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Functional roles of SPLUNC1 in innate immune response against gram negative bacteria

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Abstract

Palate, **lu**ng, and **n**asal epithelium **c**arcinoma associated (PLUNC) 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 to BPI, a 456-residue cationic protein produced by precursors of polymorphonuclear leukocytes, that has been shown to possess both bactericidal and LPS binding activities. The human PLUNC is also known as lung specific X protein (LUNX), nasopharyngeal carcinoma-related protein (NASG), and secretory protein in upper respiratory tracts (SPURT). 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 head and neck region. The functional role of SPLUNC1 in innate immunity has been suggested but not clearly defined. This paper will describe recent findings that support antimicrobial and anti-inflammatory functions of SPLUNC1 in gram negative bacteria induced respiratory infection.

Keywords

SPLUNC1; bacteria; LPS; antimicrobial; serous; mucous

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 cystic fibrosis, chronic bronchitis and chronic obstructive pulmonary disease (COPD). The conducting airway serves as a first line of defense 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 to the adaptive immune system that takes days to become effective [2]. Both respiratory epithelial cells and inflammatory cells

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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 defense against invading pathogens. Therefore, epithelial cell regulated antimicrobial activity is regarded as the first line of defense 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 defense 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-like family and their tissue expression patterns

The human PLUNC-like gene sub-family now contains eight members located on chromosome 20q11 (for nomenclature detail, see the other article in this issue). Tissue distributions of the PLUNC-like family gene expression include the major salivary glands, the submucosal glands (SMGs) 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 neck region, SPLUNC1 (PLUNC, LUNX, NASG, and SPURT) 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.

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 airway surface liquid (ASL) 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 favored 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 serous cells. 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 end, LPLUNC1 is usually expressed in mucous cells and its expression 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 defense 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 to a neutrophil protein, bactericidal/permeability-increasing protein (BPI) [24], that mediates Gram-negative bacteria (GNB) endotoxin lipopolysaccharide (LPS)-induced bacterial killing. Based on this homology to 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 publications through *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 defense role was proposed in this paper, no direct antimicrobial activity was demonstrated. Chu et al. subsequently showed that a two hour incubation of the recombinant mouse SPLUNC1 (mSPLUNC1) protein at various concentrations significantly reduced *Mycoplasma pneumoniae* (Mp) growth in a dose-dependent manner [26]. The authors also showed an increased Mp burden and neutrophil count in bronchoalveolar lavage fluid (BALF) when a SPLUNC1 neutralizing antibody was intranasally administered into mice lung prior to the Mp infection. Another *in vitro* study used recombinant human SPLUNC1 protein at various concentrations, which also demonstrated the reduced growth of *Pseudomonas aeruginosa* (Pa) in a dose-dependent manner [27]. A recombinant chinchilla ortholog of human SPLUNC1 (cSPLUNC1) has also been shown to have antimicrobial activity in killing *Haemophilus influenzae* [28]. These studies suggest that SPLUNC1 orthologs from different species may all possess antimicrobial activity against various strains of bacteria. Gakhar et al. 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 *P. 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 expose the mice to GNB. Our lab has generated a CCSP-SPLUNC1 transgenic mouse model that is driven by the mouse Clara cell secretory protein (CCSP) promoter to over-express human SPLUNC1 in mouse airway epithelial secretory cells. Two types of GNB, Pa and

Klebsiella pneumoniae (Kp), were used to infect the mouse lungs through an intra-tracheal instillation. Both wild-type and the CCSP-SPLUNC1 transgenic mice were exposed to GNB to determine their sensitivities 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 to their wild-type littermates was correlated with lower bacterial colony formation unit (CFU) counts and reduced cell numbers in total inflammatory cells and neutrophils in BAL (manuscript submitted). Histological evaluations of lung pathology also demonstrated attenuated lung inflammation and injury in CCSP-SPLUNC1 transgenic mice after GNB induced respiratory infection (data 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 Mp infection in the CCSP-SPLUNC1 transgenic mice when compared with their wild-type littermates (manuscript in press). Results from these *in vivo* studies further confirmed the antimicrobial activity of SPLUNC1. [Survival Study: CCSP mice survived longer]

The binding of SPLUNC1 to LPS provides protection against excess inflammation

GNBs like *P. aeruginosa* and *K. pneumoniae* can produce the endotoxin LPS that alerts the host to the invading bacteria and triggers defensive innate immune responses [29–31]. The NH2-terminal fragment of human BPI has been shown to bind lipid A and antagonize some LPS-mediated effects [32, 33]. We and other researchers have demonstrated that SPLUNC1, similarly to BPI, can also bind to LPS [34, 35]. 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 a LPS scavenger to suppress LPS-induced inflammatory response. Because LPS is a known agonist for Toll-like receptor 4 (TLR4) [36], we also examined the activation of TLR4 signaling 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 interleukin-1 receptor-associated kinase 4 (IRAK-4) after an intranasal instillation of LPS when compared with wild-type mice (manuscript submitted). 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 IRAK4 kinase activity, and decreased NFκB activation.

Conclusion

Bacterial infection in the lung is a major cause of mortality and morbidity, especially in high-risk groups, such as immuno-compromised patients, the elderly, and those with other underlying pulmonary diseases such as CF and COPD. The antimicrobial function of

SPLUNC1 likely plays a critical role in host defense against pathogens not only in maintaining homeostasis 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 defense 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.

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Abbreviations used in this paper

SMGs	submucosal glands
BAL	bronchoalveolar lavage
PLUNC	palate, lung and nasal epithelium carcinoma associated
Mp	Mycoplasma pneumoniae
PA	Pseudomonas. Aeruginosa
Kp	Klebsiella pneumoniae

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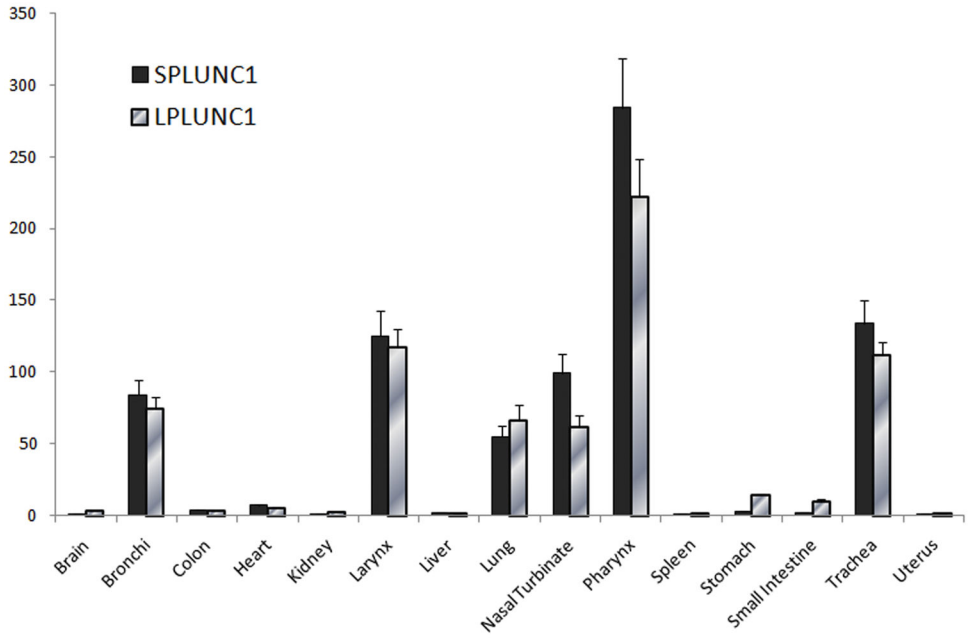

Figure 1. The relative abundance of SPLUNC1 and LPLUNC1 gene expression in various human tissues

Taqman based real time RT-PCR analysis of human *SPLUNC1* and *LPLUNC1* mRNA abundance in various human tissues samples. Relative expression was determined by the ΔΔCt method using human GUS-B RNA as a control. (mean ± S.D., $n = 3$). Human SPLUNC1 is highly expressed in tissues from nasal, laryngeal, pharyngeal, tracheal, and bronchial regions.

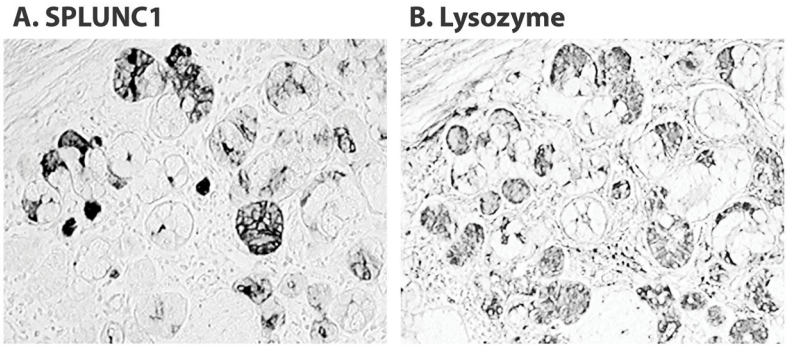

Figure 2. SPLUNC1 and lysozyme were co-localized in a subset of serous cells in SMGs

Cellular localization of SPLUNC1 (A) and lysozyme (B) proteins was assessed by immuno-histochemistry on lung sections of human trachea. Signal was not detected when parallel sections were incubated with pre-immune serum (data not shown). Original magnification, 100X.