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Structural Features Essential to the Antimicrobial Functions of Human SPLUNC1

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Abstract

SPLUNC1 is an abundantly secreted innate immune protein in the mammalian respiratory tract that exerts bacteriostatic and antibiofilm effects, binds to lipopolysaccharide (LPS), and acts as a fluid-spreading surfactant. Here, we unravel the structural elements essential for the surfactant and antimicrobial functions of human SPLUNC1 (Short Palate Lung Nasal Epithelial Clone 1). A unique α-helix (α4) that extends from the body of SPLUNC1 is required for the bacteriostatic, surfactant, and LPS-binding activities of this protein. Indeed, we find that mutation of just four leucine residues within this helical motif to alanine is sufficient to significantly reduce the fluid spreading abilities of SPLUNC1, as well as its bacteriostatic actions against the Gram-negative pathogens *Burkholderia cenocepacia* and *Pseudomonas aeruginosa*. Conformational flexibility in the body of the SPLUNC1 is also involved in the bacteriostatic, surfactant, and LPS-binding functions of the protein as revealed by disulfide mutants introduced into SPLUNC1. In addition, SPLUNC1 exerts antibiofilm effects against Gram-negative bacteria, although α4 is not involved in this activity. Interestingly, though, the introduction of surface electrostatic mutations away from α4 based on the unique dolphin SPLUNC1 sequence, and confirmed by crystal structure, are shown to impart antibiofilm activity against *Staphylococcus aureus*, the first SPLUNC1-depenent effect against a Gram-positive bacterium reported to date. Together, these data pinpoint SPLUNC1 structural motifs required for the antimicrobial and surfactant actions of this protective human protein.

Graphical abstract

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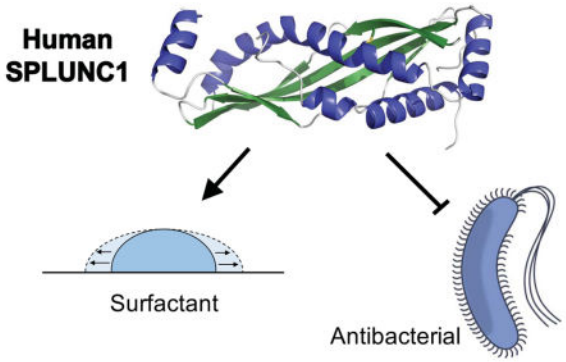
ACCESSION NUMBERS

Coordinates and structure factors for the crystal structures of the variant human SPLUNC1 presented here can be found at the RCSB with the following PDB ID codes: 517L for M2, 517J for M3, and 517K for D1.



Human Short Palate Lung Nasal Epithelial Clone 1 (SPLUNC1) is a 256-residue protein member of the PLUNC family that includes SPLUNCs 1–3 and LPLUNCs 1–5. [1] SPLUNC1, also called BPIFA1, is expressed in upper airways of the lungs, where it is one of the most abundantly secreted proteins, as well as in the colon and kidneys, and has established roles in several aspects of pulmonary function and innate immunity. [2–4] It regulates the volume of airway surface liquid that covers the pulmonary epithelia by inhibiting the actions of the epithelial sodium channel (ENaC), which is responsible for transepithelial movement of sodium and water. In the context of cystic fibrosis (CF) inactivating mutations of the CFTR channel, ENaC's actions exacerbate the dehydration of CF pulmonary tissues. [5] Eighteen amino acids in the N-terminal region of SPLUNC1 (S18) were found to be necessary and sufficient for regulating ENaC in normal conditions *in vitro*; however, in the low pH of CF tissues, both S18 and the body of SPLUNC1 were required to control ENaC. [6] SPLUNC1 has also been shown to act as a surfactant to spread fluids at the air-water interface, and an innate antibacterial factor against Gram-negative bacterial pathogens. [7–15] For example, SPLUNC1 was demonstrated to reduce *Mycoplasma pneumoniae* and *Klebsiella pneumoniae* infections in mouse models, and to prevent the growth of *Pseudomonas aeruginosa* both *in vitro* and *in vivo.* [13,16–21] The protein was also reported to associate with lipopolysaccharides (LPS) from *E. coli* and *P. aeruginosa*, albeit through unknown mechanisms. [7,18,22–25] To date, while SPLUNC1's structural mechanism in regulating ENaC is relatively well understood, the structural basis of SPLUNC1's actions as a surfactant and antibacterial factor have remained less clear.

The crystal structure of human SPLUNC1 was first reported in 2013, and by a second group in 2014, and both reveal a "super-roll" fold composed of a curved β-sheet flanked by α-helices that together enclose a hydrophobic core consisting largely of leucine residues (Figures 1A, B). [6,25] The 2013 report focused on the role the body of SPLUNC1 played in presenting the S18 peptide, which is disordered in all reported structures, for ENaC inhibition in both normal and CF tissues in vitro. By contrast, the 2014 report highlighted the ability of SPLUNC1 to associate in vitro with lipids of established pulmonary functions, specifically dipalmitoylphosphatidylcholine (DPPC). While SPLUNC1 shares structural similarity to two human innate protein proteins, the lipopolysaccharide binding protein (LBP) and bacteriocidal permeability-increasing protein (BPI), which are twice the size of SPLUNC1, its closest structural homologs are equine latherin and the dust mite allergen derP7 which are roughly SPLUNC1's size. [3,26] Latherin is an established surfactant that

spreads fluid over the animal's pelt for cooling. [27] The NMR structure of equine latherin was reported in 2013 and shares 3.9 Å rmsd over 172 equivalent Cα positions and 27% sequence identity with human SPLUNC1. DerP7, an allergen of the *Dermatophagoides pteronyssinus* European house dust mite, shares 3.4 Å rmsd over 147 equivalent Cα positions and only 5% sequence identity with human SPLUNC1. In addition to their similarity in overall fold, SPLUNC1, latherin and derP7 all contain leucine- or isoleucine-rich loops that extend from the body of each protein. In SPLUNC1, this forms α-helix 4 (α4), while in latherin and derP7 they form relatively long and short loops, respectively, with at least four Leu or Ile residues (Figures 1C, 1D). An elegant model for the surfactant actions of latherin has been proposed wherein this Leu-rich loop seeds the unrolling of the protein's super-roll fold to expose its Leu-rich hydrophobic core at the air-water interface. [28,29] Here, we test this hypothesis for human SPLUNC1, and in doing so also address the structural basis of its ability to act as an LPS-binding and innate antibacterial factor. Together, the data presented support the conclusion that improved SPLUNC1s could be designed to act as highly effective protective factors of potential use in the diseased lungs of patients with CF or other pulmonary disorders.

EXPERIMENTAL PROCEDURES

Protein Mutagenesis, Expression and Purification

Protein mutagenesis, expression and purification were carried out as previously described. [6] In addition to our previous protocol, Rosetta-gami 2 competent cells (EMD Millipore, MA, USA) were used and ion exchange chromatography was included for pDest566 (MBP Derp7 fusion) following the first Ni-NTA His-Trap gravity column purification. Both His-tagged and non-His-tagged proteins were examined and gave similar results. pDest566 was a gift from Lars Pedersen (NIEHS) and pET32-sLatherin (Thioredoxin Latherin fusion) gift from Brian Smith (University of Glasgow). Curosurf was purchased from Chiesi USA, Inc (Cary, NC). Lysozyme was purchased from MP Biochemicals, LLC. Fatty acid free BSA purchased from Sigma.

Protein Surfactant and Stability Properties

Air-liquid equilibrium surface tension measurements were performed using the Wilhelmy plate method, with a Sigma 701 force tensiometer (Biolin Scientific, Stockholm Sweden). Samples measured were at 2uM in 50mM Hepes, pH 8.0 and 150mM NaCl. Briefly, 20 ml of sample was measured every minute for 25 minutes at room temperature using a flame-cleaned platinum plate. Static contact angles were measured using a KSV Instruments LCD CAM 200 optical contact angle meter at room temperature. All measurements were carried out with drops dispensed from a Hamilton 500ul glass syringe (Hamilton CO, Reno NV) onto Parafilm (Bemis, Oshkosh WI).

Protein stability was determined using the Circular Dichroism method. [30] 10uM of rSPLUNC1 wt and mutant proteins in CD buffer containing 10mM potassium phosphate (pH 7.4), 100mM potassium fluoride and +/− 5 mM DTT were loaded into a 1-mm cuvette. Using a Chirascan-plus instrument (Applied Photophysis Limited), spectra from 185 to 280 nM were recorded at 20 ± 1.0 °C. Measurements were corrected for background signal using

a CD buffer sample. The melting profile of the sample was monitored at 221 nM from 20 °C to 94 °C.

Protein Crystallization and Structure Determination

Crystals of purified rhSplunc1 disulfide bond mutants were grown at 37 °C in 6M ammonium nitrate, and 0.1M Tris-HCL (pH 8.5), and cryoprotected in this condition with 15% glycerol. Crystals were flash-frozen in liquid nitrogen in preparation for X-ray data collection. Diffraction data were collected on the 23-ID beamline at GM/CA-CAT (Advanced Photon Source, Argonne National Laboratory). Data were processed using standard methods and structures determined by molecular replacement using the rhSplunc1 structure as a search model (PDB:4KGH). [31–33] Structures were refined using standard methods. Coordinates and structure factors can be found at PDB: 517J, 517K and 517L

Primary Human Bronchial Epithelial Cultures (HBEC)

HBECs were obtained and harvested from freshly excised bronchial specimens from normal subjects (n = 4–5 donors) following the protocol approved by the University of North Carolina Institutional Review Board. [34] HBECs were cultured at an air-liquid interface in a modified bronchial epithelial growth medium with 5% CO_2 at 37°C and were used 3–4 weeks after the seeding on 12-mm T-clear inserts (Corning-Costar, Corning, NY, USA). During image acquisition, HBECs were maintained in a modified Ringer Solution as described previously. [35]

Confocal Microscopy Measurement and Analysis of ASL

ASL labeling with Dextran-tetramethylrodamine (Life Technologies, D-1817) was performed as described previously. [6,35] Acquiring ASL images using a Leica SP8 confocal microscope with a 63x/1.3 Numerical Aperture glycerol immersion lens and analyzing acquired ASL images were performed as described previously. [36]

Bacterial Growth and Biofilm Assays

B. cenocepacia J2315 (obtained from Dr. John J. Lipuma, CFF *Burkholderia cepacia* Research Laboratory and Repository, University of Michigan Medical School), *P. aeruginosa*, and *S. aureus* (obtained from Dr. Matthew Wolfgang, University of North Carolina at Chapel Hill) were grown in Luria broth (LB) at 37°C for 24 h with shaking 300 rpm. CFU/ml were determined by serial dilution plating on LB agar plates. The bacterial cultures were adjusted to an optical density at 600 nm (OD_{600}) of ~ 0.600, and 10^6 CFU/ml bacteria was co-incubated with varying concentrations of rSPLUNC1 wt and mutant proteins in flat bottom 96-well plates (Corning Incorporated) for 24 h at 37°C. For antimicrobial activity, bacterial growth was measured at 24 h at $OD_{600\ nm}$. Samples were collected at 24 h, serial diluted in Ringer's solution and plated on LB agar plates to determine the number of CFU per milliliter. For antibiofilm activity, plates were washed and biofilms were fixed with methanol and stained with 1% crystal violet. After rinsing with distilled water, the stained biofilms were resolubilized with 33% acetic acid. Biofilm formation was measured at $OD_{590\ nm}$.

LPS Binding Assays

A modified, enzyme-linked immunosorbent assay (ELISA)-based LPS binding method was used to detect interaction between LPS and SPLUNC1 as described previously. [23] Briefly, a 96-well plate was coated overnight with purified LPS (400 ng/well) from *Pseudomonas aeruginosa* 10 or *Escherichia coli* 055:B5 (Sigma, St. Louis, MO). Wells were washed and blocked with 1% bovine serum albumin (BSA)–phosphate-buffered saline (PBS) for 1 h; then, 2-fold dilutions of purified SPLUNC1 (400 ng) were added to each well in duplicate. PBS was used as a control for this experiment. An antibody specific to human SPLUNC1 (Hycult, Plymouth Meeting, PA) was used to detect the LPS-bound SPLUNC1. After the secondary antibody reaction performed using horseradish peroxidase (HRP)-conjugated anti-mouse IgG, enzyme activity was detected using a TMB Ultra 1-step assay (Pierce Biotechnology, Inc., Rockford, IL). Absorbance was detected at $OD_{450\ nm}$ in a BioTek spectrophotometer (BioTek, Winooski, VT). This experiment was performed three times, and the data were presented as means ± SD.

RESULTS

Human SPLUNC1 is a Strong Protein Surfactant

The ability of human SPLUNC1 to act as a surfactant was examined using the Wilhelmy plate method. Purified human SPLUNC1 at 2 μM (50 μg/mL) in 50 mM HEPES pH 8.0 buffer with 150 mM NaCl exhibited a surface tension of 38 mN/m (Figure 2A; Table 1). Water and buffer (50 mM HEPES pH 8.0 buffer with 150 mM NaCl) demonstrated a surface tension of 72 mN/m, which is standard for solutions lacking surfactants. Lysozyme at 2 μM (45 μg/mL) also exhibited a surface tension of 72 mN/m, indicative of a lack of surfactant activity for this protein, while BSA at 2 μM (133 μg/mL) exhibited a moderate ability to act as a surfactant, reducing surface tension to 56 mN/m. Two structural homologues of SPLUNC1, the equine pelt cooling factor Latherin [28] and the dust mite allergen derP7 [29], were also tested at 2 μM and exhibited moderate surfactant actions, reducing surface tension to 52 mN/m and 55 nM/m, respectively. The pulmonary surfactant Curosurf, a mixture of lipid purified from porcine lungs and employed clinically to treat respiratory distress, was tested at 1.5 mg/mL and reduced surface tension to 32 mN/m. [37] However, Curosurf at the same mass to volume ratio as SPLUNC1, 50 μg/mL, reduced surface tension to only 52 mN/m. Thus, human SPLUNC1 is a strong protein-only surfactant and is superior to two proteins, latherin and DerP7, that share a common fold. Furthermore, SPLUNC1's surfactant activities are on-par with that of the standard of care for respiratory distress, even when employed at 30-fold less concentration on a weight per volume basis.

SPLUNC1's α4 is Critical to Surfactant Activity

To determine the molecular basis of SPLUNC1's surfactant activity, we designed several mutant forms of the protein using the human SPLUNC1 crystal structure (see Table 1 for SPLUNC1 proteins examined), and tested their ability to reduce surface tension. All variant proteins were expressed and purified as described, and were found using circular dichroism (CD) and CD-monitored thermal denaturation to be similar to wild-type SPLUNC1 in terms of overall structure and stability (*e.g.,* see below). [30] First, eliminating SPLUNC1's ENaC inhibitory domain (the S18 region) did not alter the surfactant activity of SPLUNC1 (ΔS18,

Figure 2B; all proteins are at 2 μM; Table 1). Second, it has been hypothesized that a surface-exposed leucine-rich loop in equine Latherin would be critical to surfactant activity. [28] In human SPLUNC1, that region corresponds to α4 (see Figs. 1C–D). Thus, we created a form of SPLUNC1 lacking α4 (Δα4) and found that this variant exhibited significantly diminished surfactant activity, only reducing surface tension to 47 mN/m (SPLUNC1Δα4 in Figure 2B). Thus, α4 in SPLUNC1 is crucial to the surfactant actions of human SPLUNC1.

Leucines in and around SPLUNC1's α4 are conserved and present in the structural homologs Latherin and derP7 (Fig. 1; Table 1). We mutated four leucines in that region (L87, L88, L91, L92) simultaneously to alanines (LA*x*4) and to serines (LS*x*4; see Fig. 1B) in SPLUNC1. We found that these proteins each showed the same diminishment of surfactant activity as Δα4, reducing surface tension to only 46–47 mN/m (Figure 2B). A control mutant in which five surface-exposed leucines (L191, L192, L195, L203, L204) located away from α4 on human SPLUNC1 were mutated to alanine (LA*x*5; Table 1; see Fig. 1B) did not show an impact on its surfactant activity, reducing surface tension to the wild-type level of 38 mN/m in the Wilhelmy plate assay. Thus, the leucines in the α4 region are necessary for the surfactant activities of human SPLUNC1.

We employed contact angle measurements of SPLUNC1 and specific variants (Figure 2C; all proteins at 2 μM) as a secondary assessment of surfactant activity. Contact angles below 90° are considered to be "wetting" and to be indicative of surfactant activity (the spreading of fluid across a surface), while those above 90° are "non-wetting". Human SPLUNC1 exhibited a contact angle of 86°, similar to that observed for SPLUNC1 ΔS18 and LA*x*5 (Figure 2C). By contrast, SPLUNC1 Δα4 and LAα4 both exhibited significantly higher contact angles of 93° and 96°, respectively (Figure 2C), similar to that observed for the weak surfactant protein BSA (94°). By this measure, SPLUNC1, ΔS18, and LA*x*5 can be categorized as wetting with intact surfactant abilities, while SPLUNC1 Δα4 and LAα4 are relatively non-wetting with reduced surfactant actions. These data are similar to those obtained in the Wilhelmy plate assay, and support the conclusion that α4 and the leucines therein are crucial to SPLUNC1's surface tension lowering activities.

Designed Disulfide Bonds Reduce SPLUNC1's Surfactant Activity

SPLUNC1 is composed of a "super-roll" fold formed by relatively parallel α-helices that come together adjacent to a rolled β-sheet (see Fig. 1A) to create a hydrophobic core composed largely of leucine residues. Therefore, it has been hypothesized that such proteins act as surfactants by partially or completely unrolling at the helical interface (grey dotted line in Fig. 1B) to expose their internal leucines to the air. [28] To test this hypothesis, we generated mutant forms of human SPLUNC1 in which disulfide bonds were added to the interface between α5 and α-helices 1-3. The addition of disulfides requires specific proximities for the amino acids to be mutated; thus, we chose two sets of residues expected to place newly-introduced cysteines close enough to form disulfide bonds. M3 (I76C +V214C) was expected to diminish the surfactant actions of SPLUNC1 given its proximity to α4, while M2 (A48C+V253C), distant from α4, was expected to serve as a control (Figures 1, 3; Table 1). The M2 and M3 SPLUNC1 variants were expressed recombinantly

in *E. coli* and purified to homogeneity, and then the structures of each were determined by x-ray crystallography to 2.6 and 2.5 Å resolution, respectively (Table 2). [31–33] Even in the initial electron density maps obtained after molecular replacement, the novel disulfides designed into each protein variant were evident. Upon refinement, the M2 (A48C+V253C) and M3 (I76C+V214C) designed disulfides were confirmed in the final models (Figure 3A and 3B).

We first confirmed that the native disulfide in SPLUNC1 did not contribute to the protein's surfactant activities. A mutant form of human SPLUNC1 in which this native disulfide was eliminated (C180A+C224A) exhibited the same surface tension of 38.5 mN/m as wild-type SPLUNC1 in a Wilhelmy plate assay (Figure 3C; all proteins at 2 μM). The M2 form of SPLUNC1 gave a surface tension of 40 mN/m while the M3 mutant significantly diminished surfactant activity, with a reduction of surface tension to only 44 mN/m (Figure 3C). Thus, locking α3 and α5 together with a disulfide impacts the surfactant action of this human protein. Addition of the reducing agent dithiothreitol (DTT) to the M3 mutant of human SPLUNC1 returned the protein to its wild-type surfactant activity (Figure 3C). Combining M3 with LA*x*4 produced a small reduction in SPLUNC1's surfactant activity: LA*x*4 is 45.5 mN/m (Figure 2B), while M3+LA*x*4 is 47 mN/m (Figure 3C). Upon DTT addition to M3+LA*x*4, reduction of the M3 disulfide bond only eliminated the small M3 contribution and left the protein with the 45.5 mN/m value expected for LA*x*4 alone Figure 3C). Thus, a disulfide at the α5 and α-3 interface reduced the surfactant actions of SPLUNC1 beyond what eliminating critical leucines near α4 achieved.

These observations were confirmed using contact angle measurements. SPLUNC1 and native disulfide-eliminated (C180A+C224A) SPLUNC1 exhibited wetting contact angles of 86° and 87°, respectively, similar to the M2 disulfide mutant at 86° (Figure 3D). M3 showed a slightly wetting contact angle of 88° in this assay. However, combining M3 with LA*x*4 produced a non-wetting contact angle of 96° (Figure 3D), higher than the contact angle of 93° measured for LA*x*4 alone (Figure 2C). Taken together, these results pinpoint the molecular features of human SPLUNC1 necessary for its surfactant activity and further support the conclusion that SPLUNC1 employs the α4-adjacent leucines and the disassociation of α5 and α-2–3 interface to lower fluid surface tension.

Impact of SPLUNC1 Variants on Airway Surface Liquid and LPS Binding

We assessed biophysically the following proteins as representative of the total panel examined above: SPLUNC1, Δα4, LA*x*4, M3, and LA*x*4+M3. We first examined the melting temperatures (T_m) using CD-monitored unfolding at increasing temperatures. [30] SPLUNC1 did not unfold even at 95 °C in the absence of reducing agent; upon the addition of 5 mM fresh DTT, SPLUNC1 exhibited a T_m of 84 °C. For comparison, the C180A +C224A native disulfide mutant SPLUNC1 demonstrated a relatively close T_m of 79 °C both with and without DTT. The remaining SPLUNC1 variants were all tested with 5 mM fresh DTT and exhibited the following values: >95 °C for Δα4, 84 °C for LA*x*4, 85 °C for M3, and 83 °C for for LA*x*4+M3. In addition, CD spectra of these mutants showed no differences in secondary structure relative to SPLUNC1. Thus, the SPLUNC1 variants examined do not differ from the native protein in overall structure or stability based on T_m

values or secondary structure, with the exception of the Δα4 which does not unfold up to 95 °C in these conditions. However, Δα4 SPLUNC1 stability was not decreased relative to SPLUNC1, indicating that using this protein reagent in subsequent studies would produce data not clouded by instability.

We studied a set of human SPLUNC1 variants for their ability to control airway surface liquid height in cultured human bronchial airway epithelial cell cultures (HBECs), an established function of SPLUNC1. 6 SPLUNC1 maintains airway surface liquid (ASL) height in HBECs grown at the air-water interface (Figure 4A). The Δα4 and LA*x*4 forms of human SPLUNC1, which were deficient in surfactant activity, also maintained ASL at a level significantly above vehicle (Figure 4A). The M3 disulfide form of SPLUNC1 exhibited a slightly reduced ability to regulate ASL, but the LA*x*4+M3 form of SPLUNC1 recovers the ability to maintain ASL (Figure 4A). Together, these data indicate that mutations that diminish the surfactant activities of human SPLUNC1 do not impact the ability of this factor to control ASL.

We also examined this set of SPLUNC1 variants for their ability to bind to LPS from *P. aeruginosa* and *E. coli* (Figure 4B, 4C).[12] We found that the forms of SPLUNC1 that exhibit wild-type surfactant activities (SPLUNC1, ΔS18, LA*x*5, and M2) all were able to bind to LPS either from either of these Gram-negative organisms. By contrast, the four forms of SPLUNC1 found to be deficient in surfactant activity (*i.e.*, Δα4, M3, LA*x*4, and M3+LA*x*4) were all significantly reduced in binding to both forms of LPS (Figure 4B, 4C). Therefore, we conclude that SPLUNC1's LPS binding is functionally linked to its surfactant activity.

SPLUNC1 Variants Against Gram-Negative Bacteria

We next examined the ability of native and variant SPLUNC1 proteins to act as antibiofilm and bacteriostatic factors against the cultured airway-relevant bacteria *Staphylococcus aureus, Pseudomonas aeruginosa* and *Burkholderia cepacia* complex. SPLUNC1 has previously been shown to act as an innate antibacterial agent against Gram-negative bacteria.[7,8] As expected, our human SPLUNC1 proteins were ineffective against both growth or biofilm formation by the Gram-positive pathogen *S. aureus*, where growth was examined by both optical density (OD) and colony forming units (CFU) and biofilm was assessed using the crystal violet assay (not shown).

SPLUNC1 and its designed mutants were found to have no effect by OD (Figure 5A) but did exert effects by CFU when measuring the growth of the Gram-negative pathogen *P. aeruginosa* (Figure 5B). Specifically, native SPLUNC1 at 10–100 μg/mL significantly reduced *P. aeruginosa* growth, and a similar result was observed with LA*x*4 SPLUNC1 (Figure 5B). Δα4 SPLUNC1 showed no effect on *P. aeruginosa* growth, while both the M3 and LA*x*4+M3 SPLUNC1 variants demonstrated enhanced *P. aeruginosa* anti-growth activity starting even at 1 μg/mL (Figure 7B). We conclude that α4 is necessary for the bacteriostatic activity of SPLUNC1 against *P. aeruginosa*, and that the M3 variant is capable of improving this activity. While the optical density (OD) measurements did not reveal changes in bacterial growth with SPLUNC1, the CFU assay, which depends on growth, clearly delineated specific SPLUNC1 effects with *P. aeruginosa*. All forms of SPLUNC1

showed essentially the same anti-biofilm activity against this Gram-negative pathogen, reducing biofilm biomass starting at 1–3 μg/mL (Figure 5C).

SPLUNC1 exerted a bacteriostatic effect in both the OD and CFU assays starting at 10 μg/mL against *B. cepacia* (Figure 6A). By contrast, all SPLUNC1 mutants exhibited diminished bacteriostatic activities in the OD assay, showing either no effect up to 100 μg/mL for Δα4 SPLUNC1, or effects only at 100 μg/mL for LA*x*4 and LA*x*4+M3 SPLUNC1 (Figure 6A). By CFU, native SPLUNC1, M3 and LA*x*4+M3 SPLUNC1s showed bacteriostatic activity at concentrations equal to or greater than 10 μg/mL, while the Δα4 and LA*x*4 forms of SPLUNC1 exhibited significantly diminished bacteriostatic activity against *B. cepacia* complex (Figure 6B). Similar to the trend observed for *P. aeruginosa*, native SPLUNC1 and all variant forms of SPLUNC1 examined demonstrated significant reduction in biofilm formation starting at 10 μg/mL (Figure 6C). Therefore, these results support the conclusion that the surfactant activity of SPLUNC1 is important for the bacteriostatic activity of this protein against Gram-negative bacteria, but is not involved in its anti-biofilm effects on either of the pulmonary pathogens *P. aeruginosa* or *B. cepacia*.

Enhanced Antibiofilm Functions Against Gram-Positive S. aureus

Finally, we noted that the mutants examined above had not impacted the antibiofilm functions of SPLUNC1 (Figs. 5C, 6C). Thus, we sought to explore that aspect of the protein's activity using a unique set of protein derived from the following observation: the GL-rich region between residues 58 and 88 (α1-α4) in the mammalian SPLUNC1s are highly similar in sequence, with the noted exception of the water mammals (*e.g.,* the SPLUNC1s from dolphin, orca and manatee; Fig. 7A). The SPLUNC1s from land mammals are neutral in this area, while the SPLUNC1 orthologs in the water mammals place charged amino acids in this region, particularly three anionic residues E62, D63 and D66 (Fig. 7A). To test the potential importance of this region toward SPLUNC1 function, we created two novel mutant forms of human SPLUNC1: D1 (G58A, S61A, G62E, G63D, G66D, I67T), and Dolphinized SPLUNC1, with the full 58-88 region of dolphin SPLUNC1 in place of the 58-88 region of human SPLUNC1 (Fig. 7A). The crystal structure of D1 SPLUNC1 was determined to 2.6 Å resolution (Table 2) and revealed the addition of a significant electronegative patch on the protein's surface, created by the three anionic residues (Fig. 7A). We examined D1 and Dolphinized SPLUNC1 proteins in bacteriostatic and antibiofilm assays with *S. aureus*, *P. aeruginosa* and BCC. For the latter two species, the proteins acted like native human SPLUNC1 (not shown). For *S. aureus*, however, while the effect on *S. aureus* growth was similar to SPLUNC1 and remained unchanged, we found that both D1 and Dolphinized SPLUNC1 exhibited antibiofilm activity above 30 μg/mL protein concentration (Fig. 7B, C). Thus, the addition of negatively charged residues in the α1-α4 region of SPLUNC1 reduced levels of *S. aureus* biofilm, and highlights this region of the protein as important for antibiofilm activity, at least against a Gram-positive bacterium.

DISCUSSION

The abundantly secreted humoral pulmonary factor SPLUNC1 is multifunctional, with established roles in regulating ASL volume, as a surfactant in lowering the surface tension at

the air-water interface, and as an innate antimicrobial protein against Gram-negative bacteria. [4,7,8,38] We hypothesized that specific molecular features of human SPLUNC1 would be responsible for each function, and we tested this hypothesis by creating site-specific mutations in the protein and determining their effects on ASL, surfactant, and antimicrobial functions. Structural comparisons between human SPLUNC1, equine latherin and *Dermatophagoides pteronyssinus* derP7 reveal similarities in their overall "super-roll" fold but differences in the region around SPLUNC1's α4. Equine latherin, whose structure was resolved by NMR in 2013, contains a leucine-rich loop in the area where SPLUNC1 places its α4, and this L-rich region was proposed by Vance *et al.* to be crucial for latherin's surfactant activities by bringing the protein to the air-water interface. [28] DerP7 also contains isoleucine and leucine residues in the same area (I62, I64, I65, L67). [29]

Because SPLUNC1's α4 region also contains several leucine residues, we chose to test the latherin hypothesis by eliminating SPLUNC1's α4, and by making specific leucine-to-alanine and leucine-to-serine mutations in this area (LA*x*4, LS*x*4). Our results both with Wilhelmy plate and contact angle methods support the conclusion that α4 and the leucines in that region are crucial for SPLUNC1's surfactant activities (Figs. 2B–C). Thus, we suggest that the α4 and L's in that region "seed" SPLUNC1's localization to the air-water interface. We term this area of human SPLUNC1 the "L-Loop". A model of the α4 region extended from the body of SPLUNC1 is shown in Figure 8 and reveals that the largely conserved leucines 87–88 and 91–92 can be placed at the terminus of a long tether composed of flexible and conserved glycine residues, and suggests a L-Loop seeding mechanism for SPLUNC1. Liu *et al.* previously showed that SPLUNC1 expression reduces the surface tension of epithelial secretions from human bronchial epithelial cell cultures, and furthermore that the level of SPLUNC1 expression correlates with reduction of that surface tension. [13]

Vance *et al.* proposed equine latherin an unrolling at the air-water interface to expose the hydrophobic core of this protein. [28] We also tested this prediction in human SPLUNC1 by engineering disulfide bonds designed to lock together the α1-α6 and α3-α5 secondary structural elements of SPLUNC1 in oxidizing conditions (see Fig. 1B). We conclude that the two different types of mutants examined here (*e.g.,* M3 and LA*x*4) reveal two distinct mechanisms used by SPLUNC1 to reduce surface tension. First, we propose that the α4 region unwinds to position the leucines of the L-Loop at the air-water interface (Fig. 4), and second that the super-roll fold partially unrolls to expose a portion of the leucine-rich hydrophobic core of SPLUNC1 to air. Together, these mechanisms work in concert to provide SPLUNC1 with potent surfactant activities, on par with the complex lipid-protein mixture CuroSurf.

To further examine our over-arching hypothesis that different regions of SPLUNC1 impart distinct functions, we tested the impact these surfactant mutations have on the ability of SPLUNC1 to control the airway surface liquid (ASL) levels in cultured human bronchial epithelial cells. It has been established previously that the N-terminal S18 region of SPLUNC1 inhibits the epithelium sodium channel (ENaC), reducing the influx of sodium and water into pulmonary epithelial cells and maintaining the height of the airway surface liquid. [5,6] We found that the Δα4, LA*x*4 and M3+LA*x*4 forms of human SPLUNC1 were not

significantly different from SPLUNC1 in ASL height (Fig. 4A). By contrast, the M3 form of SPLUNC1 exhibited a reduced ability to regulate airway liquid height, although this effect was not maintained in the M3+LA*x*4 SPLUNC1 variant (Fig. 4A). These data support the conclusion that the presentation of the S18 region of SPLUNC1 to ENaC, which has been shown to be impacted by the body of the SPLUNC1 protein, is not eliminated by mutations to the protein that affect its surfactant activity. Therefore, it appears that the ENaC-regulatory and surfactant motifs of SPLUNC1 are both physically and functionally distinct.

However, we found that surfactant activities of SPLUNC1 were required for LPS binding by the protein, as mutants that lack surface tension-lowering activities were deficient in LPS binding (Figs. 4B, 5C). We conclude that, while the ASL- and surfactant-regulating regions of SPLUNC1 are distinct, the LPS-binding and surfactant-regulating regions overlap and are functionally related. We propose that LPS molecules bind to SPLUNC1 at the air-water interface in a cleft that can form between helices 2 and 5 in Figure 4; access to this cleft would be expected to be significantly reduced in the surfactant-deficient variants Δα4, and M3+/−LA*x*4.

We examined SPLUNC1's impact on growth and biofilm formation by the Gram-positive bacterium *Staphylococcus aureus* and the Gram-negative microbes *Pseudomonas aeruginosa* and *Burkholderia cepacia*. We found that SPLUNC1 and surfactant mutants had no effect on S. *aureus*. However, SPLUNC1 exerted a bacteriostatic effect on both *P. aeruginosa* and *B. cepacia* at 10 μg/mL over 24 hours. Furthermore, human SPLUNC1 inhibited biofilm formation by *P. aeruginosa* and *B. cepacia* at 1 μg/mL and 10 μg/mL, respectively. The effects on *Pseudomonas* growth and biofilm had been reported previously [16,18,20,21,23,39], but this provides an important advance on the effects of SPLUNC1 on *Burkholderia*. These data establish that SPLUNC1 contains innate antibacterial activities against two Gram-negative pulmonary pathogens of the Proteobacteria phylum. We found that our surfactant-reducing mutations had no effect on anti-biofilm activity against *P. aeruginosa* and *B. cepacia*. Thus, we conclude that anti-biofilm activity of SPLUNC1 is independent of the α4 region.

In contrast to the biofilm results, we found that the α4 region of SPLUNC1 was necessary for the protein to reduce the growth of *P. aeruginosa* and *B. cepacia*. For *P. aeruginosa*, it has been shown previously that SPLUNC1 binds to this bacterium and produces holes in its outer membrane; together, these actions are expected to impact bacterial growth. 23 Δα4-SPLUNC1 may lack one or both of these functions, leading to its loss of bacteriostatic activity, and it is attractive to speculate that the leucine-rich α4 of SPLUNC1 may serve as the pore-forming "warhead" of this protein toward Gram-negative pathogens. An alternative hypothesis is that, because Δα4-SPLUNC1 lacks wild-type surfactant abilities, this protein variant is unable to spread bacteria at the air-water interface to prevent growth. However, the data outlined below for other SPLUNC1 variants with reduced surfactant activities makes this hypothesis less likely, as they show differential effects depending on the mutation and the bacterial species tested.

Indeed, we find for *P. aeruginosa* that the M3 and M3+LA*x*4 forms of SPLUNC1 exhibits enhances bacteriostatic activity by 10-fold over SPLUNC1; both M3-containing SPLUNC1s

significantly reduces *P. aeruginosa* growth at 1 μg/mL, while the native protein exerts this effect at 10 μg/mL. We speculate that the M3 disulfide bond may restrict the conformation of the L-Loop region of SPLUNC1 in a manner that enhances the protein's ability to bind to or form pores in Gram-negative bacteria, leading to enhanced bacteriostatic effects. Future studies will focus on testing these predictions. However, because the LA*x*4 form of SPLUNC1, which is also deficient in surfactant activity, does not exhibit a difference in *P. aeruginosa* bacteriostatic effects, we conclude that the inherent surface tension-lowering qualities of SPLUNC1 are not responsible for its ability to reduce bacterial growth. Interestingly, however, for *B. cepacia* the Δα4 and LA*x*4 forms of SPLUNC1 were significantly diminished in their bacteriostatic activities, while the M3 and M3+LA*x*4 SPLUNC1s were closer to native SPLUNC1. These results indicate that there are unique features between these two Proteobacterial pathogens that lead to differential effects by the same forms of SPLUNC1. Future studies will be undertaken to determine how different SPLUNC1s exert alternative effects on different Gram-negative bacteria.

Because the surfactant mutants had no effect on the antibiofilm activities of SPLUNC1, we probed the SPLUNC1s that were most sequence divergent in the α1-α4 region, those of the water mammals. These forms of the protein encode anionic residues in the 58–68 region in place of the neutral side chains maintained by the land mammals. Thus, we hypothesized that a variant human SPLUNC1 with these residues would exhibit a distinct function. Indeed, we found that inclusion of just six amino acid changes (D1 SPLUNC1) was sufficient to impart antibiofilm activity toward the Gram-positive pathogen *S. aureus*, albeit via an enigmatic mechanism given the anionic character of the *S. aureus* cell envelop.[40] Still, these results highlight the importance of this α1-α4 region in the antibiofilm activity of SPLUNC1, at least toward Gram-positive microbes, and suggest that additional protein variants may be created that further improve their effects against *S. aureus* and other bacterial pathogens.

Taken as a whole, the data presented define for the first time the molecular foundations of human SPLUNC1's ability to act as a surfactant and antimicrobial factor. The L-Loop and α4 regions appear essential for surfactant, LPS-binding, and bacteriostatic activities, and each of these functions is distinguishable from the ASL-regulating actions of SPLUNC1, indicating that this multifunctional protein has multiple functional sites. Furthermore, because enhanced antibacterial actions were observed for specific forms of SPLUNC1 (*e.g.,* M3, LA*x*4) that lack statistically significant effects on regulating ASL height, it appears that SPLUNC1s with improved antimicrobial actions could successfully be designed. With development, such reagents have the potential to be employed as prophylactics to prevent infections by opportunistic pulmonary pathogens in at-risk patient populations, such as those with cystic fibrosis, chronic obstructive pulmonary disorder, or are immune compromised.

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ABBREVIATIONS

LPS	Lipopolysaccharide
SPLUNC1	Short Palate Lung Nasal Epithelial Clone 1
ENaC	epithelial sodium channel
CF	cystic fibrosis
DPPC	dipalmitoylphosphatidylcholine
LBP	lipopolysaccharide binding protein
BPI	bacteriocidal permeability-increasing protein
CD	circular dichroism
Δα4	SPLUNC1 lacking α4
LA*x*4	leucines L87, L88, L91, L92 simultaneously mutated to alanines
LS*x*4	leucines L87, L88, L91, L92 simultaneously mutated to serine
LA*x*5	surface-exposed leucines L191, L192, L195, L203, L204 located away from α4 on human SPLUNC1 simultaneously mutated to alanine
M2	A48C+V253C SPLUNC1
M3	I76C+V214C SPLUNC1
DTT	dithiothreitol
T_m	melting temperatures
HBECs	human bronchial airway epithelial cell cultures
ASL	airway surface liquid
OD	optical density
CFU	colony forming units
D1	human SPUNC1 with five Dolphin SPLUNC1 mutations [G58A, S61A, G62E, G63D, G66D, I67T]
BCC	*Burkholderia cenocepacia* complex.

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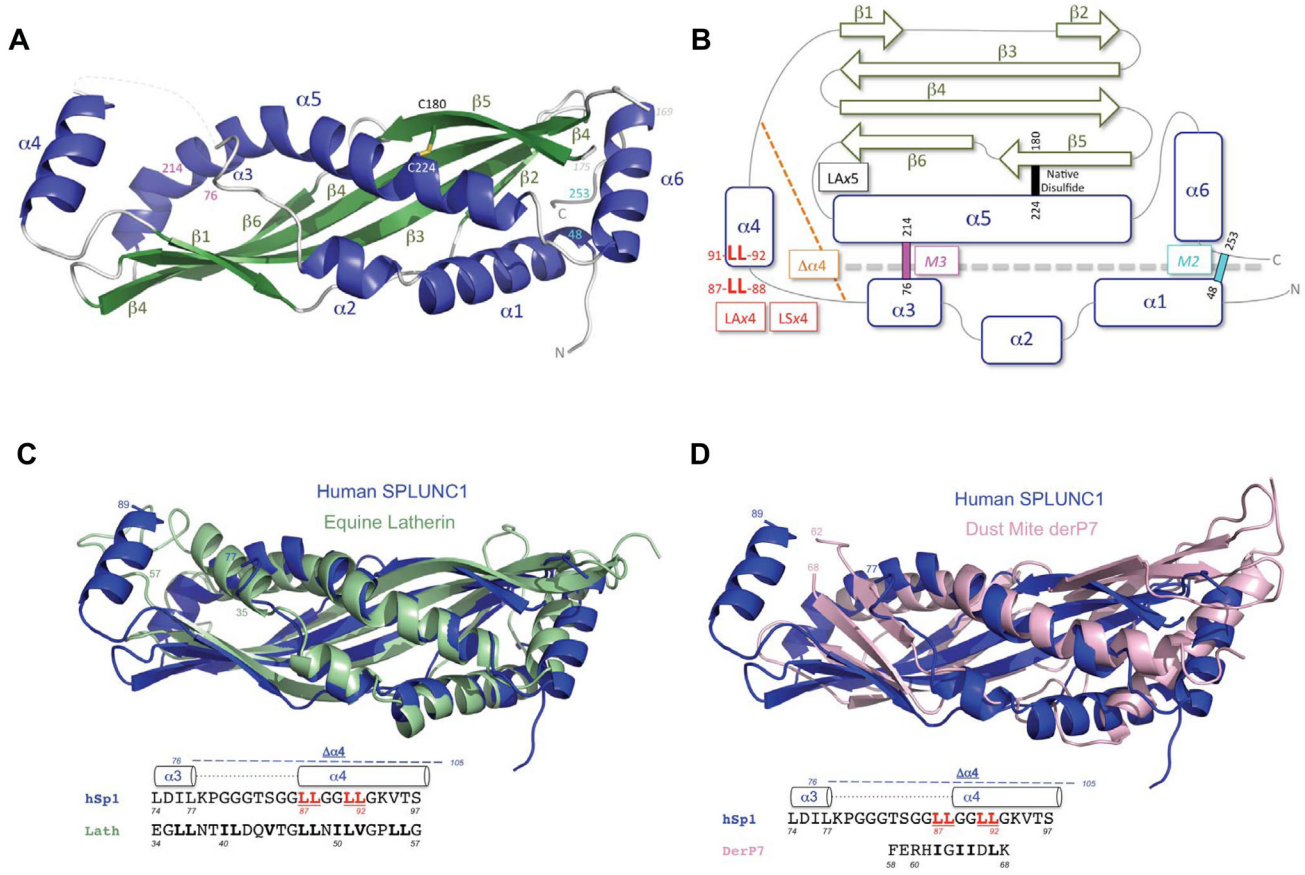

Figure 1. Structural Features of Human SPLUNC1

A. N- and C- termini are indicated (gray) as are the secondary structural elements (blue, green). The native disulfide bond is shown and labeled, and the amino acid positions used to create the two novel disulfides presented here are indicated in cyan (48, 253 for M2) and magenta (76, 214 for M3). **B.** Secondary structural elements are shown in schematic, as are the locations of the native disulfide and two novel disulfides presented (M2, M3). In addition, a schematic view of the Δα4 mutant is indicated (orange), and the locations of the Leu-Ala or -Ser mutations in the LA*x*4 and LS*x*4 variants are also shown (red). The location of the five Leu-Ala changes in the control LA*x*5 mutation is also shown (black). Finally, the gray dotted line indicates secondary structural elements that may disassociate during a potential “unrolling” of the SPLUNC1 super-roll fold. **C.** Human SPLUNC1-equine Latherin superposition with the sequences of each protein in the α4 region of human SPLUNC1 also shown, and hydrophobic Latherin residues highlighted in bold. The Δα4 mutant of SPLUNC1 is indicated with dotted line. **D.** Human SPLUNC1-dust mite derP7 superposition with the sequences of each protein in the α4 region of human SPLUNC1 also shown, and hydrophobic derP7 residues highlighted in bold. The Δα4 mutant of SPLUNC1 is indicated with dotted line.

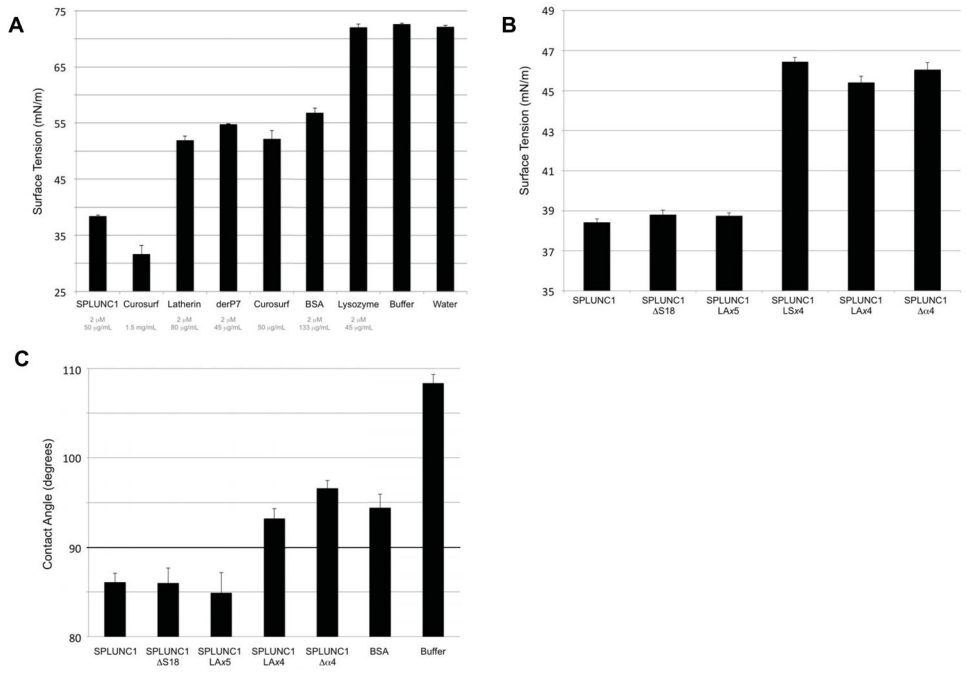

Figure 2. SPLUNC1Surfactant Activity

A. Surface tension was measured using the Wilhelmy plate method for the indicated proteins or other solutions at the indicated concentrations. **B.** *Surfactant Activities of SPLUNC1 Variants.* SPLUNC1, as well as the Δ18 and LA*x*5 SPLUNC1 variants, reduce surface tension using the Wilhelmy plate method to ~39 mN/m, while the LS*x*4, LA*x*4 and Δα4 forms of SPLUNC1 are relatively deficient in surfactant activity and only reduce surface tension to ~46 mN/m. All proteins at 2 μM. **C.** *Surfactant Activity by Contact Angle of SPLUNC1 and other Solutions.* The same SPLUNC1 proteins shown in panel B to be optimal surfactant are “wetting” (contact angle <90°) in their actions in this contact angle assay, while the LS*x*4, LA*x*4 and Δα4 forms of SPLUNC1, as well as BSA and buffer, are “non-wetting” (contact angle >90°). All proteins at 2 μM. For all data in this figure, experiments were performed ten times, and means ± SEM are shown.

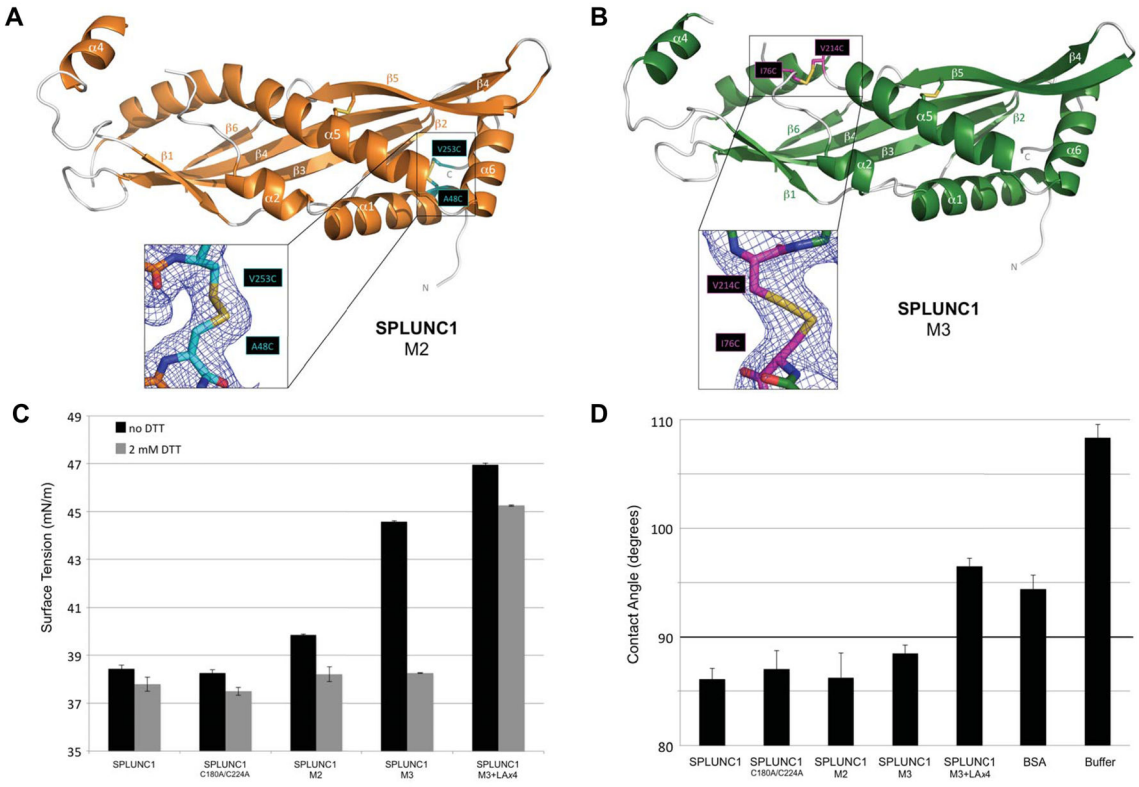

Figure 3. Designed SPLUNC1 Disulfide Mutants

A. *Crystal Structure of M2 SPLUNC1.* Overall 2.6 Å resolution structure and 2|Fo−Fc| electron density contoured at 1.5σ for designed M2 disulfide bond. **B.** *Crystal Structure of M3 SPLUNC1.* Overall 2.54 Å resolution structure and 2|Fo−Fc| electron density contoured at 1.5σ for designed M3 disulfide bond. **C.** *Surface Tension for Disulfide SPLUNC1 Mutants.* The M2 mutant and SPLUNC1 with the native disulfide eliminated (C180A/C224A) show surface tension by Wilhelmy plate close to native SPLUNC1, ~38 mN/m, while the M3 and M3+LA*x*4 mutants exhibit relatively deficient surfactant activity, with surface tensions >44 mN/m. Additional of DTT reduced surface tension of the M3 mutant back to 38 mN/m, but did not impact M3+LA*x*4, which exhibited the surface tension expected for LA*x*4 alone (see Fig. 2B). All proteins at 2 μM. **D**. *Surfactant Activity by Contact Angle for Disulfide Mutants.* Most SPLUNC1 proteins are “wetting” (contact angle <90°) in their actions in this contact angle assay, while the M3+LA*x*4 form of SPLUNC1, as well as BSA and buffer, are “non-wetting” (contact angle >90°). All proteins at 2 μM. For all data in this figure, experiments were performed ten times, and means ± SEM are shown.

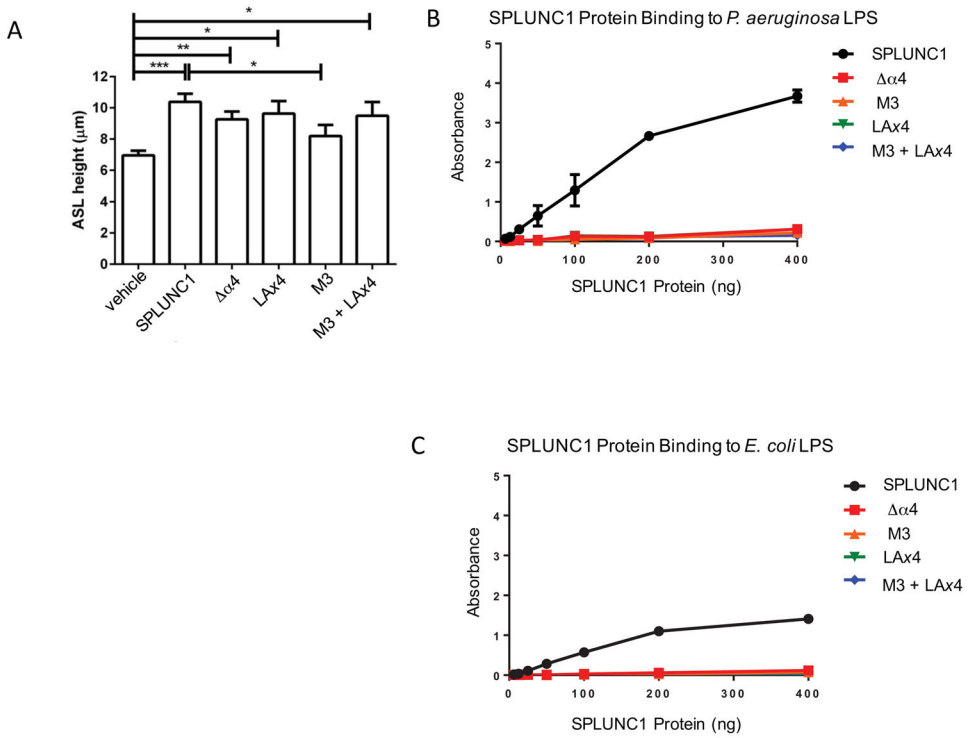

Figure 4. Impact of SPLUNC1 Variants on ASL and on LPS Binding

A. Indicated SPLUNC1 proteins were tested for their impact on airway surface liquid (ASL) levels above cultured human airway epithelial cell cultures. **B**. SPLUNC1 Binding to *Pseudomonas* LPS as assessed by ELISA. **C.** SPLUNC1 Binding to *E. coli* LPS as assessed by ELISA.

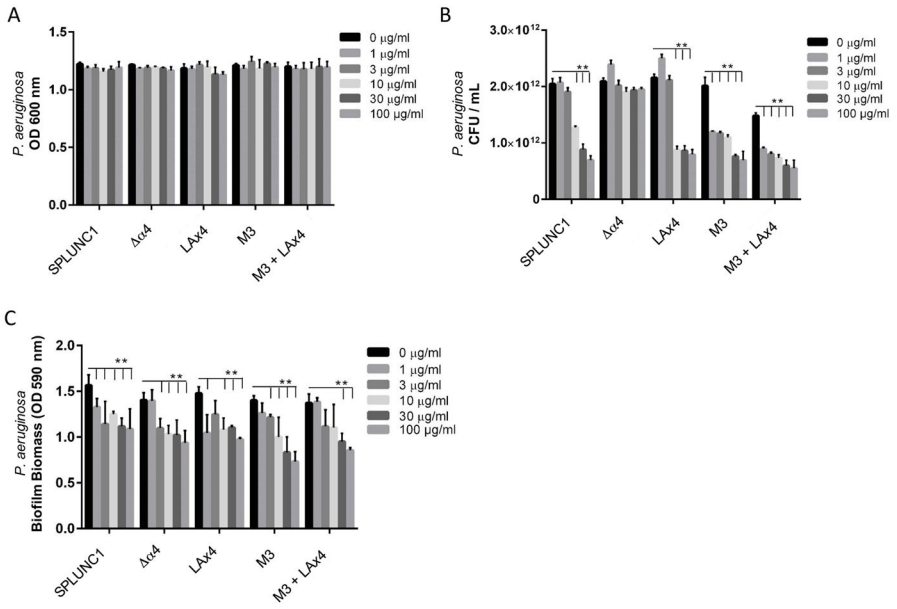

Figure 5. SPLUNC1 Variants as Antimicrobial Agents against against *Pseudomonas aeruginosa*

A. The indicated SPLUNC1 variants tested at listed concentrations had no impact on this measure of *P. aeruginosa* growth as measured by OD_{600nm}. **B.** All SPLUNC1 variants tested, except for Δα4, significantly reduced *P. aeruginosa* growth as measured by colony forming units (CFU). **, $p<0.01$. **C.** All SPLUNC1 variants tested significantly reduced *P. aeruginosa* biofilm formation. **, $p<0.01$.

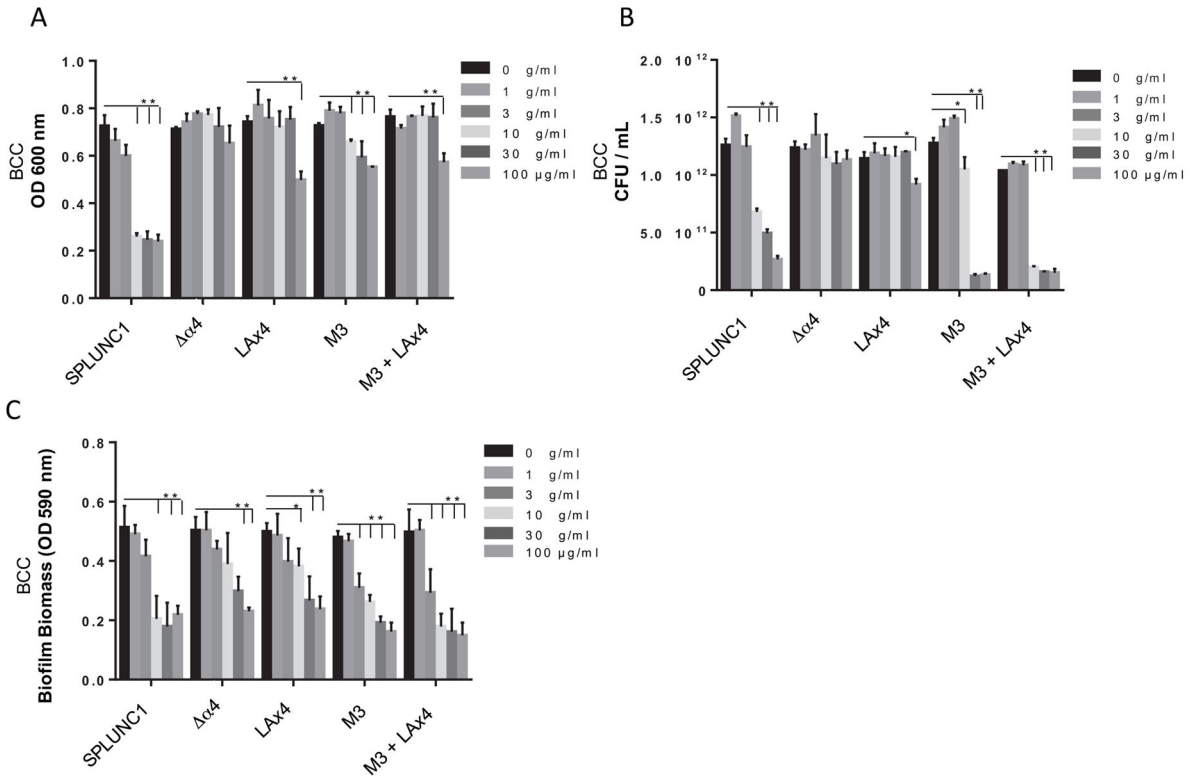

Figure 6. SPLUNC1 Variants as Antimicrobial Agents against against *Burkholderia cenocepacia* complex (BCC)

A. All SPLUNC1 variants tested, except for Δα4, significantly reduced BCC growth as measured by $OD_{600\ nm}$. **, p<0.01. **B.** All SPLUNC1 variants tested, except for Δα4 and LA*x*4, significantly reduced BCC growth as measured by colony forming units (CFU). *, p<0.05; **, p<0.01. **C.** All SPLUNC1 variants tested significantly reduced BCC biofilm formation. **, p<0.01.

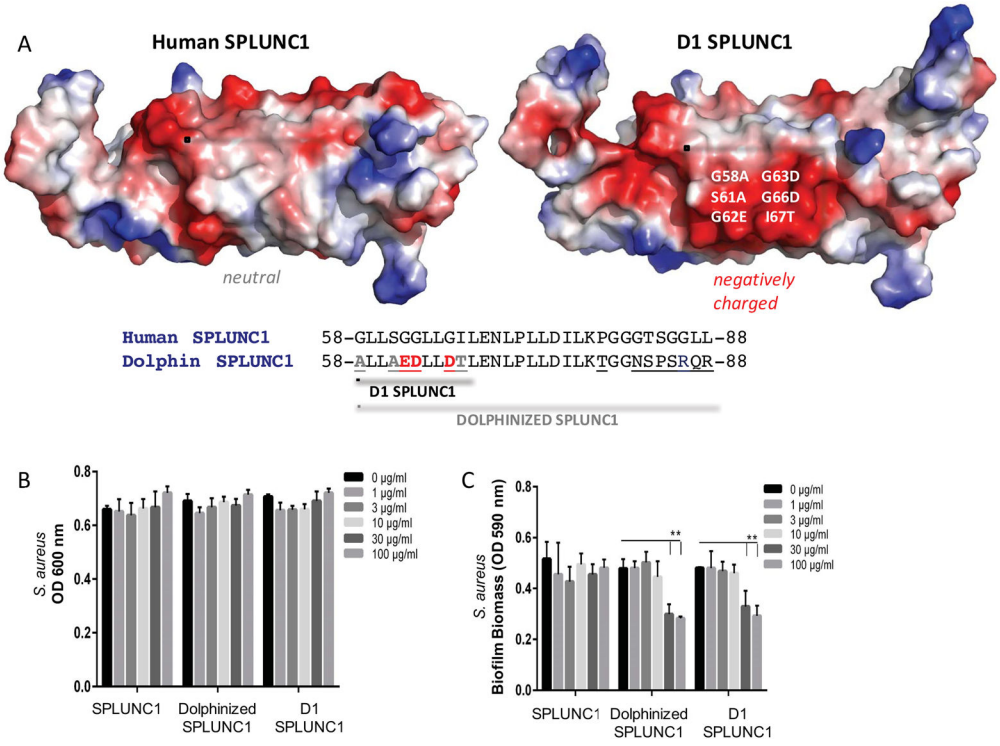

Figure 7. Human SPLUNC1 with Mutations from Dolphin SPLUNC1

A. *Surface of human SPLUNC1 D1 Mutant of Human SPLUNC1 with Indicated Mutations.* Six mutations were introduced and led to a marked increase in negative charge on the protein surface. The sequences of human and dolphin SPLUNC1s are indicated, with mutations that form D1 and Dolphinized SPLUNC1s indicated. **B.** The SPLUNC1 variants tested did not impact *S. aureus* growth by $OD_{600\ nm}$. **C.** Both Dolphinized and D1 SPLUNC1s significantly reduced biofilm generation by *S. aureus*. **, $p<0.01$.

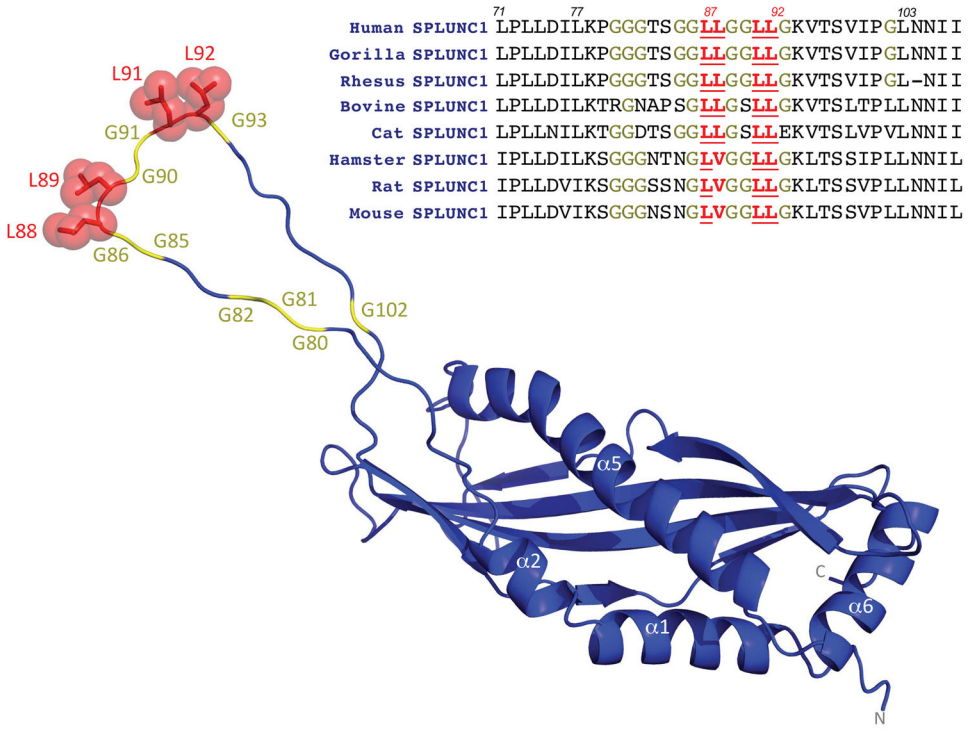

Figure 8. Model for Human SPLUNC1 with Extended α4 Region

Largely conserved glycine (yellow) and leucines (red) in mammalian SPLUNC1 sequences are shown. This flexible region can be modeled to present the key leucines examined here to the air-water interface to break fluid surface tension.

Table 1

SPLUNC1 Variants Examined

Name	Amino Acids	Function	Notes
SPLUNC1	20-256		See reference [6]
ΔS18	45-256		See reference [6]
Δα4	20-256 *with* Δ76-105	• Reduced surfactant activity • Reduced LPS binding • Reduced bacteriostatic activity against *P. aeruginosa* and *B. cepacia*	77-105 replaced with GSGS
LAx4 (LSx4)	20-256 *with* L87A (L87S) L88A (L88S) L91A (L91S) L92A (L92S)	• Reduced surfactant activity • Reduced LPS binding • Reduced bacteriostatic activity against *B. cepacia*	
LAx5	20-256 *with* L191A L192A L195A L203A L204A		
C180A/C224A	20-256 *with* C180A C224A		
M2	20-256 *with* A48C V253C		
M3	20-256 *with* I76C V214C	• Reduced surfactant activity • Reduced LPS binding	
M3+ LAx4	20-256 *with* both M3 and LA*x*4	• Reduced surfactant activity • Reduced LPS binding	
Dolphinized	20-256 *with* Δ58-88	• Increased antibiofilm activity against *S. aureus*	58-88 replaced with equivalent residues from dolphin SPLUNC1; see Fig. 7A
D1	20-256 *with* G58A S61A G62E G63D G66D I67T	• Increased antibiofilm activity against *S. aureus*	See Fig. 7A

Table 2

Crystallographic Statistics for SPLUNC1 Variant Structures

	M2	M3	D1
Resolution (highest shell), Å	38.8–2.60 (2.69–2.60)	28.3–2.54 (2.63–2.54)	29.6–2.55 (2.64–2.55)
Space group	$C222_1$	$C222_1$	$C222_1$
Unit Cell, Å	47.1, 203.6, 120.2	47.8, 206.2, 117.6	48.0, 204.9, 118.7
Total reflections ($F>0$)	94111	68073	67696
Unique reflections	18284	19395	19300
Multiplicity	5.1 (5.1)	3.5 (3.4)	3.5 (3.2)
Completeness, %	99.6 (96.7)	98.7 (94.5)	98.7 (89.4)
Mean I/sigma(I)	21.3 (4.34)	14.5 (2.3)	13.8 (2.1)
Wilson B-factor, $Å^2$	57.6	58.95	54.92
R_{merge}	0.062 (0.580)	0.055 (0.71)	0.057 (0.657)
R	0.2198	0.1995	0.2122
R_{free}	0.2837	0.2677	0.2744
No. of atoms per AU:			
protein, water	2933, 17	2934, 13	3005, 33
rms bonds, Å	0.009	0.010	0.009
rms angles,°	1.51	1.48	1.50
Ramachandran favored, %	89	93	92
Ramachandran outliers, %	3.4	3	2
Clash score	15.61	11	11
Average B-factor, $Å^2$	67	70.2	65
RCSB ID	517L	517J	517K