SPLUNC1 Deficiency Enhances Airway Eosinophilic Inflammation in Mice

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Short palate, lung and nasal epithelium clone 1 (SPLUNC1) is enriched in normal airway lining fluid, but is significantly reduced in airway epithelium exposed to a Th2 cytokine milieu. The role of SPLUNC1 in modulating airway allergic inflammation (e.g., eosinophils) remains unknown. We used SPLUNC1 knockout (KO) and littermate wild-type (C57BL/6 background) mice and recombinant SPLUNC1 protein to determine the impact of SPLUNC1 on airway allergic/eosinophilic inflammation, and to investigate the underlying mechanisms. An acute ovalbumin (OVA) sensitization and challenge protocol was used to induce murine airway allergic inflammation (e.g., eosinophils, eotaxin-2, and Th2 cytokines). Our results showed that SPLUNC1 in the bronchoalveolar lavage fluid of OVA-challenged wild-type mice was significantly reduced ($P < 0.05$), which was negatively correlated with levels of lung eosinophilic inflammation. Moreover, SPLUNC1 KO mice demonstrated significantly higher numbers of eosinophils in the lung after OVA challenges than did wild-type mice. Alveolar macrophages isolated from OVA-challenged SPLUNC1 KO versus wild-type mice had higher concentrations of baseline eotaxin-2 that was amplified by LPS (a known risk factor for exacerbating asthma). Human recombinant SPLUNC1 protein was applied to alveolar macrophages to study the regulation of eotaxin-2 in the context of Th2 cytokine and LPS stimulation. Recombinant SPLUNC1 protein attenuated LPS-induced eotaxin-2 production in Th2 cytokine-pretreated murine macrophages. These findings demonstrate that SPLUNC1 inhibits airway eosinophilic inflammation in allergic mice, in part by reducing eotaxin-2 production in alveolar macrophages.

Keywords: SPLUNC1; asthma; alveolar macrophage; Th2 cytokines; eotaxin-2

Asthma is a complex inflammatory syndrome afflicting millions of people worldwide (1). Persistent airway inflammation and acute exacerbations, the hallmarks of severe asthma, pose significant challenges to healthcare. Although the underlying mechanisms of asthma exacerbations are unclear, eosinophils appear to play a major role at least in a subset of patients (2). Clinical studies demonstrated strong correlations between enhanced endotoxin (LPS) responsiveness and disease severity in asthma (3–5). LPS, a ubiquitous component of dust and air pollution, has been proposed to activate eosinophils in the bronchial tissues of patients with asthma, in some cases provoking inflammatory processes (6). Alveolar macrophages, comprising one of the first lines in lung host defense, play a central role in the maintenance of lung homeostasis, although an excessive

CLINICAL RELEVANCE

Th2 cytokines significantly reduce short palate, lung and nasal epithelium clone 1 (SPLUNC1) expression in human airway epithelial cells. However, whether reduced SPLUNC1 in turn modulates airway allergic inflammation is unknown. This study demonstrates that allergic inflammation reduces SPLUNC1 in murine lungs. SPLUNC1 deficiency enhances airway eosinophilic inflammation in ovalbumin-challenged mice, in part by increasing eotaxin-2 production in alveolar macrophages. Restoring SPLUNC1 concentrations in allergic airways may reduce asthma severity by reducing eosinophilic inflammation.

immune response from alveolar macrophages can exaggerate airway inflammation (7). Previous studies showed that alveolar macrophages isolated from subjects with asthma are hyperresponsive to LPS, compared with alveolar macrophages from healthy control subjects (8), but the underlying mechanisms remain unclear.

Short palate, lung and nasal epithelium clone 1 (SPLUNC1) is an abundant protein present in the airway lining fluid of healthy humans and mice, and is mainly produced by large airway epithelia and submucosal glands. SPLUNC1 was predicted to exert host defense functions, based on its sequence homology with other known antimicrobial proteins such as bactericidal permeability increasing protein and LPS-binding protein (LBP) (9). We previously demonstrated that the Th2 cytokine IL-13 significantly reduces SPLUNC1 expression and secretion from well-differentiated human primary airway epithelial cells, rendering a host susceptible to bacterial infections (10). Recent studies by our group and others demonstrated that SPLUNC1 is critical for clearing respiratory pathogens such as *Mycoplasma pneumoniae* and *Pseudomonas aeruginosa* from murine lungs (10–12). In addition to its antimicrobial function, we have shown that murine recombinant SPLUNC1 protein inhibits the Toll-like receptor 2 (TLR2) agonist-induced production of IL-8 in human airway epithelial cells (10). These observations led us to hypothesize that SPLUNC1 has the potential to affect airway allergic inflammation. The present study investigated whether SPLUNC1 is important in regulating airway eosinophilic inflammation by using our recently generated SPLUNC1 knockout (KO) murine model and recombinant SPLUNC1 protein.

MATERIALS AND METHODS

Murine Model of Ovalbumin-Induced Airway Allergic Inflammation

The SPLUNC1 KO mice generated in our laboratory and littermate wild-type mice (C57BL/6 background) were bred under pathogen-free housing conditions (11). All mice (6–8 weeks old) were sensitized by an intraperitoneal injection of 20 μ g ovalbumin (OVA; Sigma-

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Aldrich, St. Louis, MO) emulsified in aluminum hydroxide (Imject Alum; Thermo Scientific, Rockford, IL) on Day 0 and Day 7, and challenged with aerosolized 1% OVA or saline for 30 minutes each day for 3 consecutive days (on Days 14, 15, and 16). Mice were killed 24 hours after the last challenge (13). Bronchoalveolar lavage (BAL) was performed with 1 ml saline. All of the experimental protocols were approved by the Institutional Animal Care and Use Committee at National Jewish Health.

Macrophage Cell Culture

Murine primary alveolar macrophages (AMs) were obtained by lavaging murine lungs. AMs were isolated by adherence and cultured in RPMI-1640 medium supplemented with 10% FBS and penicillin/streptomycin. Because of the limited numbers of murine primary AMs, a murine macrophage cell line Raw 264.7 was similarly cultured to study the mechanisms of eotaxin-2 regulation in the presence or absence of Th2 cytokines and LPS. In some experiments, cells were pretreated for 1 hour with human recombinant SPLUNC1 protein before LPS stimulation. At the indicated time points, cell samples were collected for protein and mRNA determination.

Quantitative Real-Time PCR

An equal amount of RNA (1 μ g) was used to prepare cDNA. Murine SPLUNC1, MUC5AC, and eotaxin-2 (CCL24) mRNA concentrations were determined by quantitative real-time PCR, and normalized to the housekeeping gene 18S rRNA. The comparative threshold cycle method was applied to determine the fold change in target gene mRNA expression after different treatments, compared with untreated control conditions.

ELISA

Eotaxin-2 ELISA (R&D Systems, Minneapolis, MN) was used to quantify eotaxin-2 in murine BAL fluid and lung tissues. Lung tissue eotaxin-2 was normalized with total proteins in the lung homogenates.

Western Blot Analysis

Western blot analysis was performed to examine SPLUNC1 and c-Jun. Primary antibodies against SPLUNC1 (R&D Systems), phospho-c-Jun (Cell Signaling Technology, Danvers, MA), c-Jun (Abcam, Cambridge, MA), and a corresponding secondary horseradish peroxidase-conjugated

antibody with an enhanced chemiluminescent substrate were used to detect respective proteins.

Generation of Human Recombinant SPLUNC1 Protein in Mammalian Cells

The full-length cDNA of human SPLUNC1 with a histidine (His) tag at the C-terminus was cloned into a pRK7-Neomycin (pRK7-Neo) expression vector, and the protein was expressed in the human embryonic kidney-293 (HEK293) cell line. HEK293 cells transfected with SPLUNC1 cDNA secreted His-tagged SPLUNC1 protein that was purified by affinity column chromatography, using a Ni-NTA column (Qiagen, Valencia, CA). High concentrations of salts from the elution buffer were removed using 10-kD spin filter columns (Amersham, Piscataway, NJ). Purified recombinant SPLUNC1 (rSPLUNC1) protein (> 95%) was stored at -80°C in sterile PBS with 10% glycerol solution. All reagents used for protein purification were prepared in endotoxin-free water.

Statistical Analyses

Statistical significance was determined by applying an unpaired Student *t* test or ANOVA, followed by the Tukey *post hoc* test among various treatment groups. A correlation analysis was performed by using the Pearson correlation coefficient.

RESULTS

Reduced SPLUNC1 in OVA-Challenged, Wild-Type Murine Lungs

We previously showed that the Th2 cytokine IL-13 significantly reduced SPLUNC1 expression in cultured human airway epithelial cells. We also demonstrated that OVA-induced allergic inflammation in BALB/c mice significantly reduced SPLUNC1 mRNA expression in airway epithelium (10). However, a systemic investigation of reduced SPLUNC1 in allergic lungs and of its impact on airway inflammation was not previously reported, to the best of our knowledge. Using an acute OVA challenge protocol, we reproduced our previous observations by showing that allergic inflammation down-regulated SPLUNC1 concentrations in the BAL fluid of wild-type (C57BL/6

Figure 1. Airway allergic inflammation reduces SPLUNC1 in wild-type murine lungs. (A) A schematic of ovalbumin (OVA) sensitizations and challenges to induce airway allergic inflammation in mice. Adult (6–8-week-old) C57BL/6 mice were sensitized intraperitoneally (i.p.) with OVA, challenged with aerosolized OVA or saline (Sal), and killed 24 hours after the last challenge. (B) Above, a representative Western blot shows reduced SPLUNC1 protein in the bronchoalveolar lavage (BAL) fluid (20 μ l/lane) of OVA-challenged wild-type (WT) mice. Below, Ponceau S staining of the nitrocellulose membrane that was used for SPLUNC1 immunoblotting in BAL fluid. The major 67-kD protein (albumin) band was used to normalize SPLUNC1 protein in BAL fluid. (C) Densitometric analysis of SPLUNC1 protein in BAL fluid samples, which was normalized to the 67-kD protein band, as detected by Ponceau S staining (51). (D) Scatterplot shows a negative correlation between BAL SPLUNC1 and eosinophils in both saline-challenged and OVA-challenged WT mice. Data are expressed as mean $\pm$ SEM ($n = 7$ –8 mice/group). * $P < 0.05$. AU, arbitrary units.

background) mice (Figures 1A–1C). Moreover, we found a significant inverse correlation between SPLUNC1 protein concentrations and eosinophils in BAL fluid (Figure 1D).

SPLUNC1 Deficiency Exaggerates OVA-Induced Murine Airway Eosinophilic Inflammation

SPLUNC1 KO mice and littermate wild-type (WT) mice were sensitized and challenged with OVA, as described in Figure 1A. OVA-sensitized/challenged SPLUNC1 KO mice, compared with WT mice, demonstrated increased airway inflammation, as indicated by increased numbers of leukocytes, including eosinophils in the lung (Figures 2A and 2B). OVA challenges in SPLUNC1 KO mice also increased lung MUC5AC mRNA expression, a hallmark of airway allergic inflammation (Figure 2C).

Figure 2. SPLUNC1 deficiency enhances airway eosinophilic inflammation. SPLUNC1 knockout (KO) and wild-type (WT) mice were sensitized and challenged with ovalbumin (OVA) or saline (Sal), as described in Figure 1A. Airway allergic inflammation was determined by quantifying BAL total leukocytes (A), eosinophils (B), and whole-lung MUC5AC mRNA (C). Data are expressed as means $\pm$ SEM ($n = 7$ –20 mice/group). * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

SPLUNC1 Deficiency Increases Murine Lung Eotaxin-2 Production

Because OVA-sensitized/challenged SPLUNC1 KO mice exhibited an increase in lung eosinophils, we studied the inflammatory mediators responsible for enhanced eosinophil recruitment into the lung. Previous studies demonstrated that eotaxins, including 1 and 2, display eosinophil chemotactic properties (14). Eotaxin-2 usually peaks at 24 hours after an allergen challenge, and has been considered the major eotaxin isoform in recruiting eosinophils into murine airways (15–18). Because our study outcomes were examined 24 hours after the last OVA challenge, we determined eotaxin-2 production. OVA challenges in SPLUNC1 KO versus WT mice significantly increased the eotaxin-2 protein in BAL and whole-lung tissue (Figures 3A and 3B). Eotaxin-2 protein concentrations were positively correlated with airway eosinophilia in both strains of mice (Figures 3C and 3D).

Th2 Cytokines Enhance LPS-Induced Eotaxin-2 Production in Alveolar Macrophages

Alveolar macrophages are one of the major cellular sources of eotaxin-2 in the lung (19). Alveolar macrophages were isolated from saline-challenged and OVA-challenged WT and SPLUNC1 KO mice and cultured *ex vivo* for 24 hours, with and without LPS (10 ng/ml) stimulation. Eotaxin-2 production in the alveolar macrophages of saline-challenged WT and SPLUNC1 KO mice did not differ (data not shown). However, alveolar macrophages from OVA-sensitized/challenged SPLUNC1 KO mice produced significantly higher concentrations of eotaxin-2 at baseline compared with those from OVA-sensitized/challenged WT mice (Figure 4A). Eotaxin-2 production in the alveolar macrophages of OVA-challenged SPLUNC1 KO mice was further enhanced by LPS stimulation (Figure 4A).

Previous studies demonstrated that alveolar macrophages isolated from subjects with asthma are hyperresponsive to LPS, because asthmatic macrophages produce higher concentrations of proinflammatory mediators than do normal cells (8). The *in vitro* stimulation of murine alveolar macrophages and human peripheral blood-derived macrophages with IL-4 and/or IL-13 has been shown to increase concentrations of eotaxin-2 (20, 21). We isolated alveolar macrophages from OVA-naïve WT mice and pretreated them with murine IL-4 and IL-13 (10 ng/ml each) for 2 hours. These cells were then stimulated with LPS (10 ng/ml) or medium for 24 hours. In line with previous reports (20, 21), we observed an increase of eotaxin-2 production in murine primary alveolar macrophages after IL-4 and IL-13 treatment (Figure 4B). Moreover, LPS stimulation in IL-4/IL-13-pretreated murine primary alveolar macrophages further increased eotaxin-2 production (Figure 4B). Because of the limitations of primary murine alveolar macrophages, we used a murine macrophage cell line Raw 264.7 to study the mechanisms of SPLUNC1 immunomodulatory function. Similar to our observations in murine primary alveolar macrophages, IL-4/IL-13 enhanced eotaxin-2 production in Raw 264.7 cells, and this production was significantly enhanced by LPS treatment (Figures 4C and 4D). Thus, Raw 264.7 cells represent a valid model for studying the molecular mechanisms of eotaxin-2 regulation.

SPLUNC1 Attenuates IL-4/IL-13 and LPS-Induced Eotaxin-2 Production in Macrophages

Human and murine SPLUNC1 proteins share approximately 70% homology, and they similarly inhibited *Mycoplasma pneumoniae* growth (10, 22). Therefore, we used a mammalian protein expression system to generate endotoxin-free human

Figure 3. SPLUNC1 deficiency enhances eotaxin-2 production in mice. SPLUNC1 knockout (KO) and wild-type (WT) mice were sensitized and challenged with ovalbumin (OVA) or saline (Sal), as described in Figure 1A. Eotaxin-2 protein concentrations in BAL fluid (A) and whole-lung tissue (B) were measured by ELISA. (C and D) Scatterplot graphs show a positive correlation between BAL or lung eotaxin-2 and BAL eosinophils, respectively. Data are expressed as means $\pm$ SEM ($n = 8$ –26 mice/group). * $P < 0.05$. ** $P < 0.01$.

rSPLUNC1 protein (Figures 5A and 5B). The addition of rSPLUNC1 to murine IL-4/IL-13-pretreated Raw 264.7 cells dose-dependently reduced eotaxin-2 production after LPS stimulation (Figures 5C and 5D).

Analysis of the 5' flanking region (–2 kb) of the murine eotaxin-2 gene (GenBank accession number NC_000071.5), using the TRANSFAC database (<http://www.cbrc.jp/research/db/TFSEARCH.html>), revealed the presence of two distal activator protein 1 (AP-1) [TGA(C/G)TCA] binding motifs in the promoter region. IL-4/IL-13 and LPS were shown to activate mitogen-activated protein kinase (MAPK) signaling in various types of cells, including macrophages (23–27). AP-1

has been described as a downstream target of MAPK signaling in different cell types (28). We studied the role of c-Jun activation, a part of the AP-1 transcription factor complex, in murine macrophage eotaxin-2 production. Our results demonstrated that the combination of IL-4/IL-13 and LPS significantly increased c-Jun (AP-1) activation in Raw 264.7 cells (Figures 6A and 6B). The addition of rSPLUNC1 protein 1 hour before LPS stimulation significantly reduced c-Jun activation at 24 hours (Figures 6A and 6B). These results suggest that the inhibition of c-Jun activation by SPLUNC1 may be involved in its inhibition of eotaxin-2 production in murine macrophages.

Figure 4. Eotaxin-2 production in murine macrophages. (A) Alveolar macrophages were isolated from ovalbumin (OVA)-sensitized/challenged SPLUNC1 knockout (KO) and wild-type (WT) mice, and seeded onto a 48-well tissue culture plate (4×10^4 cells/well). Cells were stimulated with LPS (10 ng/ml) or medium (control) for 24 hours, and eotaxin-2 secretion was determined by ELISA. (B) Alveolar macrophages collected from OVA-naïve WT mice were seeded onto a 48-well tissue culture plate (10×10^4 cells/well). Cells were treated with BSA (control) or murine recombinant IL-4/IL-13 (each at 10 ng/ml) for 2 hours, and then stimulated with LPS (10 ng/ml) for 24 hours, and eotaxin-2 secretion was determined by ELISA. (C and D) Raw 264.7 cells were seeded (5×10^5 cells/well) onto a 48-well tissue culture plate. The next day, they were treated with BSA (control) or murine IL-4/IL-13 (10 ng/ml each) for 2 hours, and then stimulated with LPS (10 ng/ml) for 24 hours, and eotaxin-2 protein (C) and mRNA (D) were measured using ELISA and real-time PCR, respectively. Data are expressed as means $\pm$ SEM ($n = 3$ –4 replicates). * $P < 0.05$. *** $P < 0.001$.

Figure 5. SPLUNC1 reduces eotaxin-2 in murine macrophages. (A) Full-length human SPLUNC1 cDNA with histidine (His) tag at the C-terminus (C-ter) was cloned into a pRK7-neo-vector and expressed in human embryonic kidney-293 (HEK293) cells by transient transfection. (B) His-tagged SPLUNC1 protein was purified by affinity chromatography. Western blotting was used to verify recombinant SPLUNC1 (rSPLUNC1) protein secretion and purity. (C and D) Raw 264.7 cells were seeded (5×10^5 cells/well) onto a 48-well tissue culture plate. The next day, cells were pretreated with BSA (control) or murine IL-4/IL-13 (10 ng/ml each) for 1 hour. Thereafter, cells were treated with rSPLUNC1 at different concentrations or with the control solution purified from HEK293 cells transfected with an empty plasmid vector. One hour later, cells were stimulated with LPS (10 ng/ml)

or medium alone for 24 hours. Murine eotaxin-2 protein (C) and mRNA (D) were measured using ELISA and real-time PCR, respectively. Data are expressed as means $\pm$ SEM ($n = 3-4$ replicates). * $P < 0.05$. *** $P < 0.001$. N-ter, N-terminal.

DISCUSSION

In the present study, we demonstrate that SPLUNC1 concentrations are reduced in allergic murine lungs. Moreover, we find that SPLUNC1 deficiency significantly enhances airway eosinophilic inflammation along with increased eotaxin-2 production in the lung tissue, as well as in alveolar macrophages. Collectively, and for the first time to the best of our knowledge, our data suggest an inhibitory role of SPLUNC1 in eosinophil influx into allergic airways. Our findings may provide a novel approach to attenuating eosinophilic inflammation in patients with asthma.

Allergic asthma is characterized by variable airway obstruction, bronchial hyperresponsiveness, mucus hypersecretion, and airway inflammation. Airway inflammation is manifested by the infiltration of inflammatory cells, including eosinophils and Th2 cells, into the lungs. Th2 cytokines (particularly IL-13 and IL-4) regulate both innate and adaptive immunity (29-32). For example, we have shown that Th2 cytokines dampen airway innate immunity by reducing TLR2 signaling and SPLUNC1 expression in the airways (10, 33, 34). SPLUNC1 is a newly identified host defense protein that was shown to protect the host from infections of respiratory pathogens, including *Mycoplasma pneumoniae* and *Pseudomonas aeruginosa* (10-12). Our present study has significantly advanced previous work by showing that allergic inflammation-mediated or Th2 cytokine-mediated SPLUNC1 reduction exacerbates airway eosinophilic inflammation in OVA-challenged mice. First, after OVA challenges, SPLUNC1 KO mice demonstrated increased numbers of eosinophils in the airways, compared with WT mice. Second, we observed a significant negative correlation between SPLUNC1 and airway eosinophils in WT mice upon OVA and saline challenges. However, the correlation of SPLUNC1 and eosinophils in OVA-challenged mice alone was not as strong as that in both saline-challenged and OVA-challenged WT mice ($r^2 = 0.17$ versus $r^2 = 0.36$), which could be attributable to the relatively small sample size ($n = 8$) for OVA-challenged mice alone, and to the fact that eosinophilic inflammation can be regulated by additional mediators such as IL-5. Because the treatment of eosinophilic inflammation with anti-IL-5 antibody alone has not provided significant protection against late asthmatic

responses and acute exacerbations in humans with asthma (35, 36), SPLUNC1 therapy may offer a novel approach to attenuating eosinophilic inflammation with additional antimicrobial

Figure 6. SPLUNC1 inhibits c-Jun activation in macrophages stimulated with Th2 cytokines and LPS. Raw 264.7 cells were seeded onto a 48-well tissue culture plate (5×10^5 cells/well) and treated with BSA (control) or murine IL-4/IL-13 (each at 10 ng/ml). One hour later, rSPLUNC1 (10 μg/ml) protein or control solution was added to the cells. After another hour, cells were stimulated with LPS (10 ng/ml) for 1 hour and 24 hours to determine c-Jun activation by Western blotting. (A) Representative image of phosphorylated (p)-c-Jun (ser63) and total c-Jun Western blot. (B) Densitometric analysis of c-Jun activation, as determined by the ratio of p-c-Jun and total c-Jun proteins. Data are expressed as means $\pm$ SEM ($n = 3-5$ replicates). ** $P < 0.01$. *** $P < 0.001$.

benefits, because respiratory infections are often associated with acute exacerbations in asthma (37, 38).

Eotaxin-2 has been established as a potent eosinophil chemo-attractant in regulating airway inflammation in mice (17, 39). We observed a significant increase of eotaxin-2 production in OVA-sensitized/challenged SPLUNC1 KO mice, suggesting that SPLUNC1 may prevent airway eosinophilic inflammation by reducing eotaxin-2 production. Moreover, we observed an increase of Th2 cytokine IL-13 in OVA-challenged SPLUNC1 KO murine lungs. However, the concentration of Th1 cytokine IFN- γ was not significantly different between OVA-challenged SPLUNC1 KO and WT mice.

One of the key questions in the present study involved the way that SPLUNC1 suppresses eosinophilic inflammation in allergic lungs. To address this question, we focused on alveolar macrophage eotaxin-2 production because, for therapeutic purposes, managing innate airway cell functions, including alveolar macrophages, is relatively easier than managing adaptive immune cells such as T cells. Moreover, Pope and colleagues suggested a predominant role of eotaxin-2 in causing luminal/airway eosinophilia in mice (18). Alveolar macrophages have been described as one of the major cellular sources of eotaxin-2 in the airways (19). Th2 cytokine treatment alone has been shown to enhance eotaxin-2 production in alveolar macrophages and macrophages derived from human peripheral blood monocytes (20, 21). Our results show that alveolar macrophages pre-exposed to an allergic milieu or Th2 cytokines become hyperresponsive to LPS, because eotaxin-2 was synergistically increased in these cells. This synergistic increase in eotaxin-2 production may be attributable to LPS-induced proinflammatory cytokines such as TNF- α , IL-1 β , and TGF- β , which in collaboration with Th2 cytokines have been shown to enhance eotaxin production synergistically in fibroblasts, smooth muscle cells, and epithelial cells (40, 41). However, further studies are needed to confirm these mechanisms in macrophages.

Figure 7. A schematic illustrates how SPLUNC1 may attenuate eotaxin-2 production in LPS-stimulated macrophages that are pre-exposed to IL-4 and IL-13 or an allergic airway milieu. AP-1, activator protein 1; TLR4, Toll-like receptor-4.

By using SPLUNC1 KO mice, we demonstrate, for the first time to the best of our knowledge, that SPLUNC1 inhibits eotaxin-2 production in OVA-challenged mice, thus reducing airway allergic inflammation, because the lack of SPLUNC1 *in vivo* increased lung tissue as well as macrophage eotaxin-2 production. Furthermore, SPLUNC1 protein directly reduced eotaxin-2 production in macrophages that had been pre-exposed to IL-4/IL-13, and then stimulated with LPS (Figures 5B and 5C). Our data support a previous study showing reduced concentrations of airway eosinophils in human SPLUNC1-overexpressing mice with OVA challenges (42). However, in that study, the mechanisms of eosinophil regulation by SPLUNC1 were not examined. Our results provide a plausible mechanism for the regulation by SPLUNC1 of airway allergic inflammation. Therefore, SPLUNC1 has potential therapeutic applications in reducing airway inflammation and recurrent bacterial infections in Th2 cytokine-exposed airways.

It remains unclear how SPLUNC1 down-regulates eotaxin-2 production in macrophages stimulated with Th2 cytokines and LPS. SPLUNC1 may bind to LPS (43, 44), thus reducing LPS binding to TLR4 or its coreceptors (Figure 7). One of the signatures of TLR4 signaling activation after LPS stimulation involves the activation of c-Jun, a member of the AP-1 transcription factor family, which can be activated by MAPKs (23, 28, 45). Interestingly, Th2 cytokines also activate c-Jun (46–48). The activation of c-Jun, as determined by its phosphorylation at different sites, has been shown to regulate c-Jun binding to gene promoters, c-Jun protein stability, and its dimerization with other AP-1 family members (49, 50). Analysis of 5' flanking region of the murine eotaxin-2 gene revealed two putative AP-1 binding motifs in the distal region of the murine eotaxin-2 promoter. Our results show that LPS exposure to IL-4/IL-13-pretreated murine macrophages significantly enhanced c-Jun activation, which was suppressed by recombinant SPLUNC1 protein (Figures 6A and 6B). Whether the SPLUNC1-mediated down-regulation of c-Jun activation leads to the transcriptional repression of eotaxin-2 warrants further investigation. Together, our data suggest that SPLUNC1 attenuates eosinophilic inflammation in part by reducing eotaxin-2 production in alveolar macrophages.

The present study contains several limitations. First, because multiple transcription factors may control eotaxin-2 production, the role of other transcription factors warrants future studies. Second, the way in which SPLUNC1 modulates Th2 cytokine signaling in the absence or presence of LPS or bacteria remains to be clarified. Third, because other types of cells in the lung, such as airway epithelial cells, also produce eotaxin-2, the contribution of these cells versus macrophages to lung eotaxin-2 production in its entirety needs to be addressed. Lastly, the role of SPLUNC1 in regulating airway eosinophilic inflammation can be further investigated by using human SPLUNC1 transgenic mice.

In conclusion, our data indicate that airway allergic inflammation reduces SPLUNC1. SPLUNC1 deficiency can exacerbate airway eosinophilic inflammation by enhancing eotaxin-2 production. Our study suggests a novel mechanism for persistent airway inflammation and chronic bacterial infection, as observed in a subset of patients with asthma. The administration of SPLUNC1 in asthma patients with a Th2 phenotype may provide an alternative therapy for attenuating the excessive airway inflammation associated with (or without) bacterial infection.

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

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