Functional roles of SPLUNC1 in the innate immune response against Gram-negative bacteria

Y. Peter Di[1]

Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, PA 15219, U.S.A.

Abstract

PLUNC (palate, lung and nasal epithelium clone)-associated gene originally referred to one gene, but now has been extended to represent a gene family that consists of a number of genes with peptide sequence homologies and predicted structural similarities. PLUNC-like proteins display sequence homology with BPI (bactericidal/permeability-increasing protein), a 456-residue cationic protein produced by precursors of polymorphonuclear leucocytes that have been shown to possess both bactericidal and LPS (lipopolysaccharide)-binding activities. The human PLUNC is also known as LUNX (lung-specific X protein), NASG (nasopharyngeal carcinoma-related protein) and SPURT (secretory protein in upper respiratory tract). The gene originally named *PLUNC* is now recognized as *SPLUNC1*. Its gene product SPLUNC1 is a secretory protein that is abundantly expressed in cells of the surface epithelium in the upper respiratory tracts and secretory glands in lung, and in the head and the neck region. The functional role of SPLUNC1 in innate immunity has been suggested but not clearly defined. The present review describes recent findings that support antimicrobial and anti-inflammatory functions of SPLUNC1 in Gram-negative bacteria-induced respiratory infection.

Bacterial infection and antimicrobial activity of lung

The impact of antimicrobial activity on chronic lung illnesses is an important but little understood aspect of lung diseases that are exacerbated by infections. Such diseases include, but are not restricted to, CF (cystic fibrosis), chronic bronchitis and COPD (chronic obstructive pulmonary disease). The conducting airway serves as a first line of defence against environmental insults through its action as a barrier that prevents potentially injurious materials and infectious agents from entering the body [1]. This function is augmented by the innate immune system, which acts against pathogens effectively without prior exposure to them and is essentially instantaneous compared with the adaptive immune system that takes days to become effective [2]. Both respiratory epithelial cells and inflammatory cells contribute to airway innate immunity, while airway epithelial cell-specific antimicrobial activity usually provides an immediate response against pathogens. This antimicrobial function of epithelial cells ensures and initiates an appropriate host defence against invading pathogens. Therefore epithelial cell-regulated antimicrobial activity is regarded as the first line of defence that protects the lung from bacterial infection and helps to maintain a sterile intrapulmonary environment. If antimicrobial activities are impaired or insufficient, a second line of defence mediated through neutrophils and macrophages may kill the bacteria and release cytokines. Respiratory infectious diseases occur when the innate immune response is insufficient to combat bacterial invasion.

PLUNC (palate, lung and nasal epithelium clone)-like family and their tissue expression patterns

The human PLUNC-like gene subfamily now contains eight members located on chromosome 20q11 (for nomenclature details, see the other articles in this issue). Tissue distributions of the PLUNC-like family gene expression include the major salivary glands, the SMGs (submucosal glands) of respiratory tracts and the epithelium of the nasal, laryngeal, pharyngeal, tracheal and bronchial passages [3–9]. The relative abundance and the expressional distributions of SPLUNC1 and LPLUNC1 in various tissues are shown in Figure 1. While members of this PLUNC-like gene family are commonly expressed in overlapping regions, especially in the oral, nasal and respiratory compartments of the head and the neck region, SPLUNC1 [PLUNC, LUNX (lung-specific X protein), NASG (nasopharyngeal carcinoma-related protein) and SPURT (secretory protein in upper respiratory tract)] and LPLUNC1 (C20orf114) are the only two members to express significantly in the respiratory tracts. Since SPLUNC1 is the only protein that has a described function and has been studied in association with antimicrobial activity, this review will focus on the functional role of SPLUNC1 in respiratory infection.

Figure 1 | **The relative abundance of *SPLUNC1* and *LPLUNC1* gene expression in various human tissues**

Taqman-based real-time RT (reverse transcription)–PCR analysis of human *SPLUNC1* and *LPLUNC1* mRNA abundance in various human tissues samples. Relative expression was determined by the $\Delta\Delta C_t$ method using human *GUSB* (β-glucuronidase) RNA as a control (mean ± S.D., $n = 3$). Human SPLUNC1 was highly expressed in tissues from nasal, laryngeal, pharyngeal, tracheal and bronchial regions.
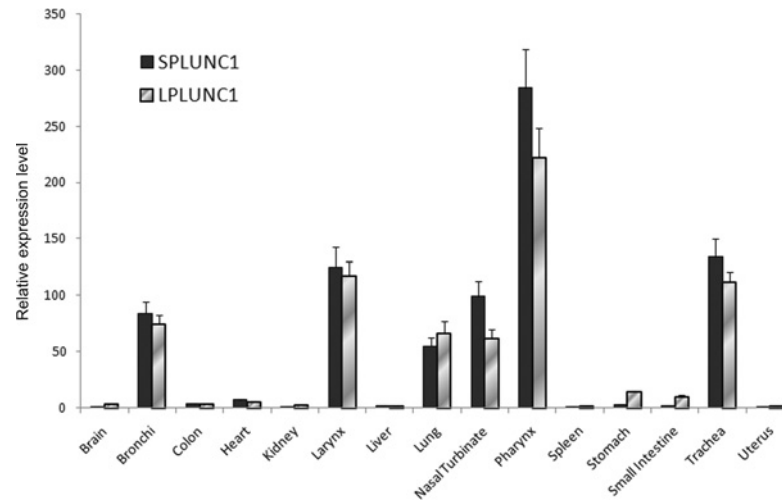
SPLUNC1 and *LPLUNC1* are expressed in serous and mucous cells respectively.

Secretory cells located in airway epithelium and SMGs are an important component of mucociliary clearance mechanisms in the normal lung, and alterations in the phenotype of these cells are associated with the pathogenesis of several lung diseases. Various secretory cells contribute to ASL (airway surface liquid) and function as important contributors to pathogen clearance [10–12]. In human large airways, goblet cells of the surface epithelium, as well as serous and mucous cells of the glands, are the principal secretory cell types [13–15]. Secretory tubules of the SMGs consist of serous cells in the acini and proximal mucous cells [16,17]. The abundance of serous cells in human airway glands (estimated serous/mucous cell volume ratio, 61%:39%) suggests that evolutionary pressures have favoured the development and persistence of the serous cell type [18]. Serous cells are prevalent on the surface epithelium of pathogen-free rodents [19], in animals lacking SMGs [20,21], in the human fetus and in pathological lung diseases such as CF and COPD. Mouse secretory cells on the surface epithelium contain phenotypic characteristics of glandular (serous and mucous) cells and express the glandular secretory proteins, suggesting that anatomical distinctions in mice are functionally compensated for with shifts in secretory cell phenotype. Serous cells are the primary defensive cells of the mucosa because they discharge bactericidal compounds that deal efficiently with pathogens [22,23]. We have found that SPLUNC1 is only expressed in serous cells and can serve as an excellent marker for them. SPLUNC1 and lysozyme were co-localized in a subset of serous cells in SMGs when serial lung sections were stained with antibodies against both proteins (Figure 2). On the other

hand, LPLUNC1 is usually expressed in mucous cells and its expression is frequently co-localized with the expression of MUC5AC, one of the most abundant airway mucins in the airway. The expression of SPLUNC1 is restricted to serous cells of the surface epithelium, secretory ducts and SMGs, sites that express high levels of host defence proteins. The unique tissue distribution of SPLUNC1 in serous cells may provide a clue regarding its function in antimicrobial processes.

In vitro antimicrobial activity of SPLUNC1

PLUNC has a sequence homology with a neutrophil protein, BPI (bactericidal/permeability-increasing protein) [24] that mediates GNB (Gram-negative bacteria) endotoxin, LPS (lipopolysaccharide)-induced bacterial killing. On the basis of this homology with BPI and the restricted expression of PLUNC in serous cells of the upper respiratory tract, SPLUNC1 is thought to have antimicrobial activity, a function now supported by several *in vitro* studies. SPLUNC1 was shown to co-localize with nanobacteria when a full-length SPLUNC1 protein was transiently expressed in nasopharyngeal carcinoma epithelia HNE1 cells [25]. Although a host defence role was proposed in that paper, no direct antimicrobial activity was demonstrated. Chu et al. [26] subsequently showed that a 2 h incubation of the recombinant mSPLUNC1 (mouse SPLUNC1) protein at various concentrations significantly reduced *Mycoplasma pneumoniae* growth in a dose-dependent manner. The authors also showed an increased *M. pneumoniae* burden and neutrophil count in the BALF [BAL (broncho-alveolar lavage) fluid] when an SPLUNC1 neutralizing antibody

Figure 2 | SPLUNC1 and lysozyme were co-localized in the serous cells of SMGs

Cellular localization of SPLUNC1 (**A**) and lysozyme (**B**) proteins was assessed by immunohistochemistry on sections of human trachea. Signal was not detected when parallel sections were incubated with pre-immune serum (results not shown). SPLUNC1 was co-localized with lysozyme in a subset of serous cells in SMGs. Original magnification, ×200. Scale bars, 10 μm.

A. SPLUNC1   B. Lysozyme
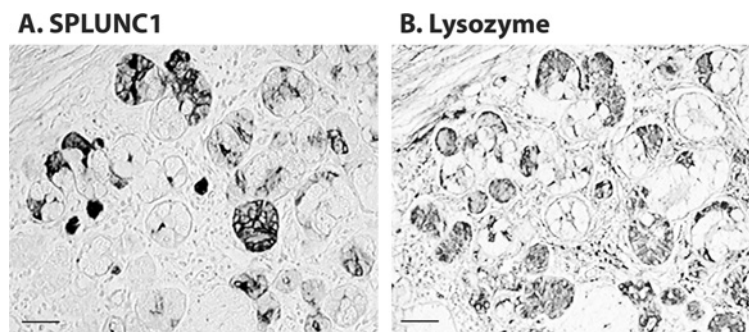
was intranasally administered into mice lung prior to the *M. pneumoniae* infection. Another *in vitro* study used recombinant human SPLUNC1 protein at various concentrations, which also demonstrated the reduced growth of *Pseudomonas aeruginosa* in a dose-dependent manner [27]. A recombinant chinchilla orthologue of human SPLUNC1 (cSPLUNC1) has also been shown to have antimicrobial activity in killing *Haemophilus influenzae* [28]. These studies suggest that SPLUNC1 orthologues from different species may all possess antimicrobial activity against various strains of bacteria. Gakhar et al. [29] further identified the sequence homology between SPLUNC1 and latherin, an equine surfactant protein that displays significant surface tension modulating activity. The authors then demonstrated that the surfactant activity of recombinant human SPLUNC1 lowers minimum surface tension and disrupts *Ps. aeruginosa* biofilm formation. This result provides additional support for a critical role of SPLUNC1 in its antimicrobial function.

In vivo antimicrobial activity of SPLUNC1

To examine the functional roles of SPLUNC1 in innate immune response against respiratory infection, we performed *in vivo* studies using a genetically modified mouse model and exposed the mice to GNB. Our laboratory has generated a CCSP (Clara cell secretory protein)–SPLUNC1 transgenic mouse model that is driven by the mouse CCSP promoter to overexpress human SPLUNC1 in mouse airway epithelial secretory cells. Two types of GNB, *Ps. aeruginosa* and *Klebsiella pneumoniae*, were used to infect the mouse lungs through an intra-tracheal instillation. Both the wild-type and the CCSP–SPLUNC1 transgenic mice were exposed to GNB to determine their sensitivies to bacteria-induced respiratory infection. We observed more enhanced antimicrobial activity in the CCSP–SPLUNC1 transgenic mice than in the wild-type mice in respiratory infections caused by both strains of GNB. The decreased susceptibility to GNB infection in CCSP–SPLUNC1 transgenic mice in

comparison with their wild-type littermates was correlated with lower bacterial CFU (colony formation unit) counts and reduced cell numbers in total inflammatory cells and neutrophils in BAL [30]. Histological evaluations of lung pathology also demonstrated decreased neutrophil infiltration, attenuated lung inflammation and injury in CCSP–SPLUNC1 transgenic mice after GNB-induced respiratory infection (results not shown). These data indicate that human SPLUNC1 is a BPI-like antimicrobial protein that possesses a functional role in innate immune response against GNB. Furthermore, we also observed increased resistance to *M. pneumoniae* infection in the CCSP–SPLUNC1 transgenic mice when compared with their wild-type littermates [31]. Results from these *in vivo* studies further confirmed the antimicrobial activity of SPLUNC1.

The binding of SPLUNC1 to LPS provides protection against excess inflammation

GNB such as *Ps. aeruginosa* and *K. pneumoniae* can produce the endotoxin LPS that alerts the host to the invading bacteria and triggers defensive innate immune responses [32–34]. The N-terminal fragment of human BPI has been shown to bind lipid A and antagonize some LPS-mediated effects [35,36]. We and other researchers have demonstrated that SPLUNC1, similarly to BPI, can also bind to LPS ([37,38] and Y.P. Di, L.Y. Chu, Y. Li, Y. Liu, S. Sayeed and J. Fan, unpublished work). The increased binding of SPLUNC1 to LPS was also observed in BALF harvested from the CCSP–SPLUNC1 transgenic mice. The CCSP–SPLUNC1 transgenic mice had an attenuated inflammatory response with decreased secretion of pro-inflammatory cytokines after LPS challenge when compared with their wild-type littermate mice. The over-expressed SPLUNC1 in mouse ASL may bind directly to LPS and acts as an LPS scavenger to suppress the LPS-induced inflammatory response. Because LPS is a known agonist for TLR4 (Toll-like receptor 4) [39], we also

examined the activation of TLR4 signalling pathway in mouse lung after both the CCSP–SPLUNC1 transgenic mice and their wild-type littermates were challenged with LPS. CCSP–SPLUNC1 transgenic mice displayed a significantly decreased kinase activity of IRAK-4 (interleukin-1 receptor-associated kinase 4) after an intranasal instillation of LPS when compared with the wild-type mice (Y.P. Di, L.Y. Chu, Y. Li, Y. Liu, S. Sayeed and J. Fan, unpublished work). These data suggest SPLUNC1-mediated anti-inflammatory activity after GNB infection may act through direct binding of SPLUNC1 to LPS that results in attenuated TLR4 activation, suppressed IRAK-4 kinase activity and decreased NF-κB (nuclear factor κB) activation.

Conclusion

Bacterial infection in the lung is a major cause of mortality and morbidity, especially in high-risk groups such as immunocompromised patients, the elderly and those with other underlying pulmonary diseases such as CF and COPD. The antimicrobial function of SPLUNC1 probably plays a critical role in host defence against pathogens not only in maintaining homoeostasis of healthy individuals but also in protecting patients whose lungs are compromised by a chronic lung disease. Results from both *in vitro* and *in vivo* studies indicate the antimicrobial function of SPLUNC1 and suggest a defensive role of SPLUNC1 in airways exposed to bacterial infection. A better understanding of an epithelial SPLUNC1-regulated antimicrobial defence mechanism that is BPI-like, but originates from respiratory epithelial cells may provide evidence that will facilitate the use of SPLUNC1 in the treatment of GNB-associated respiratory infections.

Funding

References

1　Tam, A., Wadsworth, S., Dorscheid, D., Man, S.P. and Sin, D.D. (2011) The airway epithelium: more than just a structural barrier. Ther. Adv. Respir. Dis. **5**, 255–273

2　Medzhitov, R. and Janeway, Jr, C.A. (2002) Decoding the patterns of self and nonself by the innate immune system. Science **296**, 298–300

3　Di, Y.P., Harper, R., Zhao, Y., Pahlavan, N., Finkbeiner, W. and Wu, R. (2003) Molecular cloning and characterization of *spurt*, a human novel gene that is retinoic acid-inducible and encodes a secretory protein specific in upper respiratory tracts. J. Biol. Chem. **278**, 1165–1173

4　Leclair, E.E. (2003) Four BPI (bactericidal/permeability-increasing protein)-like genes expressed in the mouse nasal, oral, airway and digestive epithelia. Biochem. Soc. Trans. **31**, 801–805

5　LeClair, E.E., Nomellini, V., Bahena, M., Singleton, V., Bingle, L., Craven, C.J. and Bingle, C.D. (2004) Cloning and expression of a mouse member of the PLUNC protein family exclusively expressed in tongue epithelium. Genomics **83**, 658–666

6　Andrault, J.B., Gaillard, I., Giorgi, D. and Rouquier, S. (2003) Expansion of the BPI family by duplication on human chromosome 20: characterization of the RY gene cluster in 20q11.21 encoding olfactory transporters/antimicrobial-like peptides. Genomics **82**, 172–184

7　LeClair, E.E., Nguyen, L., Bingle, L., MacGowan, A., Singleton, V., Ward, S.J. and Bingle, C.D. (2001) Genomic organization of the mouse *PLUNC* gene and expression in the developing airways and thymus. Biochem. Biophys. Res. Commun. **284**, 792–797

8　Zhou, H.D., Fan, S.Q., Zhao, J., Huang, D.H., Zhou, M., Liu, H.Y., Zeng, Z.Y., Yang, Y.X., Huang, H., Li, X.L. et al. (2006) Tissue distribution of the secretory protein, SPLUNC1, in the human fetus. Histochem. Cell Biol. **125**, 315–324

9　Kim, C.H., Kim, K., Jik Kim, H., Kook Kim, J., Lee, J.G. and Yoon, J.H. (2006) Expression and regulation of PLUNC in human nasal epithelium. Acta Oto-Laryngol. **126**, 1073–1078

10　Jeffery, P.K. (1987) The origins of secretions in the lower respiratory tract. Eur. J. Respir. Dis. Suppl. **153**, 34–42

11　Tarran, R. (2004) Regulation of airway surface liquid volume and mucus transport by active ion transport. Proc. Am. Thorac. Soc. **1**, 42–46

12　Ng, A.W., Bidani, A. and Heming, T.A. (2004) Innate host defense of the lung: effects of lung-lining fluid pH. Lung **182**, 297–317

13　Jeffery, P.K. and Li, D. (1997) Airway mucosa: secretory cells, mucus and mucin genes. Eur. Respir. J. **10**, 1655–1662

14　Crystal, R.G., Randell, S.H., Engelhardt, J.F., Voynow, J. and Sunday, M.E. (2008) Airway epithelial cells: current concepts and challenges. Proc. Am. Thorac. Soc. **5**, 772–777

15　Liu, X. and Engelhardt, J.F. (2008) The glandular stem/progenitor cell niche in airway development and repair. Proc. Am. Thorac. Soc. **5**, 682–688

16　Meyrick, B., Sturgess, J.M. and Reid, L. (1969) A reconstruction of the duct system and secretory tubules of the human bronchial submucosal gland. Thorax **24**, 729–736

17　Wine, J.J. and Joo, N.S. (2004) Submucosal glands and airway defense. Proc. Am. Thorac. Soc. **1**, 47–53

18　Takizawa, T. and Thurlbeck, W.M. (1971) Muscle and mucous gland size in the major bronchi of patients with chronic bronchitis, asthma, and asthmatic bronchitis. Am. Rev. Respir. Dis. **104**, 331–336

19　Jeffery, P.K. and Reid, L.M. (1981) The effect of tobacco smoke, with or without phenylmethyloxadiazole (PMO), on rat bronchial epithelium: a light and electron microscopic study. J. Pathol. **133**, 341–359

20　Pack, R.J., Al-Ugaily, L.H. and Morris, G. (1981) The cells of the tracheobronchial epithelium of the mouse: a quantitative light and electron microscope study. J. Anat. **132**, 71–84

21　Pack, R.J., Al-Ugaily, L.H., Morris, G. and Widdicombe, J.G. (1980) The distribution and structure of cells in the tracheal epithelium of the mouse. Cell Tissue Res. **208**, 65–84

22　Basbaum, C.B., Jany, B. and Finkbeiner, W.E. (1990) The serous cell. Annu. Rev. Physiol. **52**, 97–113

23　Joo, N.S., Lee, D.J., Winges, K.M., Rustagi, A. and Wine, J.J. (2004) Regulation of antiprotease and antimicrobial protein secretion by airway submucosal gland serous cells. J. Biol. Chem. **279**, 38854–38860

24　Schultz, H. and Weiss, J.P. (2007) The bactericidal/permeability-increasing protein (BPI) in infection and inflammatory disease. Clin. Chim. Acta **384**, 12–23

25　Zhou, H.D., Li, G.Y., Yang, Y.X., Li, X.L., Sheng, S.R., Zhang, W.L. and Zhao, J. (2006) Intracellular co-localization of SPLUNC1 protein with nanobacteria in nasopharyngeal carcinoma epithelia HNE1 cells depended on the bactericidal permeability increasing protein domain. Mol. Immunol. **43**, 1864–1871

26　Chu, H.W., Thaikoottathil, J., Rino, J.G., Zhang, G., Wu, Q., Moss, T., Refaeli, Y., Bowler, R., Wenzel, S.E., Chen, Z. et al. (2007) Function and regulation of SPLUNC1 protein in *Mycoplasma* infection and allergic inflammation. J. Immunol. **179**, 3995–4002

27　Zhou, H.D., Li, X.L., Li, G.Y., Zhou, M., Liu, H.Y., Yang, Y.X., Deng, T., Ma, J. and Sheng, S.R. (2008) Effect of SPLUNC1 protein on the *Pseudomonas aeruginosa* and Epstein–Barr virus. Mol. Cell. Biochem. **309**, 191–197

28　McGillivary, G. and Bakaletz, L.O. (2010) The multifunctional host defense peptide SPLUNC1 is critical for homeostasis of the mammalian upper airway. PLoS ONE **5**, e13224

29　Gakhar, L., Bartlett, J.A., Penterman, J., Mizrachi, D., Singh, P.K., Mallampalli, R.K., Ramaswamy, S. and McCray, Jr, P.B. (2010) PLUNC is a novel airway surfactant protein with anti-biofilm activity. PLoS ONE **5**, e9098

30　Lukinskiene, L., Reynolds, S., Steele, C., Stripp, B., Leikauf, G., Kolls, J. and Di, Y.P. (2011) Antimicrobial activity of PLUNC protects against *Pseudomonas aeruginosa* infection. J. Immunol. **187**, 382–390

31 Gally, F., Di, Y.P., Smith, S.K., Minar, M.N., Liu, Y., Bratton, D.L., Frasch, S.C., Michels, N.M., Case, S.R. and Chu, H.W. (2011) SPLUNC1 promotes lung innate defense against *Mycoplasma pneumoniae* infection in mice. Am. J. Pathol. **178**, 2159–2167

32 Ulevitch, R.J. and Tobias, P.S. (1995) Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Annu. Rev. Immunol. **13**, 437–457

33 Rietschel, E.T. and Brade, H. (1992) Bacterial endotoxins. Sci. Am. **267**, 54–61

34 Rastogi, D., Ratner, A.J. and Prince, A. (2001) Host–bacterial interactions in the initiation of inflammation. Paediatr. Respir. Rev. **2**, 245–252

35 Capodici, C., Chen, S., Sidorczyk, Z., Elsbach, P. and Weiss, J. (1994) Effect of lipopolysaccharide (LPS) chain length on interactions of bactericidal/permeability-increasing protein and its bioactive 23-kilodalton NH_2-terminal fragment with isolated LPS and intact *Proteus mirabilis* and *Escherichia coli*. Infect. Immun. **62**, 259–265

36 Meszaros, K., Parent, J.B., Gazzano-Santoro, H., Little, R., Horwitz, A., Parsons, T., Theofan, G., Grinna, L., Weickmann, J., Elsbach, P. et al. (1993) A recombinant amino terminal fragment of bactericidal/permeability-increasing protein inhibits the induction of leukocyte responses by LPS. J. Leukocyte Biol. **54**, 558–563

37 Zhou, H.D., Wu, M.H., Shi, L., Zhou, M., Yang, Y.X., Zhao, J., Deng, T., Li, X.L., Sheng, S.R. and Li, G.Y. (2006) Effect of growth inhibition of the secretory protein SPLUNC1 on *Pseudomonas aeruginosa*. Zhongnan Daxue Xuebao Yixueban **31**, 464–469

38 Ghafouri, B., Kihlstrom, E., Tagesson, C. and Lindahl, M. (2004) PLUNC in human nasal lavage fluid: multiple isoforms that bind to lipopolysaccharide. Biochim. Biophys. Acta **1699**, 57–63

39 Lu, Y.C., Yeh, W.C. and Ohashi, P.S. (2008) LPS/TLR4 signal transduction pathway. Cytokine **42**, 145–151