrobial activity, 100 μl Ringer's solution buffered from pH 6.0 to 7.5 with 100 mM MES (2-[*N*-morpholino]ethanesulfonic acid) or HEPES was added mucosally to CF HBECs. ASL pH was measured. These buffers maintained ASL pH at their target values for ∼8 hours. However, despite the extensive buffering, by 24 hours, CF ASL pH was ⩽6.5 (Figure 1, middle). Next, both 8 and 24 hours after the addition of pH-buffered Ringer's solution, CF ASL was collected, and this lavage was incubated with 10^3 cfu/ml J2315 for 2 hours. At 8 hours, when the pH buffering was still effective, bacterial burden was

significantly reduced, especially for the pH 7.0- and 7.5-buffered ASL. However, at 24 hours, when the buffered ASLs became acidified, bacterial burden was significantly elevated (Figure 1, right). Because pH can be hard to measure, studying the functionality of pH-sensitive proteins is an orthogonal approach to address this. Low pH reduces antimicrobial peptide/protein activity (6). Hence, acidic CF ASL may negatively affect SPLUNC1 function. Here, we demonstrated that SPLUNC1's antimicrobial activity was pH sensitive, and increasing ASL pH restored its antimicrobial function for up to 8 hours, but when pH decreased (i.e., by 24 h), antimicrobial function similarly declined.

We pretreated CF HBECs *in situ* with 10 μM wild-type recombinant SPLUNC1 (rSPLUNC1) apically for 1 hour, and then infected them with 10^3 cfu/ml *P. aeruginosa* strain PAO1 or J2315 for 2 hours at 37°C, 5% CO_2. Aliquots of HBEC ASL lavage were collected, serially diluted, and plated on Luria broth agar plates to determine bacterial burden. Consistent with the previous data, bacterial load remained high. SPLUNC1 possesses an electrostatic surface containing salt bridges that are pH sensitive (4). We used SPLUNC1 mutants with either disrupted salt bridge formation or mutated electrostatic residues near the salt bridges: 10 μM rSPLUNC1^{K156C}, rSPLUNC1^{Q140E}, and rSPLUNC1^{K138D} were able to significantly reduce both PAO1 and J2315 bacterial load in CF ASL compared with nontreated cultures (Figure 2 top). Importantly, bacterial load was reduced, by ~0.5 to 1 $\log_{10}$, to the levels seen in normal ASL, demonstrating that these SPLUNC1 mutants can maintain antimicrobial activity in CF airways.

To assess the pH-independent SPLUNC1 mutants' antimicrobial activities, sputa were collected from normal patients and patients with CF, as described in Webster and colleagues' supplement Methods (10). Normal sputum pH was ~7.2, whereas CF sputum pH was significantly reduced to ~6.8 (Figure 2, middle). In addition, CF sputum had significantly decreased SPLUNC1 levels compared with normal sputum (Figure 2, middle) (10), which may be a result of degradation by proteases in the sputum (3, 10). SPLUNC1 is present in CF HBECs but is nonfunctional (4). We propose that in CF, there is a double effect, whereby SPLUNC1 is inactivated by acidic pH and subsequently degraded as inflammation and proteases become present. We selected one mutant, rSPLUNC1^{Q140E}, for further analysis of antimicrobial effects. Normal and CF sputa were centrifuged at 4,000 × *g* for 10 minutes. Supernatants were coincubated with or without 10 μM rSPLUNC1 or rSPLUNC1^{Q140E} and with 10^3 cfu/ml PAO1 or J2315 for 2 hours at 37°C, 5% CO_2. Although CF sputum alone or with wild-type rSPLUNC1 had higher bacterial load compared with normal sputum samples, rSPLUNC1^{Q140E} significantly reduced the CF bacterial load, by ~0.5 to 1 $\log_{10}$, equivalent to normal levels (Figure 2, bottom). Importantly, we measured bacterial growth when there was still rSPLUNC1 in CF sputum, suggesting that this defect was not a result of SPLUNC1 degradation and that pH-insensitive SPLUNC1 mutants maintain antimicrobial activity in CF airway secretions.

In conclusion, we have shown that CF ASL was acidic, which rendered SPLUNC1 inactive and led to increased bacterial growth. Importantly, we demonstrated that SPLUNC1 mutants, particularly rSPLUNC1^{Q140E}, maintained pH-independent antimicrobial activity. These mutants reduced bacterial load in sputum of patients with CF, suggesting that SPLUNC1 mutants may lead to novel therapies for treating CF infections. We have developed SPLUNC1-derived peptides that are stable in the CF lung and can increase CF airway hydration (10). These peptides are currently in phase II clinical trials for CF. Our data suggest that another aspect of SPLUNC1, namely, its ability to reduce gram-negative bacterial infections, is also defective in CF, and that novel strategies to replace this deficit may lead to improved infection control in CF lungs. ■

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

Acknowledgment: The authors thank the University of North Carolina at Chapel Hill (UNC) CF Center Tissue Core for providing cells, Scott Donaldson and Margret Powell (UNC) for providing CF sputum samples, Heather Wells (UNC) for processing the sputum samples, Colin Bingle (University of Sheffield) for providing the SPLUNC1 construct, Michael Miley and Richard Feng (UNC) for purifying rSPLUNC1 and rSPLUNC1 mutants, and John LiPuma (University of Michigan) for sending the *Burkholderia cenocepacia* J2315 strain.

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Detection of Invasive Aspergillosis in Critically Ill Patients with Influenza: The Role of Plasma Galactomannan

To the Editor:

Invasive pulmonary aspergillosis (IPA) is a complication of influenza infection that has been associated with 33–67% mortality (1–3). In a recent cohort of patients with severe community-acquired pneumonia (sCAP), influenza infection was identified as a strong independent risk factor for IPA (when compared with other pathogens), yielding a 19% incidence of IPA among patients with influenza (2). However, current evidence for this association is exclusively based on retrospective studies that may have suffered from information bias resulting from the selective use of diagnostic modalities based on clinical presentation. Published data, therefore, may not accurately reflect the true association between severe influenza infection and IPA.

According to consensus criteria, IPA is diagnosed on the basis of clinical and radiological criteria in combination with the identification of *Aspergillus* species through growth in BAL fluid, histopathological evidence of lung tissue invasion, or galactomannan antigen detection in BAL or blood samples (4). Galactomannan is a cell wall component released by *Aspergillus* species during active hyphal growth. Routine determination of circulating galactomannan levels during ICU admission could thus be a valuable and noninvasive diagnostic approach in patients with severe influenza.

To better quantify the association between severe influenza and invasive *Aspergillus* disease, we accessed the Molecular Diagnosis and Risk Stratification of Sepsis (MARS) biorepository, which enrolled patients in two tertiary centers in The Netherlands between 2011 and 2017 (5). Leftover plasma samples of ICU patients were stored on a daily basis. The institutional review boards of both centers approved an opt-out method of enrolment (protocol number 10-056C). From this cohort, we selected 272 consecutive patients who presented with a high likelihood of sCAP (based on prospective adjudication by trained observers) and in whom an influenza PCR had been performed (5). Patients who had received more than 4 days of antimicrobial therapy at the time of presentation were excluded to increase homogeneity among patients with respect to their disease stage. We then performed a matched cohort study in which we compared 36 of 38 patients with sCAP with an influenza infection with a sample of 72 subjects having a negative influenza PCR (i.e., according to a 1:2 ratio). Matching criteria were age ($<$65 vs. $\geqslant$65 yr), season of presentation (yearly periods from September until May), chronic obstructive pulmonary disease, and immune deficiency. The latter was defined as a history of hematological malignancy, solid organ or stem cell transplantation, any known humoral or cellular immune deficiency, use of immunosuppressive medication, or chemotherapy/radiotherapy in the previous year. For each patient, we measured galactomannan concentrations in 300 μl ethylenediaminetetraacetic acid plasma obtained on days 1 and 7 in the ICU (or at discharge, whatever came earlier), using the Platelia assay (Bio-Rad Laboratories). An optical index $>$0.5 was considered positive. Subsequently, IPA was diagnosed according to the modified definition as proposed by Schauwvlieghe and colleagues (2). Of note, for 18 (17%) study subjects, only a single plasma sample was analyzed.

Median APACHE-IV scores were comparable between patients with sCAP with and without influenza infection (78 [interquartile range, 59–106] vs. 80 [interquartile range, 59–101]; $P = 0.81$), despite the fact that shock was more prevalent in patients with influenza (50% vs. 24%; $P = 0.006$). Three (8%) patients with and six (8%) without influenza infection received prophylactic or therapeutic antifungal regimens at the time of admission, and in a further three (8%) patients with influenza, this treatment was started during the first week in the ICU. Apparent IPA incidence (based on diagnostic evidence that had been collected as part of routine clinical practice) was 4/36 (11%) among patients with influenza and 3/72 (4%) among patients without influenza. *Post hoc* galactomannan measurements in plasma samples were positive in two additional patients at the time of ICU admission (one with and one without influenza) and in one patient with influenza at day 7. Plasma galactomannan determinations would thus increase the IPA incidence from 11% to 17% (95% confidence interval [CI], 6–33%) among patients with sCAP caused by influenza, and from 4% to 6% (95% CI, 2–14%) among patients having other causative pathogens (relative risk, 3; 95% CI, 1–10; $P = 0.08$).

Clinical characteristics of all identified IPA cases are listed in Table 1. BAL had not been performed in any of the three patients who were detected by *post hoc* galactomannan measurements. Conversely, among seven patients in whom *Aspergillus* had been identified in BAL fluid, only one had positive plasma galactomannan. Two of six patients with influenza and two of four patients without influenza had classical host factors for IPA (such as neutropenia and/or use of specific immunosuppressive medications [4]). Day 28 mortality was 67% and 23% in patients with influenza with and without IPA, respectively ($P = 0.06$). Among patients without influenza, this mortality rate was 14%.

The aggregated 17% occurrence of influenza-associated IPA observed in our study is comparable to the 19% incidence reported by Schauwvlieghe and colleagues in a study that did not systematically measure galactomannan in blood (2). However, it must be noted that the clinical relevance of the three additionally identified patients by plasma galactomannan measurements could not be sufficiently

Supported by the Center for Translation Molecular Medicine (http://www.ctmm.nl), project MARS (grant 041-201). The sponsor did not play a role in the design or conduct of the study; collection, management, analysis, or interpretation of the data; preparation, review, or approval of the manuscript; or decision to submit the manuscript for publication.

Author Contributions: K.v.d.G., D.M.V., M.J.M.B., and O.L.C. provided conceptions and design; K.v.d.G., D.M.V., F.L.v.d.V., P.-J.A.H., T.v.d.P., M.J.S., M.J.M.B., and O.L.C. provided analysis and interpretation; K.v.d.G., D.M.V., M.J.M.B., and O.L.C. provided a draft of the manuscript; and all authors revised the manuscript critically for important intellectual content.

Originally Published in Press as DOI: 10.1164/rccm.201903-0587LE on May 1, 2019