SHORT PALATE LUNG AND NASAL EPITHELIUM 1 AND AIRWAY DISEASE

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A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Chemistry (Biological Division).

Chapel Hill
2016

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ABSTRACT

Julianne Jahui Huang: Short Palate Lung and Nasal Epithelium Clone 1
and Airway Disease
(Under the direction of Matthew R. Redinbo and Stephen L. Tilley)

Airway disease such as asthma and infection is the cause substantial morbidity and mortality in the world today. Although modern medicine has developed many drugs for these conditions, these diseases remain highly prevalent and are often difficult to treat.

Short palate, lung and nasal epithelium clone 1 (SPLUNC1) is an abundant multi-functional protein in the airway. It has been reported to have immune-modulatory, surfactant and anti-microbial functions, and it regulates the airway surface liquid (ASL) height through the epithelial sodium channel (ENaC). This study focuses on utilizing SPLUNC1's protective properties in combatting airway disease.

Airway hyperresponsiveness (AHR) is a characteristic feature of asthma, yet its pathophysiology is still poorly understood. SPLUNC1 is dysregulated in allergic rhinitis and chronic rhinosinusitis with nasal polyps. However, SPLUNC1 regulation in asthmatics has not been investigated. Here, we show that in allergic asthmatic humans and house dust mite (HDM)-allergic mice, SPLUNC1 in the bronchoalveolar lavage (BAL) is reduced. We demonstrate that administration of SPLUNC1 to mice decreases their AHR and show that the molecular basis for this effect involves the coordination of the N-terminus with an electrostatic patch on the protein's body. We propose that SPLUNC1 be further investigated for use in reducing AHR.

Pseudomonas aeruginosa, a primary lung pathogen in nosocomial pneumonia and in lung diseases such as cystic fibrosis and chronic obstructive pulmonary disease (COPD), causes considerable morbidity and mortality. SPLUNC1 has been shown to neutralize and combat *P. aeruginosa in vivo* and *in vitro*. Here, we sought to establish a model for evaluating delivery of exogenous SPLUNC1 in acute lung infection and provide evidence that preemptive administration of SPLUNC1 may decrease bacterial burden.

Lastly, we suggest that the administration of SPLUNC1 which we propose for asthma and lung infection results in SPLUNC1 mediated SPLUNC1 release in the lungs, effectively increasing the local protein concentration. This effect may utilize SPLUNC1's natural protective properties to combat airway disease.

We conclude that SPLUNC1 should be investigated further for use in asthma and bacterial pneumonia.

ACKNOWLEDGEMENTS

the first publication and for the friendships you helped pave when I first arrived. Thanks to Bill and Mike for all the proteins. I'm so thankful for not having to spend very much time in that cold cold lab, waiting for my OD_{600} and for the ÄKTA to spit out my proteins. Thanks to Herodes for hanging the drops leading to my crystal structure and to Ashley for help with cloning PilY1. Rebecca, you are amazing for always keeping things organized and for having answers to tons of miscellaneous scientific and administrative questions I had from across campus.

Thanks to Kelly Chason for teaching me all I know about cell culture and ELISAs. You are a wealth of information! Also, eternal thanks to Corey Jania for all the work you put into my project. Thanks for maintaining the mouse colonies, allowing me to eat lunch on flexiVent days, and the million hours of help on the flexiVent – running it, troubleshooting it, sending it to Canada and dealing with Scireq, etc. You have been instrumental in my project in so many ways; I could not have completed all this work without your help. As Kelly always seems to have answers to cell culture questions, you always have answers to my mouse questions. Your positive attitude, lab organization, and general on-top-of-it-ness made being in the Tilley lab such a positive experience. To Lily, we entered the lab together and will exit the lab together. Your proactive attitude, ability to get things done and ability to read my mind when I've forgotten to ask you for things has been amazing. You have taken care of so many little things in lab, making my life easier. Thanks to Jessica for the cell differentials in the early days. To my undergraduate research team, Dan, Dennis, Austin and Jen, thanks for the hours of hard work and

contributions to my project. Thanks especially to Dan and Austin for your high quality work that has led to figures in my manuscript and dissertation.

To my family, for all your support. Thanks for picking up my calls and listening to my stories through all that life has thrown my way. Thanks, Mom, for always checking up on me and for listening to all my pipe leaking and appliance breaking stories and for coming to make sure my house is still intact each year. Thanks for your never-ending support in life no matter what I've chosen to get myself into. Chuck, thanks for all the car advice from across the country (I've needed a lot of advice!) and for making sure I'm okay in the event of natural disasters. Jaching, my favorite family story related to grad school was the time you mistook my "making protein" for cooking steak. Even when you didn't understand what I was doing, you always had my back and I felt that.

To all the friends I've made in graduate school, you have been integral in helping me get through this! I would not have made it out with as much sanity as I have left without you. Jet, my cubby buddy in Kenan, thanks for your friendship and all the scientific and nonscientific conversations over the years. And thanks for helping to get my butt to the gym. Thanks, Mike, for being there from quals to the busiest part of my time here. Thanks for accompanying me to campus, for the rides home and for your patience. Erica, I'm so grateful I met you during interview weekend. You went from being an atypically nice girl that weekend to my best friend in graduate school. I've loved our weekly dinners and sidesplitting laughs. I'm so glad we had each other during this time; graduate school would have been so much less fun without you. Teddy, you've been incredibly supportive in this last lap.

Thanks for adjusting my carpal bones through the hours of computer work, for

answering my miscellaneous medical questions, for all the laughs and most of all for

your patient understanding and support.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	percent
°	degree
±	plus or minus
<	less than
>	greater than
µg	microgram
µL	microliter
µmol	micromol
Å	angstrom
A	alanine
AAs	allergic asthmatics
AHR	airway hyperresponsiveness
APC	antigen presenting cell
ASL	airway surface liquid
ASM	airway smooth muscle
ATP	adenosine triphosphate
BAL	bronchoalveolar lavage
B. cenocepacia	*Burkholderia cenocepacia*
BMMC	bone marrow mast cell
BPI	bactericidal/permeability-increasing
BPIFA1	bactericidal/permeability-increasing fold containing family member A1
C	Celsius

C	cysteine
cAMP	cyclic adenosine monophosphate
CD4	cluster of differentiation 4
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CFUs	colony forming units
CO_2	carbon dioxide
COPD	chronic obstructive pulmonary disease
C-terminus	carboxyl terminus
d	day(s)
D	aspartic acid
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNP	dinitrophenyl albumin
E	glutamic acid
EDSMRF	epithelial-derived smooth muscle relaxing factor
ENaC	epithelial sodium channel
ELISA	enzyme-linked immunosorbent assay
EP	electrostatic patch
FCεRI	high-affinity receptor for the Fc region of immunoglobulin E
G	glycine
h	hour(s)
H, His	histidine

HBEC	human bronchial epithelial cell
HDM	house dust mite
H. influenzae	*Haemophilus influenzae*
His$_6$	6 x histidine affinity tag
HRP	horseradish peroxidase
IACUC	Institutional Animal Care and Use Committee
ICS	inhaled corticosteroids
ICU	intensive care unit
IgE	immunoglobulin E
IL	interleukin
i.n.	intranasal
IPTG	isopropyl-1-thio-D-galactopyranoside
IRB	Institutional Review Board
i.t.	intratracheal
K	lysine
kDa	kilodalton
kg	kilogram
LABA	long-acting beta agonist
LB	lysogeny broth
LIC	ligation independent cloning
LPS	lipopolysaccharide
M	molar
m	minute(s)

MANOVA	multivariate analysis of variance
MBP	maltose binding protein
MCC	mucociliary clearance
Mch	methacholine
MDR	multi-drug resistant
mg	milligram
mL	milliliter
mm	millimeter
mmol	millimole
N	asparagine
N-terminus	amino terminus
ng	nanogram
nm	nanometer
NVs	normal volunteers
OD_{600}	optical density at 600 nm wavelength
OVA	ovalbumin
P	p-value
PanK	pantothenate kinase
P. aeruginosa	*Pseudomonas aeruginosa*
PAGE	polyacrylamide gel
PBS	phosphate-buffered saline
pH	negative log (base 10) of the molar concentration of hydronium ions
R	arginine

RANTES	regulated on activation, normal T cell expressed and secreted; chemokine ligand 5
S	serine
S18	N-terminal 18 amino acids of SPLUNC1 protein G22-A39
S. aureus	*Staphylococcus aureus*
SDS	sodium dodecyl sulfate
SEM	standard error of mean
SPLUNC1	short palate, lung and nasal epithelium clone 1
TBSA	total body surface area
Th2	type 2 T helper cell
TMB	3,3',5,5'-tetramethylbenzidine
Tris	tris(hydroxymethyl)aminomethane
UNC	University of North Carolina at Chapel Hill
VAP	ventilator associated pneumonia
vs	versus
WT	wildtype

CHAPTER 1: INTRODUCTION

Asthma

Asthma, the most common chronic inflammatory lung disease in developed countries, affects approximately 26 million people annually in the United States alone and 300 million worldwide. Asthma has been estimated to cost 56 billion dollars in the United States and treating 20-30% of asthmatics constitutes 80% of the cost of managing all asthmatics (1, 2). The disease is influenced by both genetic and environmental components, such as pathogens and pollution, and has higher frequency and severity in boys compared to girls 0 to 14 years of age; interestingly, this prevalence reverses in adulthood with women having a higher incidence of asthma than men (3-5). The gender differences are attributed to sex hormones influencing β2 adrenoreceptors. Approximately 70% of asthmatics also have allergies and half of all asthmatics experience asthma attacks annually.

Asthma is characterized by three cardinal features: airway inflammation, airflow obstruction, and airway hyperresponsiveness (AHR). Allergic inflammation involves many steps, affects patients from mild to severe asthmatics, and results in airway remodeling that can be correlated to the severity of symptoms (6). Airway edema, mucus hypersecretion, and smooth muscle remodeling all contribute to airflow obstruction and ultimately exacerbations (7, 8). AHR or bronchial hyperreactivity is influenced by allergic inflammation causing airway remodeling but AHR is ultimately the result of smooth muscle contraction (9). These cardinal

features translate clinically into a number of symptoms including cough, wheezing, chest tightness, and shortness of breath. Untreated symptoms can progress into status asthmaticus (also termed near-fatal asthma), which results in respiratory failure and sometimes death. Approximately 4,000 people per year die from status asthmaticus in the United States.

Fortunately, a number of highly effective therapies exist to treat the underlying inflammation in asthma. These drugs control asthma well when taken on a regular basis. Inhaled corticosteroids (ICS) are the mainstay of controller medicines for asthma. They work by a number of mechanisms, including the inhibition of proinflammatory cytokine synthesis. When ineffective alone, ICSs are combined with long-acting beta agonists (LABAs), long-acting anti-muscarinics, and leukotriene receptor antagonists. In addition to acting as long-term controller medicines, beta agonists are also used acutely to reverse bronchospasm by activating beta adrenergic receptors on airway smooth muscle (ASM), leading to rises in intracellular cAMP which promotes smooth muscle relaxation. This action translates clinically to bronchodilation and a reduction of airflow obstruction.

The primary cell types mediating allergic asthma pathogenesis are dendritic cells, which are antigen presenting cells (APCs), B cells, mast cells, eosinophils, and T lymphocytes (T cells), specifically T helper 2 (Th2) cells that play a large role in allergic inflammation. Dendritic cells are the first cells activated in the pathway to allergic inflammation. They are residents on mucosal surfaces and are located near the basement membrane of the respiratory epithelium and function as cellular guards for pathogens and inhaled antigens (10). Dendritic cell numbers are amplified

in asthmatics and in an ovalbumin-sensitized allergic lung inflammation rat model (11, 12). They have been shown to play roles in AHR, increasing IgE concentration and in eosinophilia in the allergic airways. Dendritic cells are professional APCs and are key in adaptive immunity and also sensitization to antigens which results in allergic asthma via Th2 cells.

Th2 CD4$^+$ T helper cells are responsible for the release of many cytokines relevant to asthma. Although they secrete cytokines, including IL-2, IL-3, IL-4, IL-5, IL-7, IL-9, IL-13, IL-15, IL-16 and IL-17, they are most well-characterized for their secretion of IL-4, IL-5 and IL-13 in asthma. IL-4 has been reported to be responsible for skewing to a Th2 phenotype by promoting Th2 cell differentiation and inhibiting Th1 differentiation, eosinophil expansion, B-cell growth resulting in IgE production, and mucus production (5, 13, 14). IL-5 is well known for causing eosinophilia by promoting proliferation and survival of eosinophils in the airway. When IL-5 was reduced in eosinophilic asthma by anti-IL-5 antibodies, lung function was improved, exacerbations were reduced and the quality of life was improved (15). IL-13 effects have overlap with IL-4 in that they enhance IgE and mucus production, induce eosinophilic inflammation and also play a role in AHR and airway remodeling (16, 17).

The principal involvement of B cells in asthma is in the production of IgE antibodies, which is a key mediator of the allergic response and has been linked directly to disease severity (5). B cells are influenced by IL-4 to undergo immunoglobulin class switching resulting in the synthesis of IgE antibodies.

Circulating IgE, released by B cells, binds to mast cells via their high affinity FCεRI receptors.

Mast cells are mucosal sentinels which, in normal tissues, have a role in angiogenesis, homeostasis, elimination of pathogens and play a role in innate and adaptive immunity (18). Although typically residing in the mucosa and connective tissues, they additionally congregate in smooth muscles and submucosal glands in asthmatic but not normal patients. Mast cells are activated in the asthmatic lung by a number of mechanisms. The best characterized mechanism is IgE-dependent activation. Mast cells have high affinity FCεRI receptors that bind IgE which, after encountering antigens, cross-link leading to mast cell activation (19). IgE-independent activation also occurs in asthma when mast cells are stimulated by mediators such as ATP and adenosine, which activate purinergic receptors exposed on the cell surface. Mast cell activation results in degranulation, lipid mediator release, and cytokine synthesis. Mast cells, following activation by both IgE-dependent and IgE-independent mechanisms, release a number of proinflammatory mediators including histamine, serotonin, prostaglandins and leukotrienes which cause smooth muscle contraction and airflow obstruction. Additionally, mast cells secrete the asthma-related cytokines IL-4, IL-5 and IL-13 which have previously been described as playing roles in eosinophilic airway inflammation and IgE synthesis. They also secrete profibrogenic cytokines leading to airway remodeling (20).

Eosinophils are granulocytes that play a major role in the pathophysiology of allergic asthma. These leukocytes are monitored as an indication of disease severity

and inform management strategy (13). The presence of eosinophils is associated with the Th2 cytokines IL-4, IL-5, which cause eosinophil expansion and survival and IL-13 expression. Eosinophil recruitment is due to eotaxin, RANTES and IL-5 and one of the main eosinophil products, major basic protein, damages the epithelium, increases AHR and causes mast cell degranulation. Eosinophils also cause goblet cell metaplasia, matrix deposition and smooth muscle hypertrophy (21). In addition to playing a critical role in the pathogenesis of Th2-high allergic asthma, eosinophils also mediate non-allergic eosinophilic asthma. In this asthma endotype, cytokines produced by the airway epithelium (thymic stromal lymphopoietin, IL-25, and IL-33) stimulate innate lymphoid cells to produce IL-5 and IL-13. In contrast to allergic eosinophilic asthma mediated by Th2 cells, IL-4 is not produced by the innate lymphoid cells, therefore IgE levels are low. Elucidation of these biological pathways involving mast cells and eosinophils has led to the development of new therapies including anti-IgE antibodies and anti-IL-5 antibodies, typically reserved for patients with severe asthma (22-24).

Inflammation in the airway wall that develops through the mechanisms described above results in narrowing of the airway lumen. This airway narrowing, coupled with mucus hypersecretion into the lumen, leads to airflow obstruction. Clinically, this airflow obstruction manifests as wheezing and shortness of breath. In normal airways, mucus protects the epithelium by trapping inhaled particles and pathogens, but excess mucus secretion from the submucosal glands and goblet cells in asthmatics, particularly after allergen exposure, contributes substantially to airflow obstruction during asthma attacks.

While mechanisms that underlie the development of airway inflammation and airflow obstruction are well-characterized, the pathophysiology of AHR remains less well understood. AHR is defined as heightened contraction of ASM following exposure to stimuli that fail to or minimally stimulate contraction of ASM in normal subjects. Out of all the immune cells described above, only mast cells have been shown to play a critical role in AHR development. Mast cells infiltrate the ASM of asthmatics, and mediators released from mast cells are believed to contribute to AHR development. Whether or not ASM is intrinsically abnormal in asthma and in itself a major contributor to AHR development remains controversial. ASM hypertrophy and hyperplasia have been reported to contribute to AHR pathogenesis in asthma (25, 26). However, some investigations of airway smooth muscle (ASM) from asthmatic subjects suggest that it is intrinsically normal but becomes dysfunctional in the milieu of the asthmatic airway (27, 28).

Over 30 years ago, a critical role for the epithelium in producing mediators that limit AHR was demonstrated when enhanced contraction was observed after the epithelia was denuded from the bronchi of dogs (29). This observation has been confirmed in multiple species, including mice and humans (30-64). Collectively, these studies strongly support the existence of an epithelial-derived smooth muscle relaxing factor (EDSMRF) that limits AHR. However, to date the identity of this EDSMRF has remained elusive.

SPLUNC1

Short palate, lung and nasal epithelium clone1 (SPLUNC1), also known as bactericidal/permeability-increasing fold containing family member A1 (BPIFA1), is

one of the most abundantly secreted proteins in mammalian airways, comprising up to 10% of total protein found in the airway surface liquid (ASL) (65). It was first discovered in mice in 1999 in a search for genes responsible for facial development and SPLUNC1 is primarily found in the airways and oral cavity; however, here we will mostly limit the discussion to airway involvement (66, 67). SPLUNC1 is encoded by genes on the long arm of chromosome 20 in the human genome and displays sequence and structural homology to the N-terminal portion of the antimicrobial bactericidal/permeability-increasing (BPI) protein (Figure 1.1) (68). It is a 256 amino acid long approximately 25 kDa protein which has a 19 amino acid signal sequence at its N-terminus that is cleaved off prior to the delivery of the mature protein to its final destination on mucosal surfaces.

SPLUNC1 is a multi-functional protein that regulates ASL height, possesses immunomodulatory and antimicrobial properties, and demonstrates surfactant actions (69). SPLUNC1 controls airway hydration by its inhibition of the sodium epithelial channel (ENaC) (70). This regulation results in homeostatic fluid absorption in the airway and is believed to be important for maintaining normal ASL height, which is required for proper mucociliary clearance (MCC) essential for debris and pathogen removal in the lungs. SPLUNC1 accomplishes its regulation of ENaC through its N-terminal G22-A39 "S18" domain (71). Additionally, an electrostatic surface patch on the protein is responsible for presenting its N-terminal S18 region to ENaC in order to effect ASL height regulation (72).

SPLUNC1 has been reported to play an anti-inflammatory role in the context of allergic inflammation. Ovalbumin (OVA) has been used extensively to induce

allergic airway inflammation in mice (73, 74). SPLUNC1 levels are reduced in mouse lungs following OVA sensitization and knockout of the protein results in increased eosinophilic inflammation after OVA challenge (75). Additionally, Nogo-B, in the reticulon family of proteins, has been shown to regulate SPLUNC1 in the OVA model (76). Nogo-B-deficient mice demonstrate augmented eosinophilic lung inflammation in this model and SPLUNC1 was substantially reduced in the Nogo-B knockout mice. Interestingly, out of 40,000 genes examined in a genome-wide microarray comparing RNA expression from lungs of Nogo-deficient and wildtype mice, only SPLUNC1 was markedly reduced (278-fold or 95%). Restoration of SPLUNC1 by transgenic expression in the airway epithelium of Nogo-deficient mice rescued the phenotype, reverting inflammation back to wildtype levels. Collectively, these studies suggest that SPLUNC1 acts to limit the development of allergic airway inflammation, albeit by unknown mechanisms. Clinical data supporting these observations in mouse studies include studies showing that SPLUNC1 levels are low in the nasal lavage fluid and nasal tissues of patients with allergic rhinitis and chronic rhinosinusitis particularly within nasal polyps (77, 78)

Due to SPLUNC1's sequence similarity with the antimicrobial protein BPI, it was originally expected to have antimicrobial capabilities. Indeed, it has been shown to have activity against many bacteria including *Pseudomonas aeruginosa, Burkholderia cenocepacia, Haemophilus influenzae* and others (79-83). Part of its antimicrobial effects stem from its capacity to affect MCC through ASL height regulation via ENaC inhibition, as discussed above. It is also reported to have direct effects on Gram-negative bacteria through lipopolysaccharide (LPS)-binding and is

expected to disrupt biofilm formation potentially through its ability to function as a surfactant (84).

SPLUNC1 has been reported to spread the ASL at the air-liquid interface. While the purpose of liquid-spreading surfactants in the alveoli has been extensively studied, the function of surfactants, such as SPLUNC1, in the conducing airways is less well characterized. There are reports that surfactants in the conducting airways aid MCC by increasing ciliary beating. Surfactants regulate immune cells and bind allergens, and a lack of surfactant is correlated with increased airway resistance in the lungs (85). Therefore, the ability of SPLUNC1 to function as a surfactant is yet another method in which the protein is able to protect the lungs.

SPLUNC1 has been implicated in the pathogenesis of a number of disease states including cystic fibrosis (CF), lung cancer, allergy (described above) and chronic obstructive pulmonary disease (COPD). Both the cystic fibrosis transmembrane conductance regulator (CFTR) and SPLUNC1 serve to negatively regulate ENaC. In CF, mutations in CFTR results in the decreased presence of this critical chloride channel on epithelial cells. The decreased pH in the CF lung reduces SPLUNC1's ability to inhibit ENaC (72). The combination of the inability CFTR and SPLUNC1 to inhibit CFTR in CF results in the dehydration of the ASL due to excess sodium transport and leads to a dysfunction in MCC. Despite the increased SPLUNC1 in the CF lung, there is an increased incidence of bacterial colonization, particularly with *P. aeruginosa*, *B. cepacia* and *H. influenzae* (86-89).

Depending on the type of lung cancer, SPLUNC1 has been found to be upregulated and downregulated. In metastatic lung cancers, SPLUNC1 has been

detected outside its locations in the normal lung. It has been found in pleural effusions and lymph nodes and has been suspected of protectively reducing an oncogene correlated with nasopharyngeal carcinoma (69). Therefore, SPLUNC1 regulation in terms of cancer depends on the particular disease.

Finally, SPLUNC1 has been investigated in COPD and the results are varied. In one study, SPLUNC1 levels have been shown to be increased in sputum from patients with COPD (90). However, another study was not able to confirm this finding, showing instead no difference between patients with COPD and normal subjects (91). Therefore, more studies are necessary to determine whether or not SPLUNC1 may play a role in this common airway disease.

Summary

Asthma is a common chronic inflammatory airway disease affecting approximately 8% of adults and 10% of children in the United States. It is influenced by genetic and environmental components and causes wheezing, shortness of breath, coughing and chest tightness in patients with the disease. Its defining characteristics include airway inflammation resulting in airway remodeling, reversible airflow obstruction, mucus hypersecretion and AHR. Although the pathobiology of airway inflammation and airflow obstruction is well characterized, AHR is less well defined. Studies in multiple species, including mouse and human, strongly suggest the existence of an EDSMRF; however, the identity of such a factor(s) has remained elusive.

SPLUNC1 is a multifunctional protein in the airways with numerous protective abilities including ASL-height regulation (and therefore involvement in MCC), surface

tension reduction, antimicrobial function, and modulation of immune cell function. Its location in the airways facilitate these many protective abilities and its dysregulation has been indicated in several disease states including CF, lung cancer, and allergy. Although SPLUNC1 has been investigated in terms of allergic rhinitis and chronic rhinosinusitis, it has not been investigated in human allergic asthma.

Here, we report SPLUNC1 levels in allergic asthmatics and house dust mite (HDM)-allergic mice, and present data suggesting a non-redundant role for SPLUNC1 in the pathogenesis of AHR. Finally, we propose that SPLUNC1 is the EDSMRF that has been sought for decades.
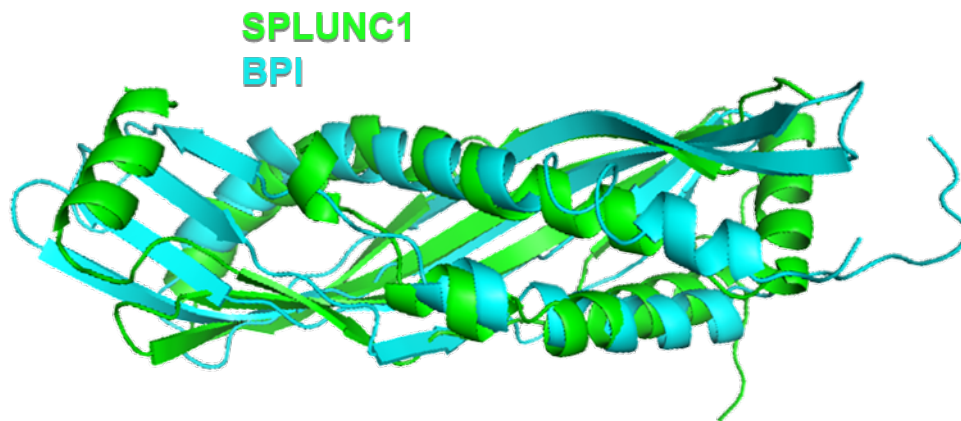
Figure 1.1 SPLUNC1 and BPI Structural Alignment
Alignment of the full length SPLUNC1 (PBD: 4KGH) in green to the N-terminal domain of BPI (PDB: 1EWF) in blue.

CHAPTER 2: SPLUNC1 AND ASTHMA/ALLERGIC INFLAMMATION

Introduction

SPLUNC1 is a multifunctional protective protein in the human airways that has been implicated in allergic airway inflammation such as in allergic rhinitis and chronic rhinosinusitis. It has also been investigated in mouse models of allergic inflammation. However, its involvement in human allergic asthma and one of the defining features of asthma, AHR, has not been investigated. Many studies involving mice and humans have pointed to the existence of an EDSMRF but have not conclusively found such a factor. In this study, we report SPLUNC1 levels in allergic asthmatics and HDM-allergic mice and suggest that SPLUNC1 is the elusive EDSMRF that has been long sought after in the literature.

Materials and Methods

Animals

Female C57BL/6 mice were purchased from The Jackson Laboratory. SPLUNC1$^{-/-}$ mice were a kind gift from Dr. Y. Peter Di and Dr. Paul B. McCray Jr. at the University of Pittsburgh. SPLUNC1$^{-/-}$ mice were backcrossed 3 generations to the C57BL/6 background. SPLUNC1$^{+/-}$ N3 heterozygote breeders were used to generate SPLUNC1$^{-/-}$ mice and SPLUNC1$^{+/+}$ littermate controls. All mice were housed in specific pathogen-free animal facilities with 12 h day and night cycles and provided food and water ad lib.

HDM-induced Allergic Airway Inflammation

Allergic airway inflammation was induced in 7-13 week old female C57BL/6 mice by intranasal (i.n.) administration of 25 µg of HDM (Greer Laboratories) for 12 days. HDM was administered once a day for 5 consecutive days followed by 2 non-treatment days, and this cycle repeated until 12 total doses of HDM were administered. Isoflurane was used for anesthesia for all i.n. instillations.

BAL Fluid from Humans and Mice

Bonchoalveolar lavage (BAL) fluid was collected from healthy and mild allergic asthmatic volunteers undergoing bronchoscopy at the U.S. Environmental Protection Agency in Chapel Hill, NC as previously described (92). BAL was performed on HDM-allergic and PBS-control mice with 0.8 mL of Hank's Balanced Salt Solution via tracheal cannula.

Human Bronchial Epithelial Cell Culture

Human Bronchial Epithelial Cells (HBECs) were obtained from freshly excised bronchial specimens from normal subjects as previously described (93). HBECs were cultured at air-liquid interface in a modified bronchial epithelial growth medium with 5% CO_2 at 37°C and were used 4-6 weeks after seeding on 12 mm Millicell inserts (EMD Millipore). The apical surface of the HBECs was washed with PBS and a media change performed. An apical wash in 500 µL PBS was collected 1 h after media change, and SPLUNC1 concentration was determined by ELISA.

Measurement of AHR

AHR was measured in tracheotomized, mechanically ventilated mice previously described (94). Briefly, mice were anesthetized with pentobarbital (70 mg/kg), tracheotomy performed and an 18 gauge tracheostomy cannula inserted. Mice were then ventilated using a small animal ventilator (FlexiVent, Scireq) and paralyzed with atracurium. Airway mechanics were measured at baseline and following increasing doses of aerosolized methacholine (Mch) using a single sinusoidal frequency applied to the airway. Lung resistance was calculated using equation of motion-single compartment model.

SPLUNC1 ELISA

A sandwich ELISA was developed and used to detect SPLUNC1 protein in BAL supernatants. Primary monoclonal and secondary biotinylated polyclonal anti-SPLUNC1 antibodies (R&D Systems) were used along with Avidin-HRP and TMB substrate (eBioscience).

Western Blot

SDS-PAGE was run on BAL supernatants and proteins transferred to a nitrocellulose membrane. Blots were incubated in primary anti-SPLUNC1 antibody and secondary HRP-conjugated antibodies (R&D Systems). Enhanced chemiluminescent substrate was used for detection. Densitometry analysis was performed using Image J software and normalized to a PonceauS 70 kDa band.

Protein Preparation

Wildtype and mutant proteins were expressed in BL21-CodonPlus cells and purified as previously described (72). BL21-CodonPlus competent cells were

transformed with the expression plasmid of interest and cultured in LB with ampicillin (100 µg/mL), chloramphenicol (34 µg/mL) and antifoam (50 µL) with shaking at 37°C until the OD_{600} reached 0.6. The cells were induced with 0.1 mM isopropyl-1-thio-D-galactopyranoside (IPTG) and the temperature was reduced to 18°C for overnight growth. Cell pellets were lysed using sonication in the presence of lysozyme, DNase 1, and protease inhibitor cocktail. Nickel and size exclusion chromatography were used for purification, and tobacco etch virus protease removed the histidine tag from the protein.

Crystallization of SPLUNC1^{K138E}

Crystals of SPLUNC1^{K138E} were grown in 6M ammonium nitrate, and 0.1M Tris (pH 8.5) and cryoprotected in 15% glycerol. Crystals diffracted to 2.7 Å with space group C222$_1$. Molecular replacement with wildtype SPLUNC1$^{\Delta19}$ was used as a search model.

Bone Marrow Mast Cell Culture

Bone barrow cells were isolated from the femurs of 8-12 week old C57BL/6 mice and grown in tissue culture for 6 weeks. Media was changed twice weekly and non-adherent cells were enriched for mast cells with IL-3. By 4 weeks, pure populations of mast cells were obtained.

Bone Marrow Mast Cell IL-13 Assay

Bone marrow mast cells (BMMCs) were treated with LPS from *P. aeruginosa* PA10 strain (0.2 µg/mL) followed by SPLUNC1 (0, 20, 100 and 200 µg/mL). IL-13 secretion was measured in the media by ELISA (eBioscience) 24 h later.

Bone Marrow Mast Cell Hexosaminidase Release

BMMCs were coated with a monoclonal IgE specific for human dinitrophenyl albumin (DNP, 100 ng/mL) and incubated overnight at 37°C. The next day, IgE-loaded mast cells were stimulated with DNP antigen (5, 50 ng/mL) and incubated for 20 m. Mast cell degranulation was assessed through detection of hexosaminidase in the media and comparing the hexosaminidase in the media to the total hexosaminidase present.

Statistics

All analyses were performed by Mann-Whitney test, Wilcoxon matched pairs-signed rank test (GraphPad Prism) or MANOVA as indicated in figure legends. For MANOVA, total airway resistance data were log2 transformed and modeled as repeated measures in a multivariate model with genotype, treatment (where applicable), and Mch dose as fixed effect, and individual mouse as random effect factors. Between-subject effects fitted by the sum of the repeated measures were tested for significant differences between genotype, treatment, and Mch dose using F-statistics (JMP v.12.0.1, SAS). All data represent mean ± SEM.

Study Approval

The IACUC and IRB of the University of North Carolina at Chapel Hill approved all experiments. Written informed consent was obtained from each human subject.

Results

SPLUNC1 is an Epithelial-derived Factor that is Reduced in Asthmatic Human and Allergic Mouse BAL

Mild allergic asthmatics (AAs) and normal volunteers (NVs) underwent bronchoscopy, and SPLUNC1 protein levels were measured in BAL fluid by ELISA. SPLUNC1 levels were markedly reduced or undetectable in samples from AAs (9.3+/-3.4 ng/mL). In contrast, most NVs had detectable SPLUNC1 at levels that were significantly higher than AAs (38.0+/-8.0 ng/mL) (Figure 2.1A). To determine if mice with asthma-like airway disease would also develop a relative SPLUNC1-deficiency in their airways, BAL fluid was collected following 12 days of mucosal sensitization with HDM. As shown in Figure 2.1B and similar to our findings in human BAL, SPLUNC1 levels in the airways of HDM-allergic mice were markedly lower than levels in PBS-treated controls (301.8+/-61.8 ng/mL vs. 885.2+/-113.8 ng/mL). Western blots confirmed the results obtained by ELISA, showing markedly reduced SPLUNC1 protein in the HDM-treated animals (Figure 2.1C).

Next, to demonstrate that airway epithelia secrete SPLUNC1, we cultured HBECs at air-liquid interface and measured SPLUNC1 levels in the apical wash by ELISA. HBECs secrete abundant SPLUNC1 (Figure 2.2). Our findings of reduced SPLUNC1 levels in HDM-allergic mice and asthmatic humans are consistent with in vitro studies on HBECs which showed that IL-13 suppresses SPLUNC1 mRNA expression, and in vivo studies in mice exposed to OVA (75, 95, 96). Together, these results strongly support the conclusion that SPLUNC1 is an epithelial-derived factor that is reduced as a result of allergic airway inflammation.

SPLUNC1 Regulates AHR

To investigate whether SPLUNC1-deficiency might contribute to AHR, airway mechanics during a graded Mch challenge were examined in naïve SPLUNC1$^{-/-}$ mice and their wildtype littermate controls. SPLUNC1$^{-/-}$ mice showed greater responsiveness to Mch, suggesting a link between SPLUNC1-deficiency and AHR (Figure 2.3). We next tested whether intratracheal (i.t.) instillation of SPLUNC1 to HDM-allergic mice would reduce AHR. Addition of SPLUNC1 1h prior to Mch challenge reduced AHR to levels similar to controls (Figure 2.4). These results indicate that SPLUNC1 plays a critical role in controlling AHR, and lead us to posit that restoration of SPLUNC1 may be therapeutic.

K138E Mutant Structure and Function

It has been reported that exercise in asthmatics leads to dehydrated or low ASL height, and that this effect on the airway produces AHR (9, 97). A well-characterized function of SPLUNC1 is its capacity to regulate and restore low ASL height through its inhibition of ENaC (70, 71). We therefore hypothesized that SPLUNC1 reduces AHR through the regulation of ASL height and sought to elucidate the structural basis for this effect. In collaboration with the Tarran Lab, we have previously reported that wildtype SPLUNC1 (Δ19) regulates ASL height at normal but not acidic pH (72). Acidification of the airways occurs in asthma and in addition to the already reduced amount of SPLUNC1 in the asthmatic airways, we expect SPLUNC1's ability to regulate ASL height to be diminished by the acidic environment (98). Data from the Tarran lab shows that a mutant, SPLUNC1^{K138E}, regulates ASL height at acidic pH whereas the wildtype protein does not (Figure

2.5A). We were interested in determining the structure of this salient point mutant to obtain insight into a structure-function relationship.

We solved the crystal structure of SPLUNC1^{K138E} to 2.67 Å which revealed no major structural differences between the wildtype protein and the SPLUNC1^{K138E} mutant (Figure 2.5B). This suggests that a structural change is not likely to cause the change in function. However, a limitation of this method is that the crystallization condition was basic (pH 8.5) rather than acidic. Since the functional differences between the wildtype and mutant protein exists in acidic rather than basic conditions, the next step would be to determine whether any structural differences exist at a more acidic condition.

The Molecular Basis for SPLUNC1's Effects on AHR

SPLUNC1's Effect on AHR is Independent of Effects on ASL Height

We next wanted to establish the molecular basis for SPLUNC1's reduction of AHR. As stated above, we hypothesized that SPLUNC1's ability to regulate ASL height was responsible for its effects on AHR. To test this hypothesis, we utilized SPLUNC1 mutants and peptides with differential effects on ASL height (71). Surprisingly, the ENaC regulating N-terminal peptide of SPLUNC1, S18, failed to attenuate AHR (Figure 2.6A). In support of this result, the ENaC-inhibitor amiloride also failed to attenuate AHR at various concentrations (Figure 2.6B, 2.6C, 2.6D). We tested a variety of concentrations to maximize the ability of amiloride to inhibit ENaC despite reports of its half-life (99-101). Therefore, we conclude that inhibition of ENaC is not likely to be the cause of AHR-reduction. We additionally tested a few mutants, SPLUNC1^{D193N} which regulates ASL height in both acidic and normal pH

(102), and SPLUNC1^{S190A} and SPLUNC1^{5XHis} which both lack the ability to regulate

ASL height (Figures 2.6C, 2.6D, 2.6E). All three mutants were able to reduce AHR,

providing further evidence that SPLUNC1's effect on AHR is independent of ENaC-

mediated changes in ASL-height.

SPLUNC1's N-terminus is Critical for AHR Reduction

Since the ENaC-regulating S18 peptide did not reduce AHR, we hypothesized

that the body of the protein, SPLUNC1$^{\Delta44}$, which is missing the 18 amino acids on

the N-terminus (S18 segment), would inhibit AHR. Surprisingly, SPLUNC1$^{\Delta44}$, similar

to S18, failed to attenuate AHR (Figure 2.7A). These data suggest that residues on

both the N-terminus and the body of SPLUNC1 are essential for reducing AHR. To

pinpoint the N-terminal residues critical for AHR reduction, we tested additional N-

terminal SPLUNC1 truncations. While SPLUNC1$^{\Delta34}$, ten amino acids longer than

SPLUNC1$^{\Delta44}$, was ineffective at attenuating AHR (Figure 2.7B), SPLUNC1$^{\Delta30}$ and

SPLUNC1$^{\Delta21}$ exhibited trends toward AHR attenuation (Figures 2.7C, 2.7D). We

then hypothesized that the aspartic acid (D) residue, the only charged residue on the

N-terminus, was critical for AHR-regulation. This residue may be involved in the N-

terminal presentation required for AHR reduction. Therefore, we removed the

negative charge by changing the D to a nonpolar hydrophobic residue alanine (A).

The SPLUNC1^{D29A} mutant reduced AHR, indicating that the charged D alone was

not responsible for the effect (Figure 2.7E). Overall, these data indicate that the N-

terminus and the body of SPLUNC1 are important for AHR reduction.

SPLUNC1's Effect on AHR is Dependent on the Coordination of its N-terminus with an Electrostatic Patch on the Protein Body

To further define the molecular basis of SPLUNC1's capacity to modulate AHR, we examined surface electrostatics. We have previously shown that a surface "electrostatic patch" is involved in the proper presentation of the N-terminus (72). Thus, we hypothesized that the electrostatic patch may also be important for the observed effects on AHR as well. To test this hypothesis, we prepared a mutant in which the N-terminal region of the human protein was attached to the mouse protein body, which by sequence alignment does not contain the surface electrostatic patch. This exchange abrogated the ability of SPLUNC1 to reduce AHR (Figure 2.8A), indicating that the N-terminal domain and electrostatic patch work together to control AHR. We hypothesized that restoring the electrostatic patch on the mouse protein with the human N-terminus would restore the protein's control of AHR. Thus, the mouse protein with the human N-terminus was modified to contain the human electrostatic patch. When we administered this protein prior to Mch challenge, the protein's AHR-reducing ability was restored (Figure 2.8B). Conversely, when we put the mouse N-terminus on the human SPLUNC1 body, AHR was reduced (Figure 2.8C) indicating that the mouse N-terminus is able to work with both the human protein body and the mouse protein body. We have not yet been able to test the full length wildtype mouse protein due to difficulties in purification, but the mouse protein body alone did not reduce AHR (Figure 2.8D). When we restored the non-charged mouse "electrostatic patch" region to the human protein body with the mouse N-terminus, AHR was reduced. (Figure 2.8E).

Lastly, as reported previously, deletions of helix 4 and helix 6 from SPLUNC1 do not alter its overall structure, and thus are expected to leave both the S18 and electrostatic regions intact (82). These two proteins reduced AHR in HDM-allergic mice to levels similar to that observed with wildtype SPLUNC1$^{\Delta 19}$ (Figures 2.8F, 2.8G). Collectively, these data indicate that the N-terminal region of SPLUNC1 and the electrostatic patch work in concert to control AHR.

SPLUNC1's Mechanism of Action for AHR Reduction

Mast cells are the most critical leukocyte in AHR development and we and others have shown that the absence of mast cells results in an inability to exhibit allergen-induced AHR (103, 104). Due to the co-localization of mast cells and SPLUNC1 in the mucosa, the capacity of mast cells to cause AHR, and the ability of SPLUNC1 to abolish AHR in allergic mice, we hypothesized that SPLUNC1 functions to reduce AHR by inhibiting the activation of mast cells. To test this hypothesis, we isolated bone marrow cells from the femurs of C57BL/6 wildtype mice and grew them in culture, enriching for mast cells with IL-3. To test whether SPLUNC1 could inhibit IL-13 production by mast cells, a Th2 cytokine important for AHR, BMMCs were treated with LPS to induce IL-13 secretion. Cells were then administered SPLUNC1 and secreted IL-13 was measured in the media 24 h later. We observed a SPLUNC1 dose-dependent trend toward reduced IL-13 secretion (Figure 2.9).

Next, we tested the potential of SPLUNC1 to inhibit antigen-mediated mast cell degranulation. Mast cells were coated with IgE overnight specific for human DNP. The next day, IgE-loaded mast cells were treated with SPLUNC1 and then

stimulated with DNP antigen and incubated for 20 m. Mast cell activation was assessed by measuring hexosaminidase in the media as a percentage of the total hexosaminidase present (105). SPLUNC1 did not alter the antigen-mediated mast cell degranulation (Figure 2.10).

These studies suggest that the presence of SPLUNC1 may reduce the secretion of IL-13 from mast cells. If the trends we observed are confirmed with more mast cell cultures, then it is possible that inhibition of mast cell IL-13 synthesis may in part be responsible for SPLUNC1's ability to attenuate AHR.

Discussion

Using samples from allergic asthmatics, we have shown that levels of the normally abundant airway protein SPLUNC1 are significantly reduced. Similarly, the induction of allergic airway inflammation in mice results in reduced SPLUNC1 levels. SPLUNC1-deficient mice are hyper-responsive to Mch, and restoring SPLUNC1 to the airway of HDM-allergic mice reverses the AHR that develops in this model. Collectively, these data suggest that SPLUNC1 is an epithelial-derived relaxing factor, something that has been sought for 30 years. Further, they suggest that SPLUNC1 deficiency may contribute to AHR development in asthma.

SPLUNC1 is a multi-functional protein in the airway with antimicrobial, surfactant, ASL-height regulating, and immunomodulatory activities. Mice lacking SPLUNC1 show enhanced eosinophilic airway inflammation and goblet cell hyperplasia following OVA sensitization (75). And allergic inflammation significantly reduces SPLUNC1 levels in the lungs (76). These studies on inflammation, collectively with our data showing low SPLUNC1 levels in asthmatics and its

importance in AHR, suggest that correction of SPLUNC1-deficiency in asthmatics may be therapeutic.

One of SPLUNC1's well-characterized functions is its ability to regulate ASL height through ENaC (70, 71). Here we report that the ENaC-regulating SPLUNC1 peptide S18 is unable to reduce AHR, giving an indication that ENaC-regulated changes in ASL height is not likely to be the route of AHR reduction. In support of this finding, the ENaC inhibitor amiloride also failed to reduce AHR. It is known that asthma exacerbations are correlated with acidification of the airways (98). Our group has previously shown that the S18 peptide, and point mutant SPLUNC1^{D193N} are able to regulate ASL height at both normal and acidic pH (72). Despite the fact that both are able to regulate ASL height independent of pH, SPLUNC1^{D193N} reduced AHR while S18 did not. We additionally tested two mutants which do not regulate ASL height (SPLUNC1^{S190A}, SPLUNC1^{5XHis}) and showed that these proteins significantly reduced AHR. Therefore, we conclude that SPLUNC1's ability to alter ASL height via ENaC inhibition is not the mechanism for its ability to affect AHR.

Since SPLUNC1$^{\Delta44}$, which lacks the first 18 amino acids of the protein, did not reduce AHR, we felt confident that the N-terminus of the protein was critical for the observed effects on AHR. Interestingly, the first two N-terminal residues of SPLUNC1 exhibit structural similarity to the muscarinic receptor antagonist, scopolamine. Both scopolamine and SPLUNC1's N-terminal residues glutamine and phenylalanine contain an amine group and an aromatic ring. We thus hypothesized that SPLUNC1 might act as a muscarinic receptor antagonist, preventing Mch from

inducing AHR when given before Mch challenge. However, when we removed the two N-terminal residues from the protein, its ability to abolish AHR was not eliminated. These data suggest that muscarinic receptor antagonism is not responsible for the effects of SPLUNC1 on AHR.

We previously reported that an "electrostatic patch" on SPLUNC1 is involved in presenting the protein's N-terminus (72). We hypothesized that the electrostatic patch may be involved in N-terminus presentation for AHR-reduction as well. Indeed, we were able to support this hypothesis by preparing a few mutant proteins. The first was a mouse-human chimeric protein that naturally does not have an electrostatic patch. This protein, containing the mouse body and human N-terminus was not able to reduce AHR. However, when the hybrid protein was conferred the human electrostatic patch by replacing amino acid sequences in the protein body, the ability to affect AHR was restored, indicating the requirement of the N-terminal coordination with the electrostatic patch for AHR-reduction. We also prepared the complementary human protein with a mouse N-terminus and the human protein with the mouse terminus which was given the mouse "electrostatic patch" region. Both of these proteins reduced AHR, indicating the mouse N-terminus may be able to coordinate with both the mouse protein body and the human protein body. To further support the hypothesis that the N-terminus and the protein body are both needed for AHR-regulation, we focused on two major alpha helices, helix 4 and 6, which flank the beta-stranded region of the SPLUNC1 protein. We deleted each alpha helix from the protein individually, which are expected to leave the electrostatic patch and S18 region intact. Both proteins significantly reduced AHR in the HDM-allergic mice.

Thus, these data provide further evidence that both the N-terminus and electrostatic patch are necessary for SPLUNC1 to effect AHR.

Defining the portions of the SPLUNC1 protein that mediate its effect on AHR reduction is critical for the development of SPLUNC1-derived proteins as therapeutics for asthma. Equally important is understanding the cellular and molecular mechanisms by which SPLUNC1 influences AHR. To begin to define this mechanism, we chose to examine SPLUNC1's effect on mast cell activation. Mast cells are critical effector cells in AHR development, in part through their synthesis of IL-13 (106, 107). Our data suggests that SPLUNC1 may inhibit IL-13 synthesis by mast cells. Since we only observed a trend toward IL-13 reduction, more experiments with additional BMMC cultures are warranted. Human mast cells can also be cultured in vitro, and similar experiments with human mast cells could further support this mechanism of action in IL-13 inhibition is seen in human cells. IL-13 has long been recognized as a key mediator in AHR development. It is tempting to speculate that the down-regulation of SPLUNC1 in the allergic airway results in increased IL-13 synthesis by mast cells, resulting in AHR development.

In summary, we have demonstrated that one of the most abundant proteins in the normal airway, SPLUNC1, is reduced in allergic asthmatics and in mice sensitized with the common aeroallergen HDM. Furthermore, we show that reduced airway SPLUNC1 enhances bronchoconstriction in response to Mch. Importantly, this AHR can be reversed by restoring SPLUNC1, and the molecular basis for this effect on airway physiology involves electrostatic coupling of the SPLUNC1 body with portions of the N-terminus. These results suggest that strategies to increase

27

endogenous SPLUNC1 levels or the administration of recombinant SPLUNC1 to the

airway be further investigated as novel approaches for treating asthma.
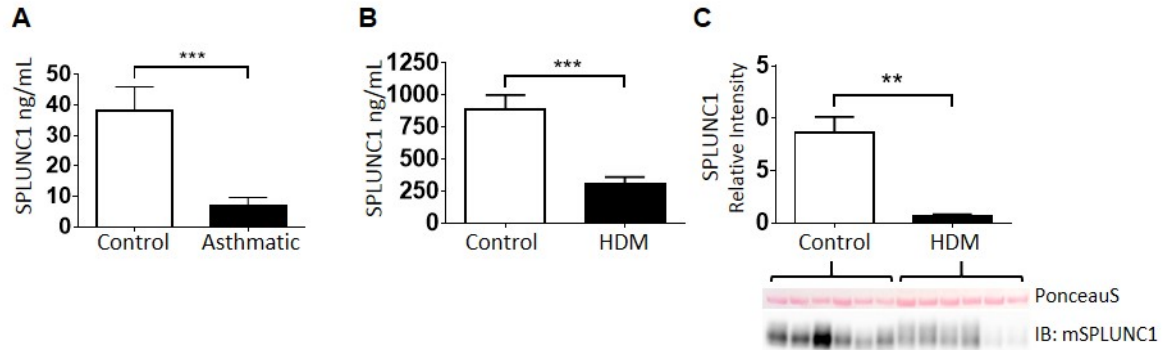
Figure 2.1. SPLUNC1 is Reduced in Allergic Airways
A. Human BAL was obtained from normal volunteers (Control) and allergic asthmatics (Asthmatic) undergoing bronchoscopy and SPLUNC1 levels were measured in BAL supernatants by ELISA. n=20 for Control group and n=21 for Asthmatic group.
B. C57BL/6 mice were treated with HDM (25 µg i.n.) or PBS (Control) for 12d, then SPLUNC1 levels were measured in BAL supernatants by ELISA. n=17 for both groups.
C. BAL supernatants from HDM-sensitized and Naïve (Control) mice were evaluated via Western blot densitometry analysis. n=6 for both groups. Mann-Whitney test. **P<0.005 ***P<0.0005
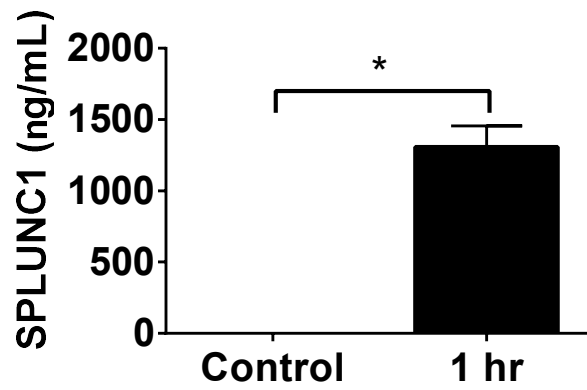
Figure 2.2 HBECs Secrete SPLUNC1
HBECs were cultured at air-liquid interface for 4-6 weeks and SPLUNC1 levels were measured in apical wash samples 1h after the addition of apical PBS (20 µL) by ELISA. n=4. Mann-Whitney test. *P<0.05
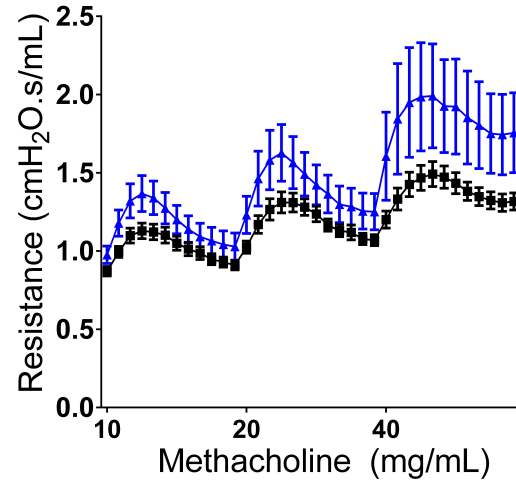
Figure 2.3 SPLUNC1^{-/-} Mice are Hyperresponsive to Methacholine
Airway resistance was measured in SPLUNC1^{-/-} (blue triangles) and SPLUNC1^{+/+}
(black squares) mice following a graded Mch challenge. n=25-27 **P<0.005 by
MANOVA.
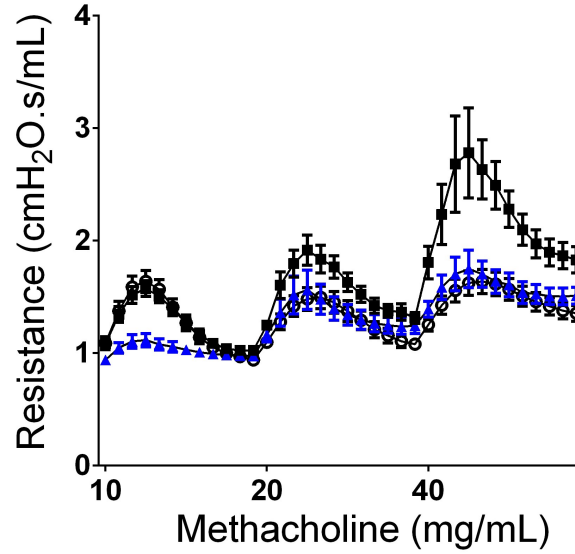
Figure 2.4 Administration of Wildtype SPLUNC1$^{\Delta 19}$ Abolishes Allergen-induced AHR

C57BL/6 mice were treated with HDM (25 µg i.n.) or PBS for 12d. One day after the last HDM challenge, mice were dosed with wildtype SPLUNC1$^{\Delta 19}$ (150 µg i.t.) or PBS and AHR to methacholine was measured 1h later. Open circles: PBS+PBS group; black squares: HDM+PBS group; blue triangles: HDM+SPLUNC1$^{\Delta 19}$. n=14-15 for all groups. *P<0.05 in B by MANOVA.
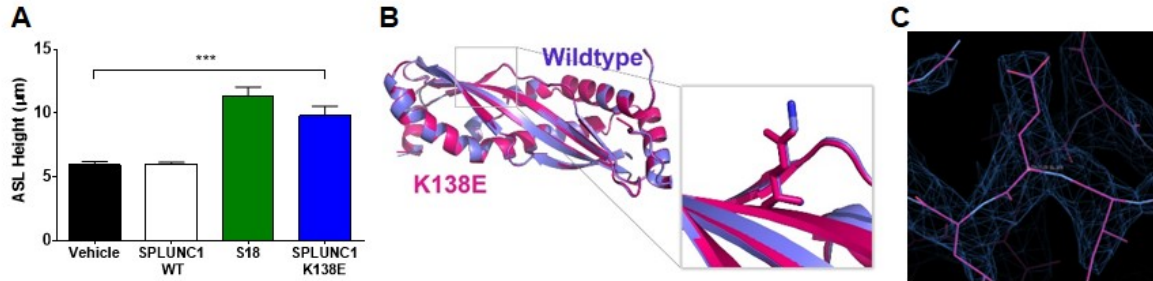
Figure 2.5 SPLUNC1^{K138E} Protein Restoration of ASL-height at Acidic pH is Not the Result of a Structural Change

A. Mean ASL height in HBEC cultures at pH 6.0 8 h after addition of wildtype (WT) SPLUNC1, S18 peptide or SPLUNC1^{K138E} (n ≥ 7 for each group). Mann-Whitney test. ***$P<0.0005$

B. Crystal structure of SPLUNC1^{K138E} mutant (pink) at 2.7 Å resolution superimposed on wildtype protein (purple), with a close-up of the normal K138 and mutant E138

C. Electron density of the glutamic acid at position 138 in the mutant structure; 2Fo-Fc map contoured at 2σ
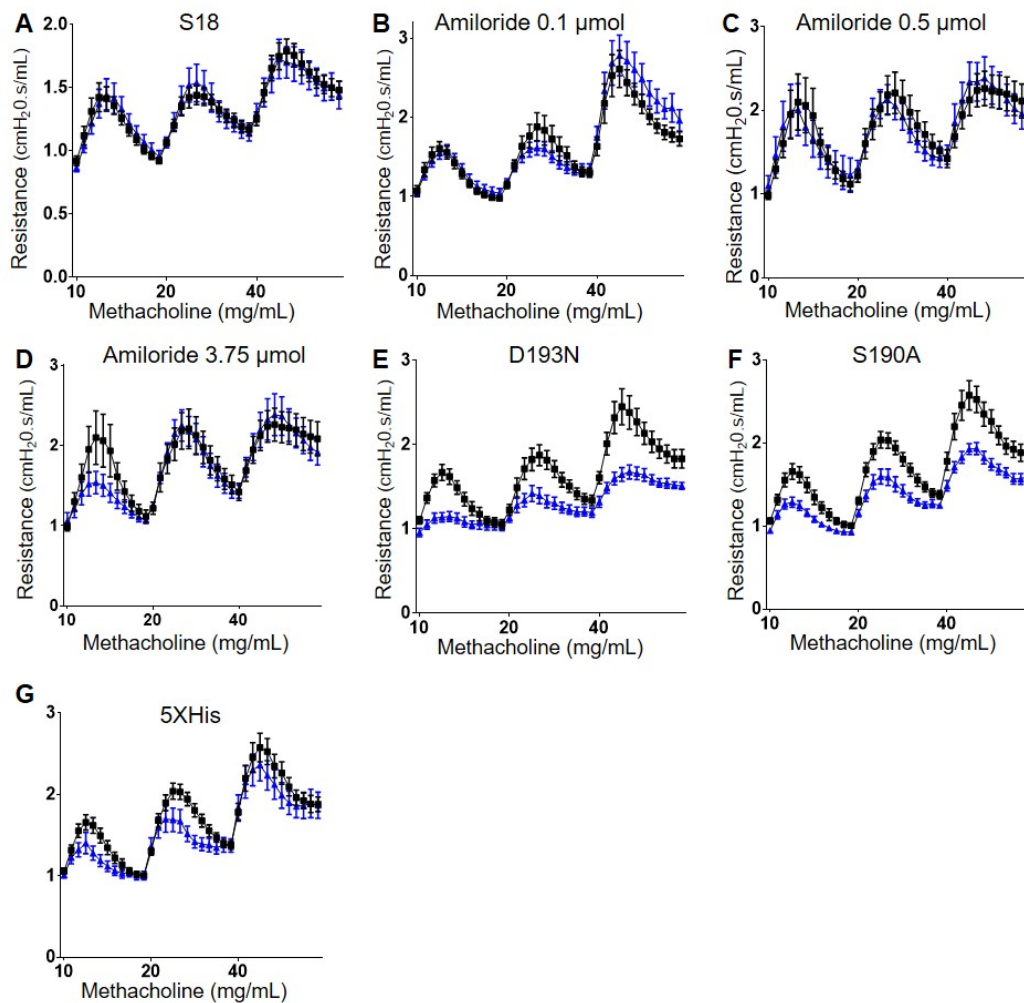
Figure 2.6. SPLUNC1's Effect on AHR is Independent of Effects on ASL Height
C57BL/6 mice were treated with HDM (25 µg i.n.) for 12d. One day after the last
HDM challenge, mice were dosed with
A. S18 (6 mmol i.t.)
B. Amiloride (0.1 µmol i.t.)
C. Amiloride (0.5 µmol i.t.)
D. Amiloride (3.75 µmol i.t.)
E. SPLUNC1^{D193N} (150 µg i.t.)
F. SPLUNC1^{S190A} (150 µg i.t.)
G. SPLUNC1^{5XHis} (150 µg i.t)
or PBS and AHR to methacholine was measured 1h later. Black squares: HDM+PBS
group; blue triangles: HDM+treatment group. n=10-15 for all groups. $P>0.05$ in A, B,
C and D * $P<0.05$ in G, ***$P<0.0005$ in E and F by MANOVA.
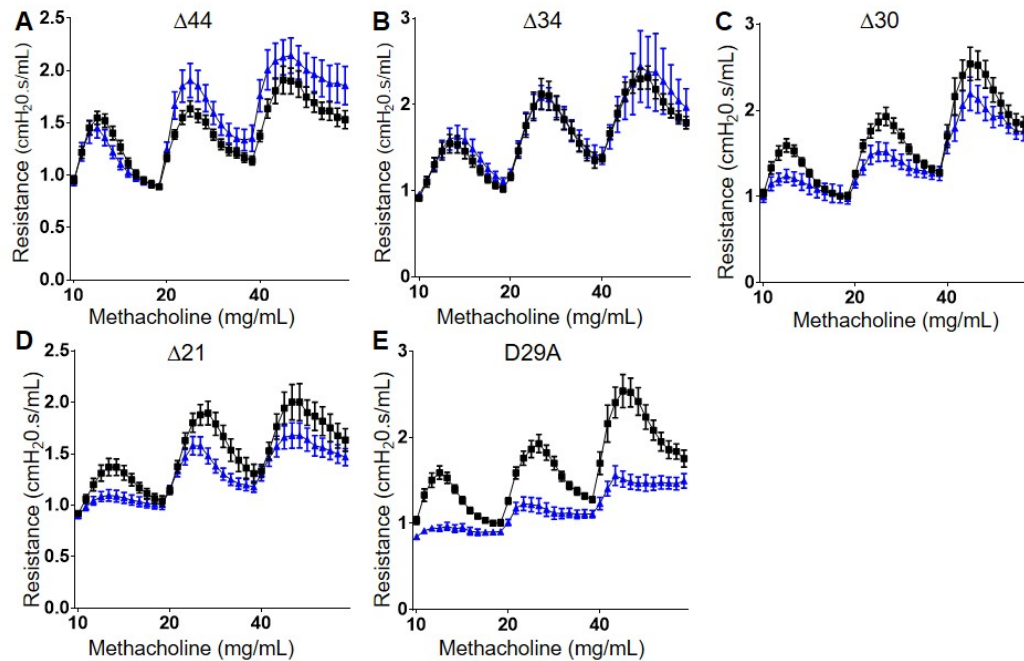
Figure 2.7 SPLUNC1's N-terminus is Critical for AHR Reduction
C57BL/6 mice were treated with HDM (25 µg i.n.) for 12d. One day after the last HDM challenge, mice were dosed with
A. SPLUNC1$^{\Delta 44}$ (150 µg i.t.)
B. SPLUNC1$^{\Delta 34}$ (150 µg i.t.)
C. SPLUNC1$^{\Delta 30}$ (150 µg i.t.)
D. SPLUNC1$^{\Delta 21}$ (150 µg i.t.)
E. SPLUNC1^{D29A} (150 µg i.t.)
or PBS and AHR to methacholine was measured 1h later. Black squares: HDM+PBS group; blue triangles: HDM+treatment group. n=9-17 for all groups. P=0.47 in A, P=0.50 in B, P=0.08 in C, P=0.06 in D, ***P<0.0005 in E by MANOVA.

A mSP1hNT

N-term GGLPVPL**D**QTLPLNVNPALPSNPT
- Charged
Neutral EP
α4 T163 Q176 α6
N217

B mSP1hNT + EP

N-term GGLPVPL**D**QTLPLNVNPALPSNPT
- Charged
+ Charged EP
α4 T163K Q176R α6
N217D

C hSP1mNT

N-term QLAGLPLPLGQLPLNVNPALPLSPT
Neutral
+ Charged EP
α4 T163K Q176R α6
N217D

D mSP1ΔNT

Neutral EP
α4 T163 Q176 α6
N217
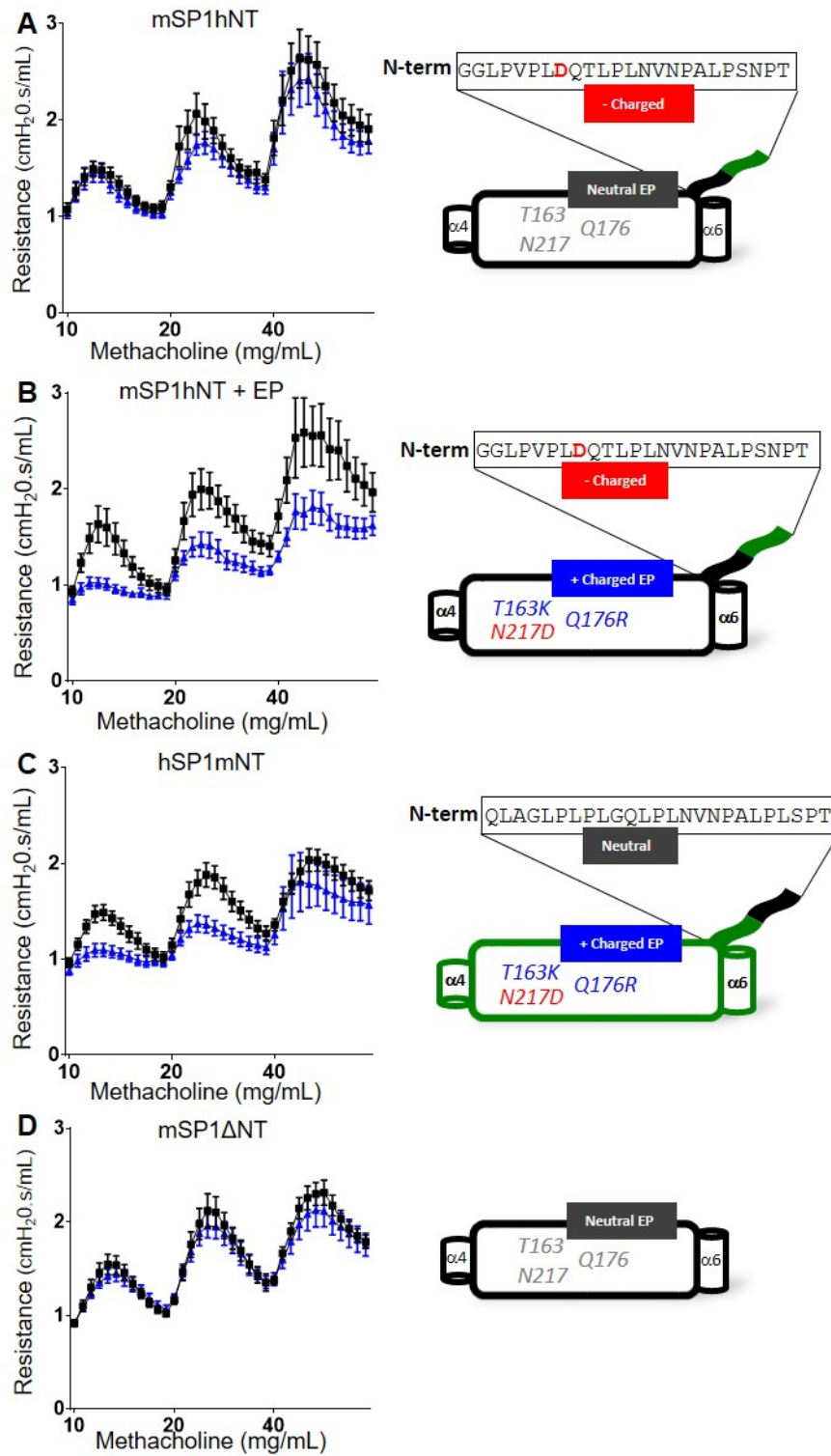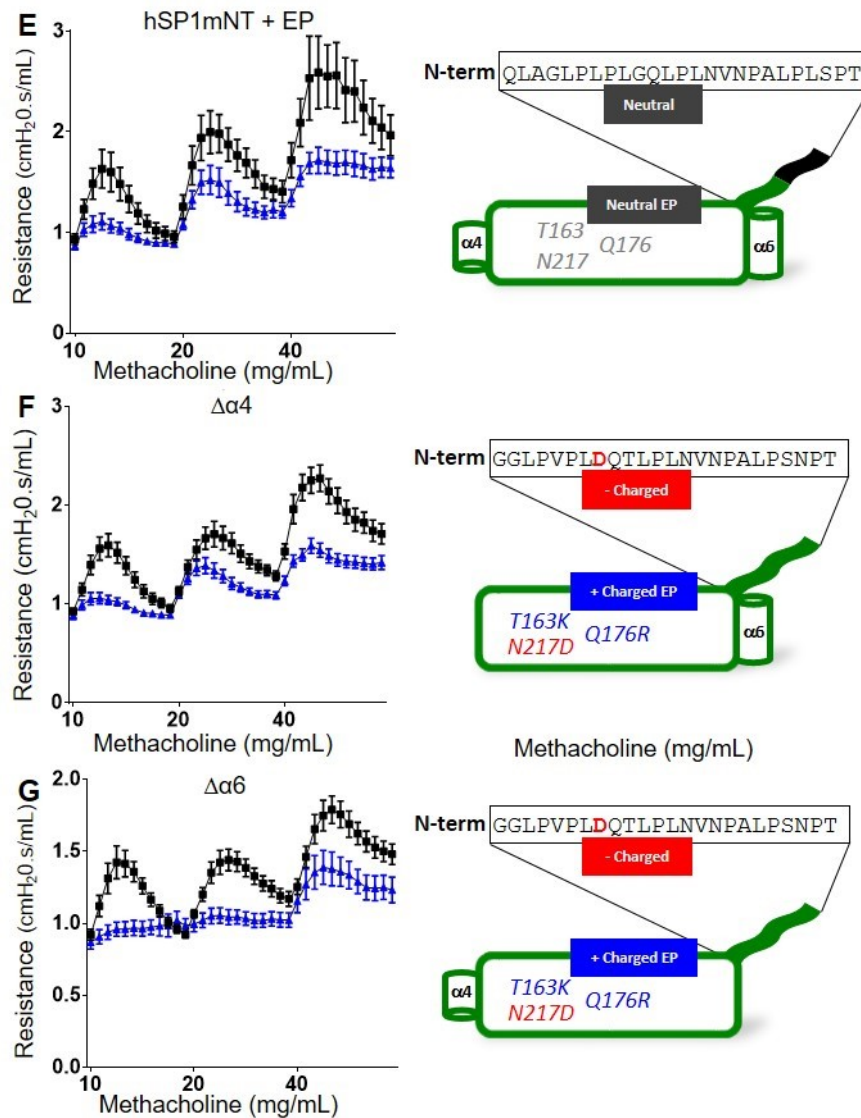
Figure 2.8 Both the N-terminus and Electrostatic Patch are Necessary for AHR-reduction

C57BL/6 mice were treated with HDM (25 µg i.n.) for 12d. One day after the last HDM challenge, mice were dosed with

A. SPLUNC1^{mSP1hNT} (150 µg i.t.)

B. SPLUNC1$^{mSP1hNT+EP}$ (150 µg i.t.)

C. SPLUNC1^{hSP1mNT} (150 µg i.t.)

D. SPLUNC1$^{mSP1\Delta45}$ (150 µg i.t.)

E. SPLUNC1$^{hSP1mNT+EP}$ (150 µg i.t.)

F. SPLUNC1$^{\Delta\alpha4}$ (150 µg i.t.)

G. SPLUNC1$^{\Delta\alpha4}$ (150 µg i.t.)

or PBS and AHR to methacholine was measured 1h later. Black squares: HDM+PBS group; blue triangles: HDM+treatment group. n=8-15 for all groups. $P>0.05$ in A, *$P<0.05$ in B, C, and G, **$P<0.005$ in D and F by MANOVA.
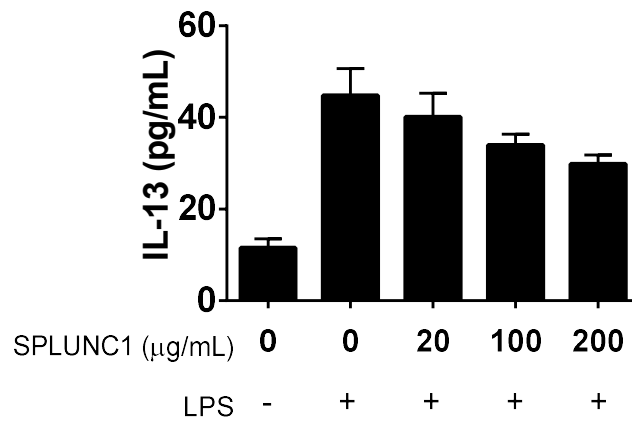
Figure 2.9 SPLUNC1 Exhibits Dose-dependent Reduction of IL-13 Secretion in Mast Cells
Mast cells were treated with PAO1 LPS (0.2 µg/mL) followed by SPLUNC1 (0, 20, 100, and 200 µg/mL) and IL-13 secretion measured in the media 24 h later. Data analyzed by Wilcoxon matched-pairs signed rank test.
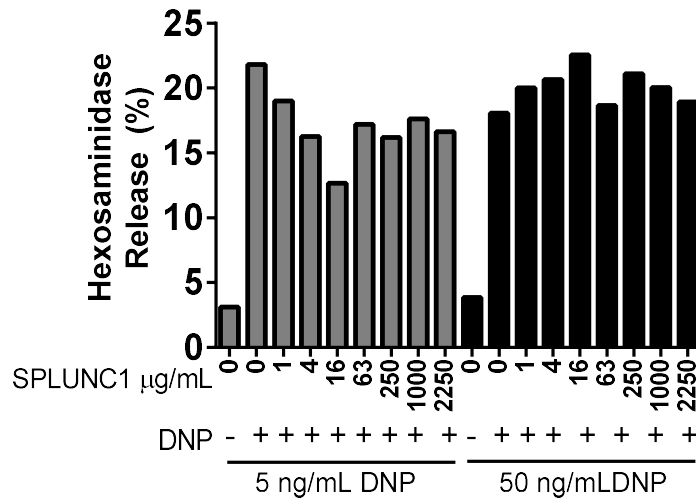
Figure 2.10 SPLUNC1 Does Not Reduce Allergen-induced Mast Cell Degranulation
Mast cells coated in anti-dinitrophenyl albumin (DNP) IgE were treated with SPLUNC1 at 0, 1, 4, 16, 250, 1000 and 2250 µg/mL and stimulated with DNP antigen (5, 50 ng/mL). Percent hexosaminidase degranulation was measured as the hexosaminidase in the media compared to total hexosaminidase present.

CHAPTER 3: SPLUNC1 AND LUNG INFECTION

Introduction

Pneumonia kills approximately 35% of patients with healthcare-associated infections in the US, and the common opportunistic pathogen *Pseudomonas aeruginosa* is the primary Gram-negative infectious bacterium cultured from the lungs of patients with hospital-acquired pneumonia (108, 109). Additionally, *P. aeruginosa* colonizes the airway of 80-85% of patients with cystic fibrosis by age 20, and infects patients with moderate, severe, and exacerbated COPD (110-112). Although antibiotics such as antipseudomonal beta-lactams, aminoglycosides and quinolones are available, the prevalence of antimicrobial resistance is extensive and growing (113-116). Thus, new methods to help eradicate *P. aeruginosa* infections are urgently needed.

SPLUNC1 has been shown to neutralize and combat *P. aeruginosa* both *in vitro* and *in vivo (79, 80, 117-119)*. The published *in vivo* studies include studies performed on knockout and transgenic SPLUNC1 mice. These data suggest that delivering SPLUNC1 to the lungs of infected subjects may be therapeutic. To test this hypothesis, we evaluated the efficacy of recombinant human SPLUNC1 to inhibit bacterial growth in *vivo* using a murine model of *P. aeruginosa* pneumonia. We present data showing that SPLUNC1 may decrease bacterial burden in an acute lung infection model with *P. aeruginosa.*

Materials and Methods

Animals

Female C57BL/6 mice bred in-house or purchased from The Jackson Laboratory were used for these studies. Mice were housed in specific pathogen-free animal facilities with 12 h day and night cycles and provided food and water ad lib.

Protein Preparation

Protein was expressed in BL21-CodonPlus cells and purified as previously described (72). BL21-CodonPlus competent cells were transformed with the expression plasmid of interest and cultured in LB with ampicillin (100 μg/mL), chloramphenicol (34 μg/mL) and antifoam (50 μL) with shaking at 37°C until the OD_{600} reached 0.6. The cells were induced with 0.1 mM IPTG and the temperature was reduced to 18°C for overnight growth. Cell pellets were lysed using sonication in the presence of lysozyme, DNase 1, and protease inhibitor cocktail. Nickel and size exclusion chromatography were used for purification, and tobacco etch virus protease removed the histidine tag from the protein.

Bacterial and SPLUNC1 Dosing

P. aeruginosa PAO1 strain obtained from the Matthew Wolfgang laboratory on the UNC campus was grown overnight in LB broth at 37°C in a sterile round bottom tube that allows for venting. A 0.5 mL aliquot of the overnight culture was diluted into 24.5 mL sterile LB and grown at 37°C in an incubated shaker until log phase growth (OD_{600} 0.6-1.2). The mice were given 0.3 mg SPLUNC1 or PBS i.t. followed by 5×10^6 colony forming units (CFU) of PAO1 in PBS + 1% protease

peptone 1 h later. Mice were kept on heating pads overnight and left lungs were harvested 1 d later.

Lung Harvest

Isoflurane euthanasia was performed and the left lung removed from each mouse. The lung was weighed, minced, and stored in PBS with 7% fetal bovine serum and 2% Triton X-100. Lungs were homogenized with metal beads in a Next Advance Bullet Blender. Lung homogenates were plated on LB agar plates overnight and colonies counted 1 d later.

Statistics

Analyses were performed by Mann-Whitney test. All data represent mean ± SEM.

Study Approval

The IACUC of the University of North Carolina at Chapel Hill approved all experiments.

Results

In order to establish an acute lung infection, we tested a 5 x 10^6 CFU *P. aeruginosa* PAO1 strain dose on C57BL/6 mice bred in-house or purchased from The Jackson Laboratory. Lungs were harvested one day later and CFU/mg of lung determined. We successfully established infection and discovered that The Jackson Laboratory animals had higher CFU/mg lung than in-house bred animals (Figure 3.1).

In order to observe a more dramatic reduction of infection with SPLUNC1 administration, we chose to proceed with The Jackson Laboratory animals which

had a higher CFU/mg lung count. We administered SPLUNC1 1 h prior to infection with PAO1 and discovered a trend toward reduction of CFU/mg of lung with SPLUNC1 administration (Figure 3.2). In summary, we show that we were able to establish a model for the delivery of exogenous SPLUNC1 to an acute lung infection and provide evidence that SPLUNC1 may reduce bacterial load in this model.

Discussion

SPLUNC1 has previously been shown to possess antimicrobial properties, which includes activity against the Gram-negative bacterium, *P. aeruginosa* (79, 80, 117-119). The published studies examined SPLUNC1's activity against *P. aeruginosa* either *in vitro,* or *in vivo* in knockout and transgenic mice. No study to date has reported administering exogenous SPLUNC1 to mice. In order to determine whether SPLUNC1 has the potential to be used therapeutically against *P. aeruginosa* infection, and to determine the molecular basis for SPLUNC1's ability to combat infection *in vivo*, we needed to be able to deliver exogenous SPLUNC1 in acute lung infection. In a model of *P. aeruginosa* pneumonia, we show that the delivery of SPLUNC1 trends towards reduction of bacterial burden in mice. Interestingly, we observed a trend toward increased bacterial burden in purchased mice compared to mice bred in our animal facility when given *P. aeruginosa* alone. One potential explanation for this observation is stress induced by travel and acclimation to a new housing environment. Stress is known to be immunosuppressive through a number of mechanisms. One mechanism is catecholamine-induced elevation of cAMP in immune cells via beta-adrenergic stimulation. Rises in intracellular cAMP inhibits immune cell migration and activation.

Using purchased mice, we were able to show a trend towards lower CFUs in mice treated with rSPLUNC1 (Figure 3.2). However, the variability in the severity of infection was high, thus contributing to the lack of statistical significance. More experiments with larger numbers of mice will be necessary to determine whether this trend that we observed is a real effect of SPLUNC1, and if so, would suggest that administration of rSPLUNC1 be evaluated further for use in treating lung infections such as *P. aeruginosa.*

Future Directions

In this study, we begin to evaluate the ability of exogenous SPLUNC1 administration to reduce bacterial burden *in vivo*. One challenge mentioned above was that the error was higher than desired in the infection group which affected our ability to detect statistical significance from the results obtained. Therefore, future directions include more practice with infectious dosing to normalize the dose delivered for a more consistent result. Once more consistent infections are established, rSPLUNC1 would be tested again and statistical significance evaluated.

Our group has published *in vitro* studies which detail the structural elements critical for SPLUNC1's antimicrobial properties (82). If anti-infective actions of rSPLUNC1 are established *in vivo*, then future directions include testing mutants with reduced surfactant functions as well as bacteriostatic and LPS-binding activity against *P. aeruginosa in vivo.* As we also have mutants with reduced activity against *Burkholderia cenocepacia,* once we obtain consistency with *P. aeruginosa* infection, we intend to establish an infectious model with *B. cenocepacia*. Wildtype and mutant proteins may then be tested for their ability to reduce *B. cenocepacia* infection.

Lastly, we have prepared a mutant protein which has gained function against the Gram-positive *Staphylococcus aureus*. Future directions include testing this mutant protein against *S. aureus* in mice.
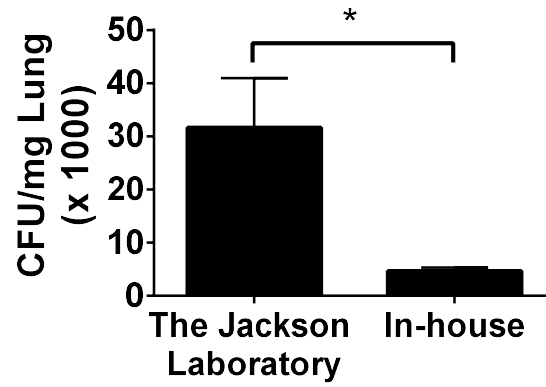
Figure 3.1. The Jackson Laboratory vs. In-house Mouse PAO1 Infection
The Jackson Laboratory and In-house bred C57BL/6 mice were treated with PAO1
(5×10^6 CFU i.t.). One day later, the left lung from each mouse was harvested,
homogenized and plated for colony counts. n = 5 for both groups.
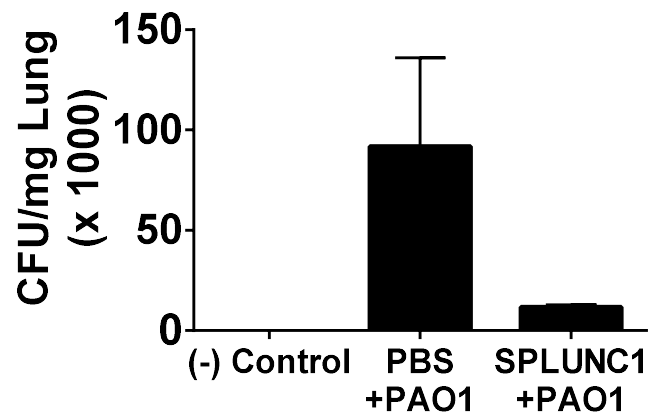
Figure 3.2 SPLUNC1 Reduces CFU Load in PAO1 Lung Infection
In-house bred C57BL/6 mice were treated with SPLUNC1 (0.3 mg i.t.) one hour
before PAO1 infection (5 x 10^6 CFU i.t.). One day later, the left lung from each
mouse was harvested, homogenized and plated for colony counts. n=2 for (-)
Control, n=7 for PBS+PAO1 and n=5 for SPLUNC1+PAO1 groups.

CHAPTER 4: ADMINISTRATION OF SPLUNC1 RESULTS IN SECRETION OF SPLUNC1

Introduction

Studies from our group and others address SPLUNC1's protective properties in the context of allergic inflammation and infection (75, 76, 79-83, 96, 118-122). Chapters 2 and 3 suggest that administration of SPLUNC1 or methods to increase the endogenous SPLUNC1 be investigated for treatment of asthma and lung infection. Overall, we have shown that the local increase in SPLUNC1 concentration in the lung acts protectively in these diseases. While the literature has shown that delivery of drugs such as α1-antitrypsin, beta-2-agonists and glucocorticoids results in an increase in SPLUNC1 concentration, we advocate that exogenous SPLUNC1 delivery should also be considered (78, 95, 119, 123). Here, we show that the delivery of exogenous SPLUNC1 results in feedback releasing endogenous SPLUNC1. We explore the possibility of utilizing this effect to assist in combatting AHR and lung infection as described in chapters 2 and 3.

Materials and Methods

Animals

Female C57BL/6 mice were purchased from The Jackson Laboratory. SPLUNC1$^{-/-}$ mice were a kind gift from Dr. Y. Peter Di and Dr. Paul B. McCray Jr. at the University of Pittsburgh. SPLUNC1$^{-/-}$ mice were backcrossed 3 generations to the C57BL/6 background. SPLUNC1$^{+/-}$ N3 heterozygote breeders were used to

generate SPLUNC1$^{-/-}$ mice and SPLUNC1$^{+/+}$ littermate controls. All mice were housed in specific pathogen-free animal facilities with 12 h day and night cycles and provided food and water ad lib.

HDM-induced Allergic Airway Inflammation

Allergic airway inflammation was induced in 7-13 week old female C57BL/6 mice by i.n. administration of 25 µg of HDM (Greer Laboratories) for 12 days. HDM was administered once a day for 5 consecutive days followed by 2 non-treatment days, and this cycle repeated until 12 total doses of HDM were administered. Isoflurane was used for anesthesia for all i.n. instillations.

SPLUNC1 Dosing and BAL Collection

SPLUNC1 was administered to mice (150 µg i.t.) and BAL performed with 0.8 mL of Hank's Balanced Salt Solution via tracheal cannula 1, 12, 24, 48 and 72 h after administration.

Cell Culture

HBECs were obtained from freshly excised bronchial specimens from normal subjects as previously described (93). HBECs were cultured at air-liquid interface in a modified bronchial epithelial growth medium with 5% CO_2 at 37°C and were used 4-6 weeks after seeding on 12 mm Millicell inserts (EMD Millipore). The apical surface of the HBECs was washed with PBS and a media change performed. SPLUNC1 (50 µg) was administered to the apical surface of the cells at media change and an apical wash in 500 µL PBS was collected 0.5, 1, 1.5, 2, 2.5 and 3 h after media change. SPLUNC1 concentration was determined by ELISA.

SPLUNC1 ELISA

A sandwich ELISA was used to detect SPLUNC1 protein in BAL supernatants and HBEC samples. Primary monoclonal and secondary biotinylated polyclonal anti-SPLUNC1 antibodies (R&D Systems) were used along with Avidin-HRP and TMB substrate (eBioscience).

Western Blot

SDS-PAGE was run on BAL supernatants and proteins transferred to a nitrocellulose membrane. Blots were incubated in primary anti-SPLUNC1 antibody and secondary HRP-conjugated antibodies (R&D Systems). Enhanced chemiluminescent substrate was used for detection.

Protein Preparation

Proteins were expressed in BL21-CodonPlus cells and purified as previously described (72). BL21-CodonPlus competent cells were transformed with the expression plasmid of interest and cultured in LB with ampicillin (100 μg/mL), chloramphenicol (34 μg/mL) and antifoam (50 μL) with shaking at 37°C until the OD_{600} reached 0.6. The cells were induced with 0.1 mM IPTG and the temperature was reduced to 18°C for overnight growth. Cell pellets were lysed using sonication in the presence of lysozyme, DNase 1, and protease inhibitor cocktail. Nickel and size exclusion chromatography were used for purification, and tobacco etch virus protease removed the histidine tag from the protein.

Statistics

All analyses were performed by Mann-Whitney and Wilcoxon matched-pairs signed rank tests as indicated (GraphPad Prism). All data represent mean ± SEM.

Study Approval

The IACUC of the University of North Carolina at Chapel Hill approved all experiments.

Results

Administration of SPLUNC1 Results in Secretion of SPLUNC1

HBECs cultured at air-liquid interface were exposed to wildtype SPLUNC1$^{\Delta 19}$ applied to their apical surface and an apical wash was collected 0.5, 1, 1.5, 2, 2.5 and 3 h after SPLUNC1 delivery. SPLUNC1 levels measured in apical wash samples by ELISA were significantly higher in SPLUNC1-treated as compared to PBS-treated cells (Figure 4.1). To determine whether this would also be the case *in vivo*, we dosed mice with SPLUNC1$^{\Delta 19}$ i.t. and collected BAL at various time points post-administration. We show here that SPLUNC1 levels were more than 90X higher 1 h after SPLUNC1 treatment as compared to PBS treatment taking into account the dose administered (Figure 4.2A). The SPLUNC1 levels remained above baseline for at least 24 h after SPLUNC1 administration. We supplement these data with Western blot results to illustrate that the increase in SPLUNC1 is not simply due to measuring the administered protein in the ELISA. First, we provide evidence that the exogenous recombinant SPLUNC1 administered runs at a smaller size than the endogenous protein (Figure 4.2B). We show this by comparing naïve wildtype mouse BAL which only contains endogenous SPLUNC1 with SPLUNC1$^{-/-}$ mouse BAL and recombinant protein. The endogenous wildtype SPLUNC1 runs at 35 kDa while the recombinant protein runs at 25 kDa. No protein was detected in the SPLUNC1$^{-/-}$ BAL. When we administered SPLUNC1$^{\Delta 19}$ to mice, the endogenous 35

kDa SPLUNC1 protein band intensity increased (Figure 4.2C). These results show both *in vitro* in HBECs and *in vivo* in mice that the administration of SPLUNC1 results in the release of SPLUNC1.

SPLUNC1 Secretion is Specific to SPLUNC1 Administration

To show SPLUNC1 secretion is specific to SPLUNC1 administration, we administered a 25 kDa protein pantothenate kinase (PanK), which was prepared in the laboratory using a similar method as SPLUNC1, and LPS. We show in Figure 4.3 that only the administration of SPLUNC1 resulted in the abundant secretion of SPLUNC1.

SPLUNC1 Concentration Increase is Not Sustained in Allergic Inflammation

Thus far, we have shown that SPLUNC1 administration results in the secretion of SPLUNC1 in normal HBECs and naïve animals and that this effect is specific to the administration of SPLUNC1. We suggest in chapter 2 that SPLUNC1 administration be evaluated for reducing AHR in allergic inflammation. We hypothesized that the release of endogenous SPLUNC1 contributes to the protective effect observed in Mch challenge and sought to evaluate the ability of mice to secrete SPLUNC1 in an allergic inflammatory state. We administered exogenous SPLUNC1$^{\Delta19}$ and SPLUNC1$^{\Delta44}$ to HDM-allergic wildtype mice and measured SPLUNC1 in the BAL at several time points after administration. We report that the administration of wildtype SPLUNC1$^{\Delta19}$ and mutant SPLUNC1$^{\Delta44}$ both result in increased SPLUNC1 in BAL but that the increase appears to be sustained for a shorter duration in HDM-allergic as compared to PBS-dosed mice (Figure 4.4A, 4.4B). Although the administration of SPLUNC1 results in the increased

concentration of SPLUNC1 in the BAL, the duration for the increase is much longer in the PBS-treated than the HDM-allergic mice. Since SPLUNC1 has been shown to be reduced in the lungs during allergic inflammation, we speculate that the allergic inflammatory milieu may diminish the ability to sustain a SPLUNC1 concentration increase through suppression of transcription or reduction of intracellular SPLUNC1 stores.

Discussion

We suggest in chapters 1 and 2 that SPLUNC1 administration or methods to increase endogenous SPLUNC1 be investigated for treatment of asthma and lung infection. We show here that administration of SPLUNC1 itself results in the release of endogenous SPLUNC1 and that this effect is specific to the administration of SPLUNC1. We also note that the increase in SPLUNC1, while sustained in naïve and PBS-treated mice, had a shorter duration of increase in our preliminary studies with allergic inflammation. Thus far, it is unclear whether this effect has therapeutic significance in terms of AHR as SPLUNC1$^{\Delta 19}$ and SPLUNC1$^{\Delta 44}$ had similar preliminary results for the release of endogenous SPLUNC1 while the two proteins had substantially differing results with regard to AHR (Figure 2.4 and 2.7A). More investigation is needed to confirm the preliminary findings and to determine what the results may mean with regard to AHR and infection.

Future Directions

Although we begin in this chapter to unravel the idea that SPLUNC1 administration results in the release of endogenous SPLUNC1, many of the reported results are preliminary findings leaving more experiments left to complete. More

mice should be given SPLUNC1 i.t. and BAL collected at the 1, 12, 24, 48 and 72 h time points (Figure 4.2A). The goals are to increase the n numbers and to see if error may be reduced with more animals especially at the 1 h time point. Additionally, more mouse BAL should be tested by Western blot and the results normalized to obtain a quantitative evaluation of the increase in SPLUNC1 through Western blot. This method could exclude the exogenously administered 25 kDa SPLUNC1 which the ELISA technique is not able to separate out due to the antibody recognition of both endogenous and recombinant proteins.

More mice also need to be given PanK and LPS in order to increase the n numbers to show that only SPLUNC1 administration results in the release of SPLUNC1 (Figure 4.3). Additionally, the drugs which have been published to increase the SPLUNC1 concentration in the lungs should be tested and BAL collected for evaluation of SPLUNC1 content. The goal is to compare the administration of SPLUNC1 with α1-antitrypsin, beta-2-agonists and glucocorticoids. We would like to investigate whether the amount of SPLUNC1 released differs between the various treatments currently shown to increase SPLUNC1. Future directions also include testing these drugs in HDM-allergic mice and the evaluation of AHR.

In figure 4.4, we show that SPLUNC1's ability to reduce AHR does not correlate with release of endogenous SPLUNC1. SPLUNC1$^{\Delta19}$ and SPLUNC1$^{\Delta44}$ administration both result in similar release of SPLUNC1 into the BAL however, SPLUNC1$^{\Delta19}$ reduces AHR while SPLUNC1$^{\Delta44}$ does not. As SPLUNC1 release does not appear to correlate with AHR outcome, we would like to determine whether or

not SPLUNC1 release correlates with an ability to reduce bacterial burden in acute

lung infection.
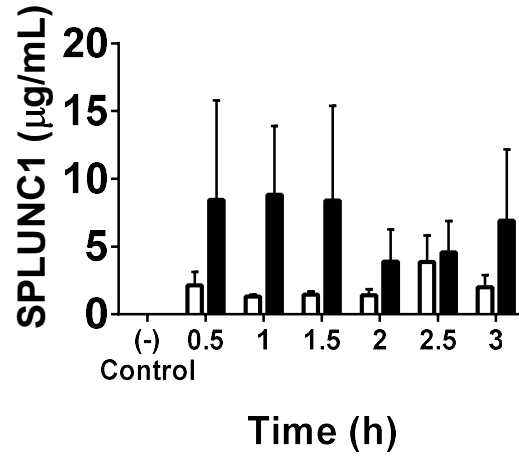
Figure 4.1. SPLUNC1 Increases SPLUNC1 Secretion by HBECs
HBECs were cultured at air-liquid interface for 4-6 weeks and SPLUNC1 levels were measured in apical wash samples 1 h after the addition of apical PBS (white bars) or SPLUNC1 (black bars, 50 μg) by ELISA. n=5. $P<0.05$ between PBS and SPLUNC1 groups by Wilcoxon matched-pairs signed rank test.
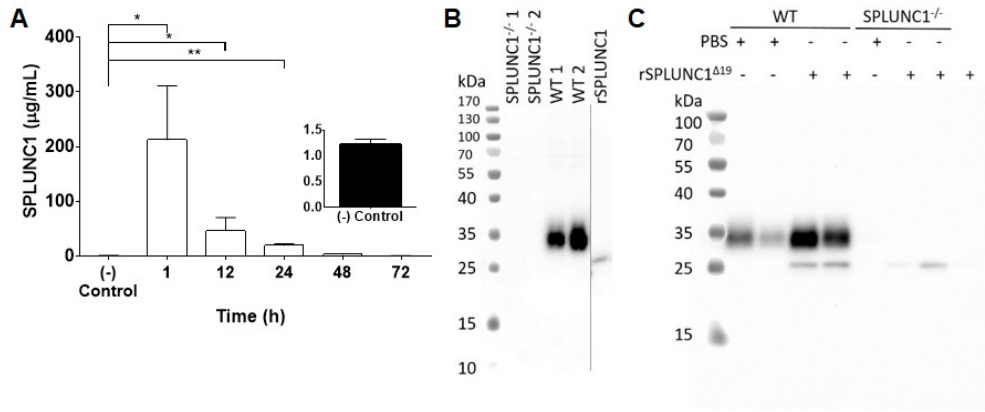
Figure 4.2. SPLUNC1 Administration Increases SPLUNC1 Secretion in Mouse BAL up to 48 Hours Post-administration

A. C57BL/6 mice were dosed with SPLUNC1 (80 µg i.t.) or PBS ((-) Control). BAL was collected at the indicated time points and SPLUNC1 levels measured in the BAL supernatants by ELISA. Inset shows combined (-) Control PBS result from all time points. n=3-4 for all SPLUNC1 groups and n=11 for (-) Control. Mann-Whitney test. *P<0.05 **P<0.005

B. BAL was collected from C57BL/6 (WT) and SPLUNC1$^{-/-}$ mice and SPLUNC1 was evaluated in BAL supernatants by Western blot. Rightmost lane shows recombinant wildtype SPLUNC1$^{\Delta19}$ alone.

C. C57BL/6 and SPLUNC1$^{-/-}$ mice were dosed with SPLUNC1 (80 µg i.t.). BAL was collected 1 h later and SPLUNC1 was evaluated in BAL supernatants by Western blot. Rightmost lane shows recombinant wildtype SPLUNC1$^{\Delta19}$ alone.
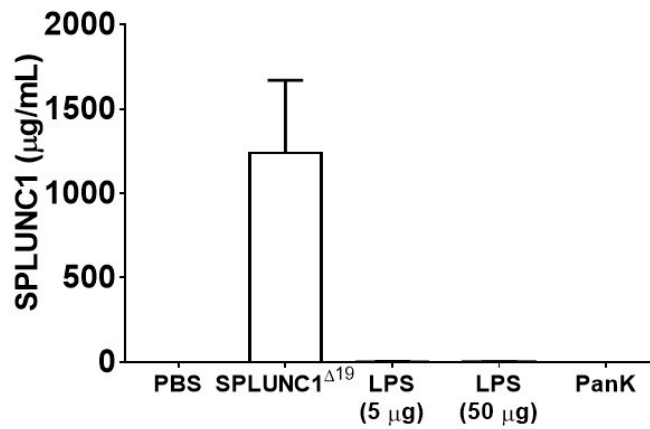
Figure 4.3 SPLUNC1 Secretion in Mice is Specific to SPLUNC1 Administration
C57BL/6 mice were dosed with SPLUNC1 (150 µg i.t.), LPS (5 µg or 50 µg i.t.), an unrelated 25 kDa protein pantothenate kinase (PanK, 150 ug i.t.) or PBS. BAL was collected one hour later and SPLUNC1 levels measured in the BAL supernatants by ELISA. n=2 for all groups except PanK n=1.
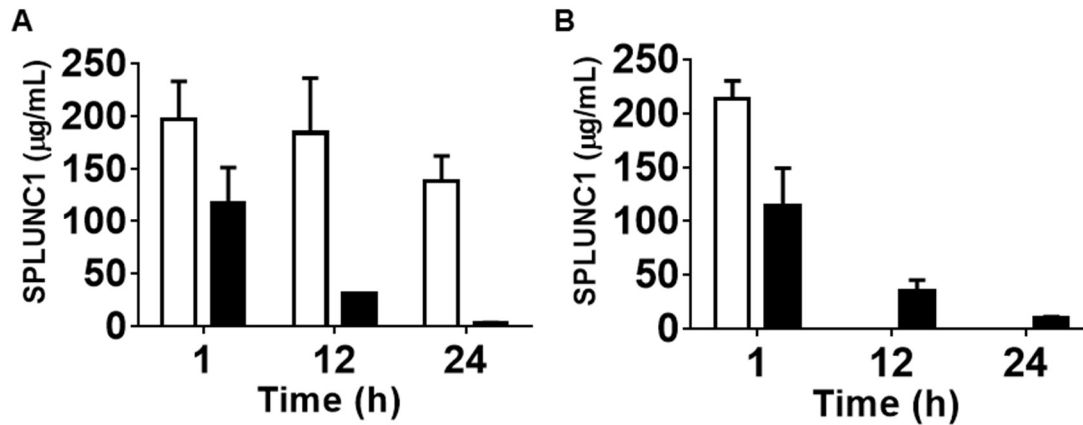
Figure 4.4 SPLUNC1 Secretion Remains High at least 24 h after SPLUNC1 Administration in Controls but Decreases Rapidly in HDM-Allergic Mice.
C57BL/6 mice were treated with HDM (25 µg i.n.) or PBS for 12 d. One day after the last HDM challenge, mice were dosed with

A. Wildtype SPLUNC1$^{\Delta 19}$ (150 µg i.t.) or

B. SPLUNC1$^{\Delta 44}$ (150 µg i.t.)

and BAL was collected at the indicated time points. Open bars: PBS+ SPLUNC1; closed bars: HDM+ SPLUNC1. n=2-3 for all groups except PBS+SPLUNC1 $^{\Delta 44}$ 12 h and 24 h time points were not tested. Mann-Whitney test.

APPENDIX: *P. aeruginosa* Infection and PilY1

Introduction

As introduced in chapter 2, *P. aeruginosa* in a common opportunistic pathogen. This bacterium routinely affects patients with burns, cystic fibrosis, COPD, those who are immunocompromised, and is a primary Gram-negative infectious bacterium in patients with hospital-acquired pneumonia. Critical care infection rates have been reported as greater than 70% in ICU patients remaining at the hospital for longer than 7 days and *P. aeruginosa* is the leading multi-drug resistant (MDR) Gram-negative organism causing ventilator associated pneumonia (VAP) in ICU and burn patients (124-126). With increasing infection rates and incidence of MDR *P. aeruginosa*, we sought to investigate new potential prophylactic agents for this pathogen.

As previously published by our group, *P. aeruginosa* binds to integrins via an RGD (arginine-glycine-aspartic acid) domain on the type IV pilus-associated PilY1 protein (127). This binding is involved in *P. aeruginosa* attachment to human cells, and consequently PilY1 has a role in mediating infection. We sought to elucidate the mechanistic details of *Pseudomonas* infection via the type IV pilus and also investigate potential prophylactic therapies for bacterial pneumonia.

Aims

The aims of the project were: 1) Use x-ray crystallography to extend the previously determined three dimensional structure of *P. aeruginosa* PAK strain PilY1

to amino acids 532-1163 and 191-1163 which both include the critical pilus RGD motif (128). An alternative is to use PA14 strain PilY1 (amino acids 200-1170). Obtaining an extended crystal structure would facilitate a detailed understanding of how the RGD is presented for integrin binding to human cells. These data are expected to lead to the development of specific, directed prophylactic measures for preventing binding and subsequent infection by *P. aeruginosa*. 2) Administer PilY1 to healthy and damaged lung epithelial cells *in vitro* in collaboration with the laboratory of Matthew Wolfgang. Artificial ventilation causes damage to lung epithelial cells and this study would help in the understanding of how pilus interaction with damaged tissue differs from healthy tissue. This aim involves observing PilY1 and integrin co-localization as well as wound healing. In order to visualize the protein, a biotin tag will be added to the protein's C-terminus. 3) Administer cyclic RGD peptides in collaboration with the Wolfgang lab to lung epithelial cells *in vitro* followed by *P. aeruginosa* to evaluate binding and infection rate. The purpose is to occupy cell surface integrins with the cyclic RGD peptide, preventing *P. aeruginosa* from establishing an infection. 4) Administer cyclic RGD peptide *in vivo* to mouse lung using i.n. or i.t. delivery to a clinically relevant burn model in collaboration with the laboratory of Bruce Cairns (UNC School of Medicine). Mice are to receive 20% total body surface area (TBSA) burns to the dorsum which would produce first pro-inflammatory and then anti-inflammatory responses. The mice would be administered cyclic RGD peptide and infected with *P. aeruginosa* i.t. If the cyclic RGD peptide is successful at inhibiting bacterial pneumonia, the mice would be expected exhibit decreased colonization of bacteria in the lungs relative to

infected mice which did not receive cyclic RGD peptide treatment. Taken together, these investigations, which extend from atomic-level to animal studies, would help clarify the mechanism of *Pseudomonas* infection, elucidate how bacteria affect damaged vs. healthy cells, and potentially identify a new structure-based prophylactic approach for the prevention of nosocomial bacterial pneumonia with the potential to overcome current mechanisms of drug resistance.

Preliminary Progress

***P. aeruginosa* PilY1 PAK Strain Amino Acids 532-1163 and 191-1163 and PA14 Strain Amino Acids 200-1170 Cloning**

PAK PilY1 532-1163 corresponding DNA was cloned into a ligation independent cloning vector with a 6X histidine (His$_6$) tag. PAK PilY1 191-1163 DNA was cloned into ligation independent cloning vectors with His$_6$ and maltose binding protein (MBP) tags. The PilY 191-1163 MBP vector was transformed into BL21-Gold and BL21-Origami™ cells and purification was performed using the BL21-Gold cells which resulted in the elution of the protein in the void volume. It is possible that the protein aggregated due to cysteines forming inappropriate disulfide bonds.

PA14 200-1170 DNA was cloned into a ligation independent cloning vector with a His$_6$ tag and transformed into BL21-AI™, BL21-Gold and BL21-Origami™ 2 cells. An expression test revealed that BL21-Gold cells had slight but visible induction of protein in the soluble fraction after one and two hours of induction. The BL21-AI™ soluble fraction did not have visible protein. Sequence confirmation revealed a C477G and a silent mutation at amino acid 645.

PAK PilY1 532-1163 Cloning into Avidity Vector for Biotinylation

PAK PilY1 532-1163 DNA was successfully cloned into the pAC6 AviTag™ vector and the sequence confirmed.

Administration of Cyclic RGD Peptides to Mice

An IACUC amendment was submitted and approved for the administration of cyclic RGD peptides to mice. A 20% TBSA burn is to be given to the dorsum of mice followed by resuscitation and analgesia. One, three, seven and fourteen days after burn procedure, between 5×10^4 and 5×10^9 of RGD molecules are to be delivered to the mice followed by between 1×10^3 and 1×10^6 CFU of *P. aeruginosa*. After one, two and three days the mice will be euthanized and blood, lung and BAL collected from each mouse for evaluation of bacterial recovery.

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