

The antimicrobial protein short palate, lung, and nasal epithelium clone 1 (SPLUNC1) is differentially modulated in eosinophilic and noneosinophilic chronic rhinosinusitis with nasal polyps

Yi Wei, MD,^{a,*} Wentong Xia, MD,^{a,*} Xingling Ye, MD,^{a,*} Yunping Fan, PhD,^b Jianbo Shi, PhD,^a Weiping Wen, PhD,^a Pingchang Yang, PhD,^c and Huabin Li, MD,^{a,d} on behalf of the Nasal Health Group, China (NHGC) *Guangzhou, Zhuhai, Shenzhen, and Shanghai, China*

Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is a highly heterogeneous disease with aberrant host defense responses. However, whether innate immunity is similarly impaired in patients with eosinophilic and those with noneosinophilic CRSwNP remains unclear.

Objectives: We sought to evaluate the expression and possible modulation of short palate, lung, and nasal epithelium clone 1 (SPLUNC1), an innate immune molecule, in the 2 CRSwNP subsets.

Methods: Polyp tissue and uncinate processes were collected from 40 patients with CRSwNP, 27 patients with chronic rhinosinusitis without nasal polyps (CRSsNP), and 22 control subjects. Expression of SPLUNC1; Toll-like receptor (TLR) 2, TLR3, and TLR4; and the proinflammatory cytokines IL-1 α , IL-4, IL-13, IL-17A, and IFN- γ was examined in nasal tissues. Additionally, SPLUNC1 expression in response to specific inflammatory stimulation was measured in cultured polyp epithelial cells and A549 cells.

Results: Polyp tissues exhibited significantly decreased expression of SPLUNC1 and other innate immune molecules compared with uncinate process tissues from patients with CRSwNP ($P < .05$), patients with CRSsNP, and healthy control subjects. Moreover, the eosinophilic CRSwNP subset exhibited significantly decreased SPLUNC1 expression and numbers of submucosal glands, as well as significantly increased IL-4 and IL-13 mRNA levels, compared with the noneosinophilic subset ($P < .05$). Accordingly, SPLUNC1 expression in polyp epithelial cells was significantly inhibited by IL-4 and IL-13 stimulation

in vitro but was significantly upregulated after stimulation with TLR agonists and glucocorticoids ($P < .05$).

Conclusion: Differential SPLUNC1 suppression between the eosinophilic and noneosinophilic CRSwNP subsets suggests that they possess distinct pathogenic mechanisms. This finding might benefit the design of appropriate therapeutic interventions targeted to each subset. (J Allergy Clin Immunol 2013;■■■■:■■■-■■■.)

Key words: Short palate, lung, and nasal epithelium clone 1, nasal polyps, chronic rhinosinusitis, Toll-like receptor, cytokine, eosinophil, submucosal gland, glucocorticoid

On the basis of whether visible nasal polyps exist in the middle meatus, chronic rhinosinusitis (CRS) can generally be classified into 2 subtypes: chronic rhinosinusitis with nasal polyps (CRSwNP) and chronic rhinosinusitis without nasal polyps (CRSsNP).¹ Although CRSsNP is dominated by neutrophilic inflammation (an antimicrobial response), CRSwNP is instead characterized by T_H2-skewed responses and profound tissue eosinophilia (an allergy-like response) that reflects a complex interplay between host defense and environmental factors that trigger these responses, including bacterial, viral, and fungal exposures.² Therefore aberrant host defenses, such as a defective physical barrier or dysregulated innate immunity, have been suggested to play a pivotal role in initiating and perpetuating inflammatory responses in patients with CRSwNP.³

Because CRSwNP is a disease characterized by significant heterogeneity, many emerging and unsolved issues currently exist surrounding its cause, pathophysiology, and treatment.⁴ Recent studies on CRSwNP have focused on exploring the underlying inflammatory mechanisms to explain the observed variable clinical manifestations. On the basis of the extent of tissue eosinophilia, clinical features, and computed tomography scans,⁵⁻⁷ CRSwNP can be further classified into eosinophilic and noneosinophilic subsets, each characterized by distinct degrees of severity, prognoses, and therapeutic strategies. Moreover, different immunopathologic mechanisms have been suggested to underlie the variable CRSwNP clinical manifestations among ethnic groups.⁷⁻¹⁰ For example, we recently revealed that some Chinese patients with CRSwNP exhibited specifically enhanced T_H1/T_H17 responses and excessive neutrophil accumulation^{7,10} and reported that patients with eosinophilic and those with noneosinophilic CRSwNP produce distinct immunopathologic profiles.¹⁰ In agreement with studies from other groups,¹¹ we also showed that some Chinese patients with CRSwNP exhibited noneosinophilic inflammation and a propensity for lower asthma

From ^athe Allergy and Cancer Center, Otorhinolaryngology Hospital, First Affiliated Hospital of Sun Yat-sen University, and the Otorhinolaryngology Institute of Sun Yat-sen University, Guangzhou; ^bthe Department of Otolaryngology, Fifth Affiliated Hospital of Sun Yat-sen University, Zhuhai; ^cthe Health Science Center, Shenzhen University, Shenzhen; and ^dthe Department of Otolaryngology, Head and Neck Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai.

*These authors contributed equally to this work.

Supported by grants from the National Natural Science Grant of China (nos. 81070771, 81070772, and 81271054), the Ministry of Hygiene (no. 201202005), Guangdong Province Natural Science Grant (nos. S2011010004634 and S2011020002295), and the Program for New Century Excellent Talents in University (no. NCET-10-0851).

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication April 21, 2013; revised September 9, 2013; accepted for publication September 12, 2013.

Corresponding author: Huabin Li, MD, Allergy and Cancer Center, Otorhinolaryngology Hospital, First Affiliated Hospital of Sun Yat-sen University, No. 58, Zhongshan 2nd Road, Guangzhou 510080, China. E-mail: allergyli@163.com.

0091-6749/\$36.00

© 2013 American Academy of Allergy, Asthma & Immunology

<http://dx.doi.org/10.1016/j.jaci.2013.09.052>

Abbreviations used

BPI: Bactericidal/permeability-increasing protein
 CRS: Chronic rhinosinusitis
 CRSsNP: Chronic rhinosinusitis without nasal polyps
 CRSwNP: Chronic rhinosinusitis with nasal polyps
 GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
 H&E: Hematoxylin and eosin
 hpf: High-power field
 LPLUNC2: Long palate, lung, and nasal epithelium clone 2
 MBP: Major basic protein
 PAS: Periodic acid–Schiff
 PEC: Polyp epithelial cell
 PLUNC: Palate, lung, and nasal epithelium clone
 qRT-PCR: Quantitative real-time PCR
 SPLUNC1: Short palate, lung, and nasal epithelium clone 1
 TLR: Toll-like receptor

comorbidity,¹² a finding that differed from white patients with CRSwNP.

The palate, lung, and nasal epithelium clones (PLUNCs) are a recently described family of proteins predicted to exert host immune defense functions.¹³ Among the 10 PLUNC proteins described thus far, short palate, lung, and nasal epithelium clone 1 (SPLUNC1) has been extensively investigated for its role in airway diseases.¹⁴ Although a functional role for SPLUNC1 in airway innate immunity has been suggested, this has not yet been clearly defined. Some studies demonstrated that recombinant SPLUNC1 protein kills bacteria, reduces biofilm formation by *Pseudomonas aeruginosa* *in vitro*,^{15–17} and inhibits airway eosinophilic inflammation in allergic mice.^{18,19} However, published data on SPLUNC1 expression in patients with airway diseases are somewhat inconsistent: although one study found that SPLUNC1 expression was reduced in patients with CRSwNP, others conversely found significantly increased SPLUNC1 expression in patients with allergic rhinitis and those with cystic fibrosis.^{20–22}

Histologic approaches to classifying eosinophilic from noneosinophilic CRSwNP represent the first logical step for improved prognostication and evidence-based management for the differing inflammatory conditions produced by this disease. To evaluate whether host defenses are similarly impaired between the 2 CRSwNP subsets, we compared expression of the antimicrobial protein SPLUNC1 in patients belonging to the eosinophilic and noneosinophilic CRSwNP subsets and investigated SPLUNC1 regulation under allergy-like or antimicrobial inflammatory conditions *in vitro*.

METHODS**Patients and tissue samples**

Adult patients with CRS (CRSwNP, n = 40; CRSsNP, n = 27) were recruited from participating hospitals in 2 major cities in southern China. CRSwNP and CRSsNP diagnoses were made according to the current European position paper on rhinosinusitis and nasal polyps.¹ Skin prick tests were performed to evaluate the atopic status of the patients. Asthma was diagnosed by a pneumologist based on disease history and evaluation of airway responsiveness.

All patients with CRS underwent an unsuccessful standardized course of medical therapy (oral and/or nasal glucocorticoids, antibiotics, antileukotrienes, and nasal irrigation for >12 weeks) and consented to undergo tissue collection at the time of surgery. During endoscopic surgery, the following nasal tissues were sampled: polyp tissue from patients with CRSwNP and

uncinate process tissue from patients with CRSwNP and those with CRSsNP. Patients undergoing septoplasty because of anatomic variations were enrolled as healthy control subjects (n = 22), and the uncinate process was sampled during septal surgery. None of the subjects used oral or nasal glucocorticoids or other medications (eg, antibiotics or antileukotrienes) for 4 weeks before sample collection, except as noted. The demographic data of all subjects enrolled in this study are listed in Table 1. This study was approved by the ethics committee boards at the First Affiliated Hospital of Sun Yat-sen University, and written informed consent was obtained from each subject.

Tissue samples were divided into 3 pieces: the first was frozen in liquid nitrogen and stored at –80°C for subsequent RNA extraction, the second was used for protein extraction, and the third was fixed overnight in a freshly prepared fixative containing 4% paraformaldehyde in PBS (pH 7.4) and embedded in paraffin wax for histologic assessment. In addition, polyp epithelial cells (PECs) were isolated and cultured from fresh polyp tissues to assess the effect of Toll-like receptor (TLR) activation and proinflammatory cytokines on SPLUNC1 expression *in vitro*.

Histologic staining

Hematoxylin and eosin (H&E), periodic acid–Schiff (PAS), and Masson trichrome staining were performed according to the manufacturer's instructions. Immunohistochemical staining for SPLUNC1, major basic protein (MBP), TLR2, TLR3, and TLR4 was performed, as previously described.²³ Detailed methods are described in the Methods section in this article's Online Repository at www.jacionline.org.

Quantitative real-time reverse transcription PCR

SPLUNC1; long palate, lung, and nasal epithelium clone 2 (LPLUNC2); bactericidal/permeability-increasing protein (BPI); TLR2; TLR3; and TLR4 mRNA expression levels were evaluated by using quantitative real-time PCR (qRT-PCR) analysis, as previously described.²³ Detailed methods are described in the Methods section in this article's Online Repository.

Western blot analysis

Protein levels of SPLUNC1 and the specific TLRs were evaluated by means of Western blot analysis. These methods are described in the Methods section in this article's Online Repository.

ELISA

The method for measuring SPLUNC1 protein levels in nasal tissue extracts by means of ELISA is described in this article's Online Repository.

In vitro cell culture and stimulation

Polyp tissues were collected from 4 randomly selected patients with CRSwNP, and PECs were isolated, as we previously described.²³ The methods for culturing and stimulating PECs and A549 cells (purchased from American Type Culture Collection, Manassas, Va) are described in the Methods section in this article's Online Repository.

Determining the effect of glucocorticoid treatment on SPLUNC1 levels in nasal tissues

Nasal secretion samples from 7 patients with eosinophilic CRSwNP with sustained nasal symptoms after endoscopic sinus surgery were obtained to determine SPLUNC1 levels in nasal tissues before and after glucocorticoid treatment, and the concentration of SPLUNC1 was determined by means of ELISA. The experimental details are described in the Methods section in this article's Online Repository.

Statistical analysis

Data were expressed as medians and interquartile ranges, and statistical significance was analyzed by using the Kruskal-Wallis *H* test and the

TABLE I. Subjects' characteristics

	Control subjects	Patients with CRSsNP	Patients with CRSwNP
No. of patients	22	27	40
Sex, male/female	14/8	15/12	22/18
Age (y)	34 (23-56)	31 (22-54)	34 (24-62)
Duration (y)	—	4 (2-7)	5 (2-11)
Skin prick test response, positive/negative	—	8/19	12/28
Asthma history, yes/no	—	3/24	5/35
Smoking, yes/no	—	7/20	10/30

Age and duration are presented as medians (ranges).

nonparametric Mann-Whitney *U* test. For the *in vitro* assays, data were expressed as the mean $\pm$ SEM from 3 independent experiments, and statistical significance was analyzed by using the paired or unpaired Student *t* test. A *P* value of less than .05 was considered statistically significant.

RESULTS

SPLUNC1 expression levels and distribution in nasal tissues

To evaluate whether host defense molecules were altered in polyp tissues from patients with CRSwNP, we first determined the expression of SPLUNC1 and other innate immune molecules (LPLUNC2, BPI, TLR2, TLR3, and TLR4) in the nasal mucosa. As shown in Fig 1, SPLUNC1, LPLUNC2, BPI, TLR2, TLR3, and TLR4 mRNA levels were significantly lower in polyp tissues from patients with CRSwNP than in uncinate tissues from control subjects, patients with CRSsNP, or patients with CRSwNP ($P < .05$). Interestingly, no significant differences in SPLUNC1, LPLUNC2, BPI, TLR2, TLR3, or TLR4 mRNA expression were observed in uncinate tissues between patients with CRSsNP and those with CRSwNP, suggesting that the reduced antimicrobial SPLUNC1 expression in polyp tissues was likely a secondary event after impaired TLR activation. SPLUNC1 protein levels were also significantly reduced in polyp tissues from patients with CRSwNP compared with those in the uncinate tissues of control subjects, patients with CRSsNP, or patients with CRSwNP ($P < .05$, Fig 2, A-C). SPLUNC1 immunoreactivity in immunohistochemistry of tissue sections was mainly localized in the submucosal glands of the nasal mucosa and was also significantly reduced in polyp tissues compared with that seen in the uncinate tissues of control subjects, patients with CRSsNP, or patients with CRSwNP ($P < .05$; Fig 2, E). Accordingly, the number and staining intensity of the submucosal glands were significantly reduced in polyp tissues compared with control tissues ($P < .05$, see Fig E1 in this article's Online Repository at www.jacionline.org). These findings collectively indicate that the expression of SPLUNC1 and other innate immune molecules is significantly suppressed in patients with CRSwNP but not in patients with CRSsNP and that a paucity of submucosal glands might account for the secondary SPLUNC1 suppression in polyp tissues.

SPLUNC1 expression is differentially expressed between polyp tissues from the eosinophilic and noneosinophilic CRSwNP subsets

Eosinophilia is more commonly associated with CRSwNP than CRSsNP. However, even within the population of patients

with CRSwNP, both the presence and extent of eosinophilia in polyp tissues are quite variable. The histologic profile of polyp tissues in patients with eosinophilic and those with noneosinophilic CRSwNP was thus characterized by evaluating submucosal glandular formation, tissue edema, and collagen deposition. We observed that the staining intensity of SPLUNC1 and the mean number and staining intensity of submucosal glands were significantly higher in polyp tissues from patients with noneosinophilic than from patients with eosinophilic CRSwNP ($P < .05$, see Fig E2 in this article's Online Repository at www.jacionline.org). Accordingly, we observed significantly enhanced tissue edema but significantly reduced collagen deposition in patients with eosinophilic CRSwNP ($P < .05$, see Fig E3 in this article's Online Repository at www.jacionline.org). Confirming the histologic findings, we found that polyp tissues from the noneosinophilic CRSwNP subset exhibited significantly higher SPLUNC1 mRNA and protein levels than those from the eosinophilic subset ($P < .05$, Fig 3). Collectively, these findings suggest that the underlying inflammatory responses differ between the 2 CRSwNP subsets.

TLRs and proinflammatory cytokines are differentially expressed in the eosinophilic and noneosinophilic CRSwNP subsets

As mentioned above, the presence and extent of eosinophilia in polyp tissues vary among patients with CRSwNP; indeed, published data indicate that many Chinese patients with CRSwNP do not demonstrate significant eosinophilia.¹⁰ On the basis of our observation that polyp tissues from patients with eosinophilic CRSwNP exhibited lower antimicrobial SPLUNC1 expression, we hypothesized that antimicrobial innate immune responses were lower in polyps from patients with eosinophilic than those with noneosinophilic CRSwNP. To establish whether any potential relationship existed between innate and/or adaptive immunity and tissue eosinophilia in patients within the 2 CRSwNP subsets, we examined the expression of TLRs (TLR2, TLR3, and TLR4) and proinflammatory cytokines (IL-1 α , IL-4, IL-13, IFN- γ , and IL-17A) in polyp tissues. As we hypothesized, mRNA and protein levels of TLR2, TLR3, and TLR4 were significantly lower in polyp tissues from patients with eosinophilic than those with noneosinophilic CRSwNP ($P < .05$, see Fig E4 in this article's Online Repository at www.jacionline.org). Accordingly, mRNA levels of the T_H2 cytokines IL-4 and IL-13 were significantly higher in eosinophilic polyp tissues than in noneosinophilic polyp tissues ($P < .05$). However, mRNA expression for the other examined cytokines (IL-1 α , IFN- γ , and IL-17A) was not significantly different between the 2 CRSwNP subsets (see Fig E5 in this article's Online Repository at www.jacionline.org). These findings suggest that T_H2-skewed responses and impaired TLR activation might play critical roles in suppressing SPLUNC1 and dysregulating mucosal immunity in the eosinophilic CRSwNP subset.

TLR agonists and proinflammatory cytokines differentially regulate SPLUNC1 expression in cultured airway epithelial cells *in vitro*

To establish a direct relationship between TLRs and innate immunity, as well as between proinflammatory cytokines and

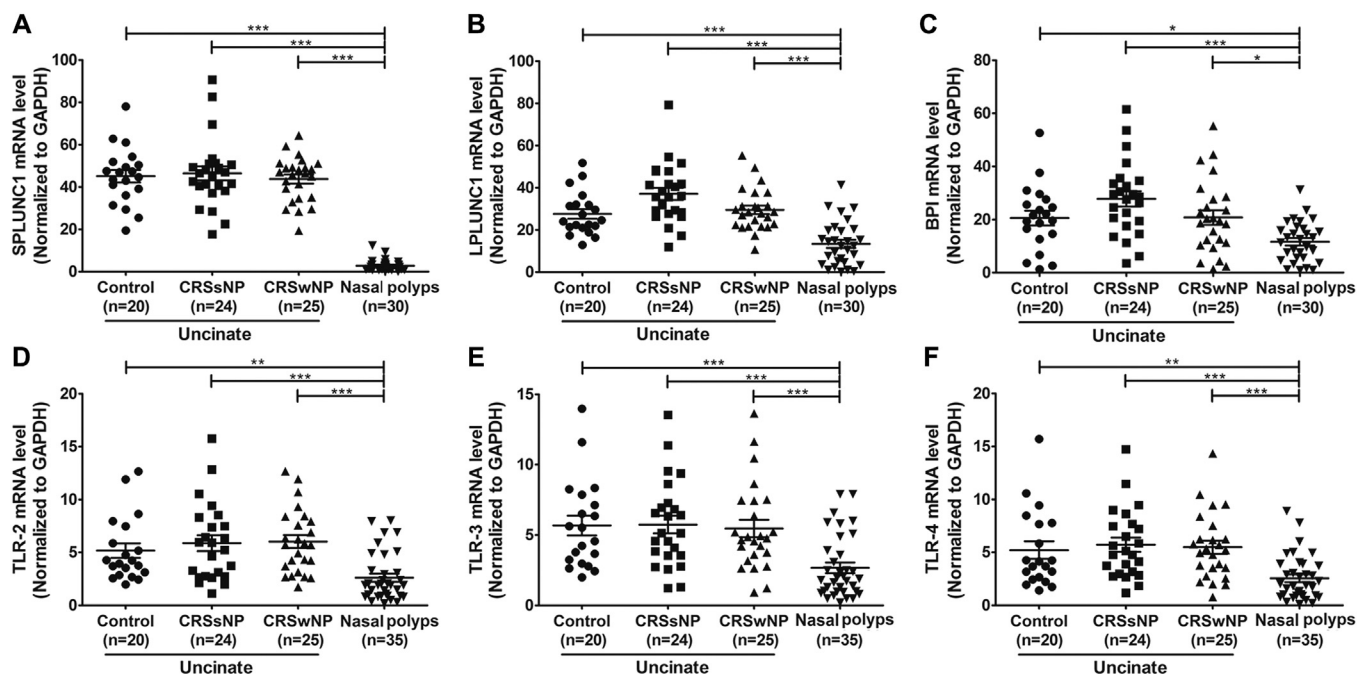


FIG 1. SPLUNC1 mRNA expression in nasal tissues. SPLUNC1 (A), LPLUNC2 (B), BPI (C), TLR2 (D), TLR3 (E), and TLR4 (F) mRNA levels were significantly reduced in polyp tissues from patients with CRSwNP compared with values seen in the other subgroups. Results are expressed as medians (interquartile ranges). * $P < .05$, ** $P < .01$, and *** $P < .001$.

innate immunity in the nasal mucosa, we examined the effects of TLR activation and proinflammatory cytokine stimulation on SPLUNC1 expression in cultured airway epithelial cells (PECs and A549 cells) *in vitro*. As shown in Fig 4, SPLUNC1 mRNA and protein levels were significantly increased after stimulating the cultured epithelial cells with TLR agonists ($P < .05$). In contrast, stimulation with the T_H2 cytokines IL-4 and IL-13 most prominently inhibited SPLUNC1 mRNA and protein levels in the cultured cells ($P < .05$) compared with stimulation by the other proinflammatory cytokines, including IL-1 α , IL-6, IFN- γ , TGF- β , and IL-17A, which also significantly inhibited SPLUNC1 expression, although to a lesser extent than the T_H2 cytokines ($P < .05$, Fig 5). These findings suggest that proinflammatory cytokines and local mucosal defenses exert opposing regulatory mechanisms on innate immune responses in the physiologic environment.

Glucocorticoid treatment increases SPLUNC1 expression *in vitro* and *in vivo*

Because glucocorticoid treatment is recommended as the primary treatment of choice for patients with CRSwNP, we examined the effect of glucocorticoids on SPLUNC1 mRNA expression in PECs *in vitro*. After incubating cells with glucocorticoids (including dexamethasone, budesonide, and fluticasone), SPLUNC1 mRNA expression was significantly upregulated in the presence or absence of proinflammatory cytokines (IL-4, IL-13, IFN- γ , and IL-17A; $P < .05$, see Fig E6 in this article's Online Repository at www.jacionline.org). This finding indicates that glucocorticoid treatment enhances SPLUNC1 expression, which is normally suppressed by T_H2

and other proinflammatory cytokines. We also examined SPLUNC1 protein levels in nasal secretions from 7 patients with eosinophilic CRSwNP (restricted to those who had persistent nasal symptoms after endoscopic sinus surgery) before and after a 2-week treatment of intranasal glucocorticoids. Intranasal glucocorticoid treatment (Flixonase; Glaxo-SmithKline, London, United Kingdom) significantly increased SPLUNC1 levels in nasal secretions and improved total nasal symptom scores (visual analog scale scores, $P < .05$, see Fig E7 in this article's Online Repository at www.jacionline.org), indicating that glucocorticoid treatment might partially exert its anti-inflammatory effect by increasing SPLUNC1 expression and enhancing local mucosal immunity, as Zhang et al²⁴ demonstrated elsewhere.

DISCUSSION

Endotyping patients with CRSwNP by histopathologic classification (eosinophilic vs noneosinophilic) rather than by simple clinical phenotypes (CRSwNP vs CRSsNP) reflects a change toward understanding the underlying inflammatory processes so as to design more appropriate approaches to disease management.²⁵ However, few studies have addressed the molecular mechanisms underlying the distinct histopathologic phenotypes of CRSwNP.^{25,26} In this study we observed that innate immunity, as indicated by expression of the antimicrobial protein SPLUNC1, was differentially impaired between the eosinophilic and noneosinophilic CRSwNP subsets. This differential suppression of SPLUNC1 might be associated with the development of T_H2 -skewed responses and tissue eosinophilia. Thus these findings broaden our understanding of the pathophysiology underlying CRSwNP.

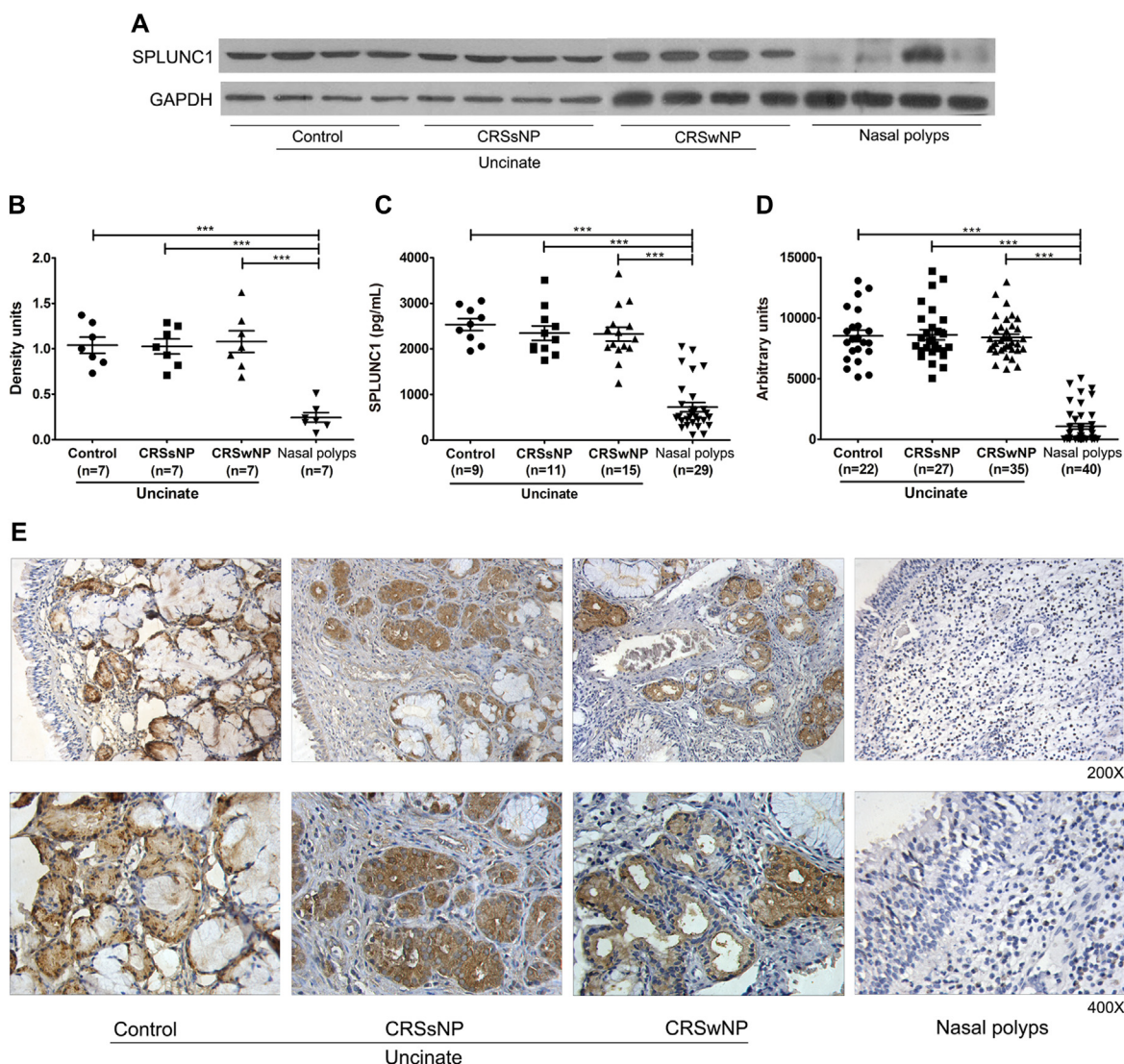


FIG 2. Protein expression and distribution of SPLUNC1 in nasal tissues. Representative Western blot results (A), densitometric analysis (B), ELISA analysis (C), quantitative analysis of SPLUNC1 staining intensity (D), and representative SPLUNC1 staining (E; $\times 200$ and $\times 400$ magnification) in nasal tissues from patients with CRSwNP and those with CRSsNP, as well as control subjects, is shown. Data are expressed as medians (interquartile ranges). *** $P < .001$.

CRSwNP is a predominantly inflammatory disease associated with dysregulated interaction between local mucosal immunity and environmental factors.³ Innate immune dysfunction is thought to contribute to CRS pathogenesis by establishing an environment that incurs increased sinonasal microbial colonization. Consistent with previous reports,^{21,27-30} we found that SPLUNC1, LPLUNC2, BPI, TLR2, TLR3, and TLR4 mRNA expression was significantly reduced in polyp tissues from patients with CRSwNP compared with uncinate tissues of control subjects, patients with CRSsNP, or patients with CRSwNP. Similarly, the number and staining intensity of submucosal glands were also significantly reduced in polyp tissues compared with values seen in control tissues. Although TLR activation is known to initiate signals that culminate in a number of host defense responses, whether the reduced expression of TLR2, TLR3, and TLR4 is associated with the

paucity of submucosal glands and polyp formation still requires thorough investigation in regard to the polyp tissues. Because we found that the antimicrobial protein SPLUNC1 was mainly distributed in the submucosal glands of the nasal mucosa and that decreased SPLUNC1 expression was generally restricted to the polyp rather than the uncinate tissues, our findings suggested that reduced SPLUNC1 expression in polyp tissues was likely to be a secondary event after impaired TLR activation. ELISA and Western blot assays also confirmed that polyp tissues exhibited reduced SPLUNC1 levels. Because SPLUNC1 has been suggested to exert profound antimicrobial and antibiofilm activities,^{15,16,31,32} it is not surprising that decreased SPLUNC1 expression is also associated with *Pseudomonas aeruginosa*-related sinus infections and poor clinical outcomes.¹⁷

In the present study we focused on investigating the differential innate immune response in the 2 distinct eosinophilic and

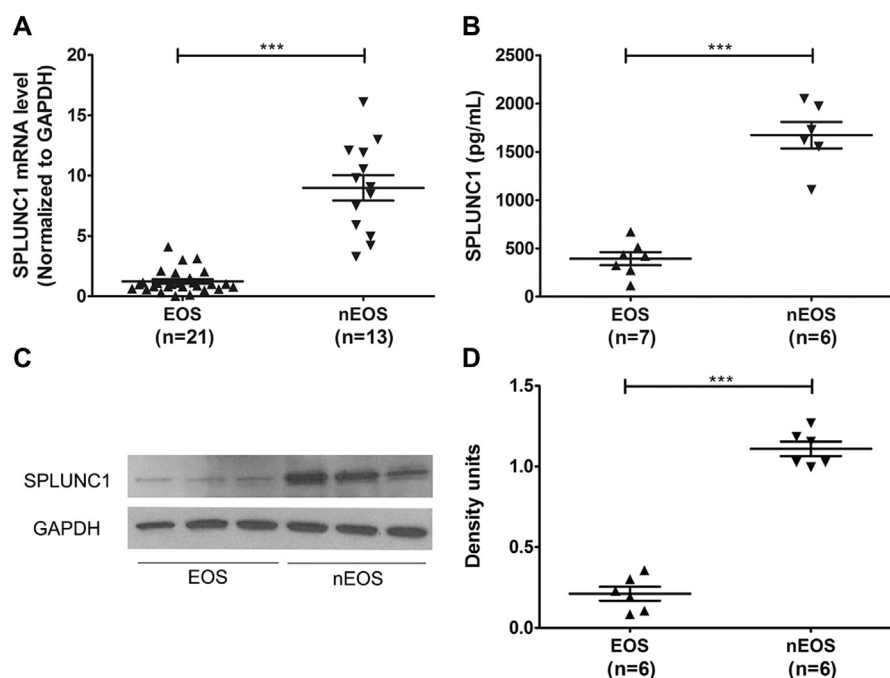


FIG 3. SPLUNC1 mRNA and protein expression in eosinophilic (EOS) and noneosinophilic (nEOS) polyp tissues. **A**, mRNA expression of SPLUNC1 in polyp tissues. **B-D**, ELISA analysis (Fig 3, B), representative Western blot results (Fig 3, C), and densitometric analysis (Fig 3, D) of SPLUNC1 in tissue extracts of nasal polyps. *** $P < .001$.

noneosinophilic CRSwNP subsets. Although several CRS guidelines distinguish patients with CRS according to the presence or absence of nasal polyps, CRSwNP is a heterogeneous disease that includes several subsets, and proper differential diagnosis is required to design appropriate therapeutic interventions. Recently, several studies examined the histologic properties of eosinophilic and noneosinophilic CRSwNP by using different study-specific criteria; consistently, the 2 CRSwNP subsets exhibited distinct features in tissue remodeling, edema, and collagen deposition.^{33,34} In this study we further characterized the inflammatory responses between the eosinophilic and noneosinophilic CRSwNP subsets by examining the innate immune response in addition to evaluating their histopathologic profiles. Interestingly, we found significant differences in the number and staining intensity of the submucosal glands, tissue edema, and collagen deposition between the 2 CRSwNP subsets. It was previously demonstrated that the nasal mucosa possessed 2 distinct histologic changes in patients with CRS, and tissue eosinophilia was associated with a paucity of submucosal glands.³⁵ Here, we consistently found that SPLUNC1 immunoreactivity, as well as mRNA and protein levels, in polyp tissues of patients with eosinophilic CRSwNP were significantly lower than in patients with noneosinophilic CRSwNP. On the basis of a report by Seshadri et al,²⁰ decreased SPLUNC1 expression in polyp tissues was thought to be associated with decreased glandular formation. In our study we further showed a significant difference in SPLUNC1 expression, glandular formation, tissue edema, and collagen deposition between the eosinophilic and noneosinophilic CRSwNP subtypes, adding novel information to our understanding of the pathophysiology of CRSwNP. Our findings suggest that innate immune responses in patients belonging to the 2

CRSwNP subtypes are differentially impaired and that this difference might relate to the observed histopathologic differences between these subtypes.

To date, SPLUNC1 regulation in nasal tissues under physiologic or pathologic conditions remains poorly understood. Some investigators have recently addressed the importance of TLR signaling on SPLUNC1 production.³⁶ TLRs are important components of innate immunity suggested to function as pivotal elements in mucosal host defense; however, their importance in CRSwNP pathogenesis is not well understood. In a report by Rammanathan et al,²⁷ epithelial cells isolated from patients with CRSwNP displayed decreased expression of multiple antimicrobial innate immune markers, including TLR9, after exposure to T_H2 cytokines, such as IL-4 and IL-13. This finding suggests a possible opposing regulatory mechanism between proinflammatory cytokines and local mucosal defenses in inflammatory environments. However, the association between aberrant mucosal defenses and excessive T_H2 responses with tissue eosinophilia remains unclear. To address this issue, we examined the expression of several TLRs (TLR2, TLR3, and TLR4) and proinflammatory cytokines (IL-1 α , IL-4, IL-13, IFN- γ , and IL-17A) in patients belonging to the 2 CRSwNP subsets. As we hypothesized, relatively enhanced mucosal immunity and weakened T_H2 responses were observed in the noneosinophilic compared with the eosinophilic subset. Together with other reports,²⁷ our findings suggest that T_H2 -skewed responses might play a role in suppressing SPLUNC1 expression and dysregulating mucosal immunity in patients with CRSwNP. Alternatively, SPLUNC1 induction after TLR activation might serve as an innate defense mechanism used by airway epithelial cells to fight invading

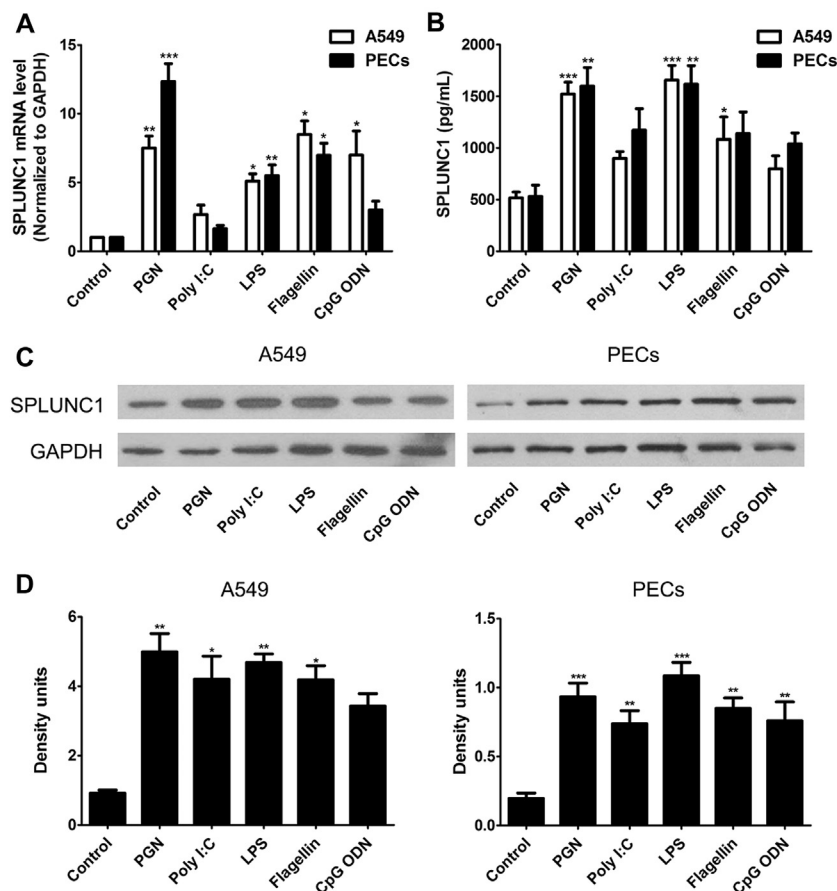


FIG 4. SPLUNC1 mRNA and protein levels in cultured PECs and A549 cells in response to TLR agonists. **A**, SPLUNC1 mRNA expression after a 12-hour stimulation. **B**, ELISA analysis of SPLUNC1 levels in supernatants after a 24-hour stimulation. **C** and **D**, Representative Western blot results of cell lysates (Fig 4, C) and densitometric analysis (Fig 4, D) after a 24-hour stimulation. Results represent mean values from 3 independent experiments. Data are expressed as means (SEMs). * $P < .05$, ** $P < .01$, and *** $P < .001$. PGN, Peptidoglycan; PolyI:C, polyinosinic-polycytidylic acid.

pathogens. Our hypothesis is supported by recent findings by Thaikoottathil et al¹⁸ showing that SPLUNC1 inhibits airway eosinophilic inflammation in allergic mice partly by reducing eotaxin-2 production in alveolar macrophages.

Although the regulation of antimicrobial innate immune molecules, such as SPLUNC1, is an active area of research, the direct role of TLR signaling and proinflammatory cytokines on regulating SPLUNC1 production in nasal epithelial cells is poorly understood. In this study SPLUNC1 mRNA and protein levels were significantly upregulated after TLR agonist stimulation, suggesting that antimicrobial signaling is essential for inducing SPLUNC1 expression. Conversely, the presence of T_H2 cytokines (IL-4 and IL-13) *in vitro* most prominently inhibited SPLUNC1 mRNA and protein expression. Therefore these findings demonstrate that TLRs and proinflammatory cytokines (particularly T_H2 cytokines) differentially modulate SPLUNC1 expression in nasal epithelial cells. Notably, we found that glucocorticoids, including dexamethasone, budesonide, and fluticasone, universally increased SPLUNC1 mRNA expression independent of the presence or absence of proinflammatory cytokines (IL-4, IL-13, IFN- γ , and IL-17A). From studies performed in several *in vitro* and *in vivo* model systems, glucocorticoids have been suggested to enhance innate immunity while suppressing adaptive immunity by increasing the sensitivity of

the epithelial surface to TLR ligands and inducing expression of innate immune molecules, such as surfactant proteins A and D.³⁷ Although the underlying molecular mechanisms require further characterization, this finding demonstrates that glucocorticoid treatment not only directly induces SPLUNC1 expression but also rescues proinflammatory cytokine-induced SPLUNC1 suppression. Correspondingly, we found that glucocorticoid treatment (Flixonase) significantly increased SPLUNC1 levels in nasal secretions while improving total nasal symptom scores in patients with eosinophilic CRSwNP. Therefore SPLUNC1 regulation in patients with CRSwNP might represent a novel target for attenuating disease severity and improving surgical prognoses.

In summary, we reveal here the differential inflammatory responses underlying SPLUNC1 suppression and the distinct histologic features underlying the observed clinical properties in patients with eosinophilic and those with noneosinophilic CRSwNP. These findings potentially reflect the differentially impaired innate immune responses resulting from dysregulated antimicrobial and allergy-like responses in polyp tissues. The molecular mechanisms identified here will help to better understand the pathogenic processes involved in these 2 CRSwNP subsets, as well as in designing novel therapeutic strategies to improve clinical outcomes.

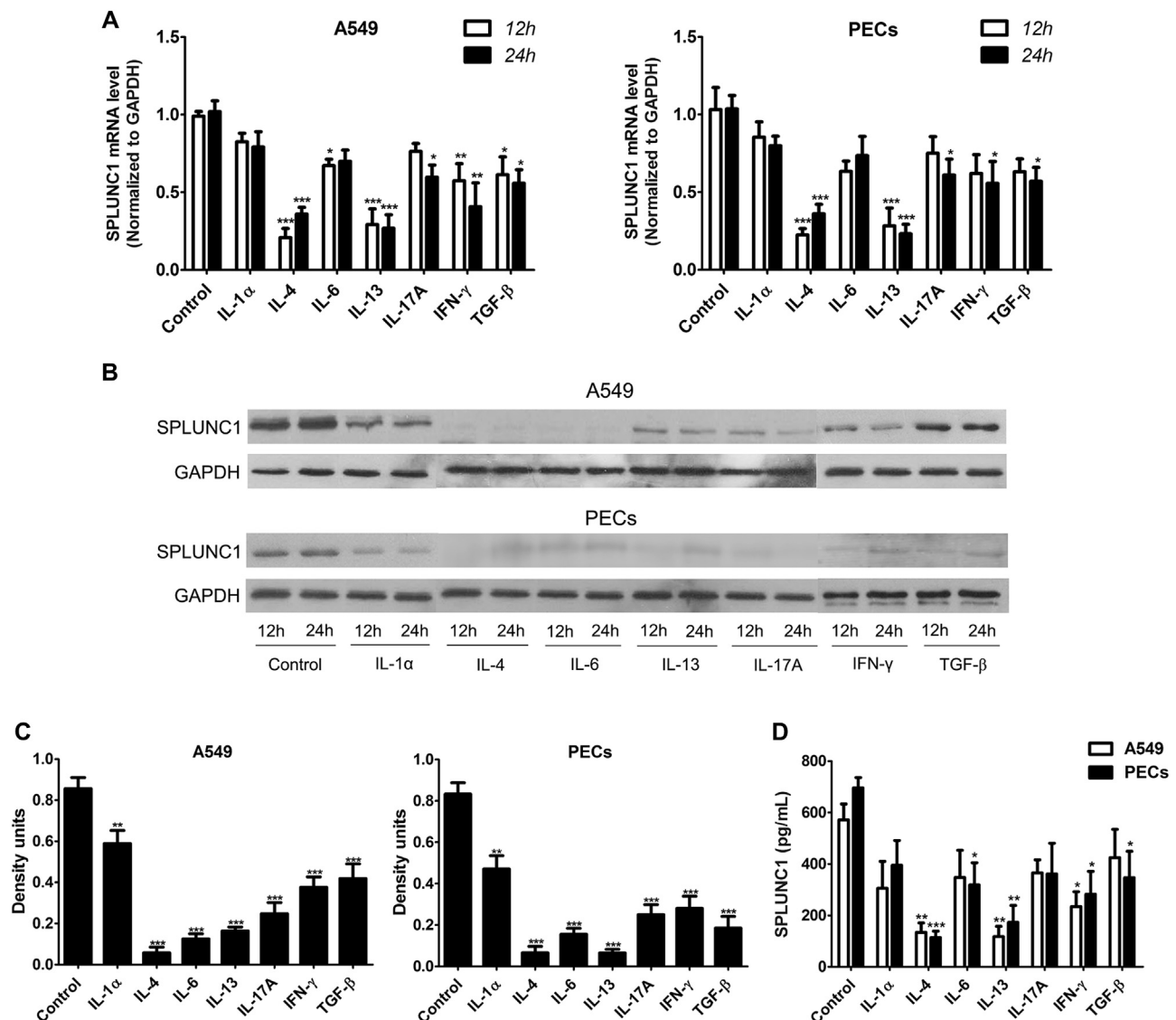


FIG 5. SPLUNC1 mRNA expression in cultured PECs and A549 cells in response to proinflammatory cytokines. **A**, SPLUNC1 mRNA expression after a 12- or 24-hour stimulation. **B-D**, Representative Western blot results (Fig 5, B), densitometric analysis (Fig 5, C), and ELISA analysis (Fig 5, D) of SPLUNC1 levels in supernatants after a 24-hour stimulation. Results represent mean values from 3 independent experiments. Data are expressed as means (SEMs). * $P < .05$, ** $P < .01$, and *** $P < .001$.

Clinical implications: Differential SPLUNC1 suppression in polyp tissues from patients in the eosinophilic and noneosinophilic CRSwNP subsets implicates distinct pathogenic processes in each subset. This finding might contribute to designing appropriate therapeutic interventions targeted to each subset.

REFERENCES

1. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012. A summary for otorhinolaryngologists. *Rhinology* 2012;50:1-12.
2. Hsu J, Peters AT. Pathophysiology of chronic rhinosinusitis with nasal polyp. *Am J Rhinol Allergy* 2011;25:285-90.
3. Tieu DD, Kern RC, Schleimer RP. Alterations in epithelial barrier function and host defense responses in chronic rhinosinusitis. *J Allergy Clin Immunol* 2009;124:37-42.
4. Payne SC, Borish L, Steinke JW. Genetics and phenotyping in chronic sinusitis. *J Allergy Clin Immunol* 2011;128:710-20.
5. Sakuma Y, Ishitoya J, Komatsu M, Shiono O, Hiramata M, Yamashita Y, et al. New clinical diagnostic criteria for eosinophilic chronic rhinosinusitis. *Auris Nasus Larynx* 2011;38:583-8.
6. Ishitoya J, Sakuma Y, Tsukuda M. Eosinophilic chronic rhinosinusitis in Japan. *Allergol Int* 2010;59:239-45.
7. Wen W, Liu W, Zhang L, Bai J, Fan Y, Xia W, et al. Increased neutrophilia in nasal polyps reduces the response to oral corticosteroid therapy. *J Allergy Clin Immunol* 2012;129:1522-8.e5.
8. Zhang N, Van Zele T, Perez-Novo C, Van Bruene N, Holtappels G, DeRuyck N, et al. Different types of T-effector cells orchestrate mucosal inflammation in chronic sinus disease. *J Allergy Clin Immunol* 2008;122:961-8.
9. Kim JW, Hong SL, Kim YK, Lee CH, Min YG, Rhee CS. Histological and immunological features of non-eosinophilic nasal polyps. *Otolaryngol Head Neck Surg* 2007;137:925-30.
10. Cao PP, Li HB, Wang BF, Wang SB, You XJ, Cui YH, et al. Distinct immunopathologic characteristics of various types of chronic rhinosinusitis in adult Chinese. *J Allergy Clin Immunol* 2009;124:478-84, e1-2.
11. Bachert C, Zhang N, Holtappels G, De Lobel L, van Cauwenberge P, Liu S, et al. Presence of IL-5 protein and IgE antibodies to staphylococcal enterotoxins in nasal

- polyps is associated with comorbid asthma. *J Allergy Clin Immunol* 2010;126:962-8.
12. Fan Y, Chen S, Qu X, Zuo K, Li X, Huang J, et al. A lower prevalence of asthma among patients with chronic rhinosinusitis in southern China. *J Allergy Clin Immunol* 2011;127:520-2, e1-5.
 13. Bartlett JA, Gakhar L, Penterman J, Singh PK, Mallampalli RK, Porter E, et al. PLUNC: a multifunctional surfactant of the airways. *Biochem Soc Trans* 2011;39:1012-6.
 14. Di YP. Functional roles of SPLUNC1 in the innate immune response against Gram-negative bacteria. *Biochem Soc Trans* 2011;39:1051-5.
 15. Lukinskiene L, Liu Y, Reynolds SD, Steele C, Stripp BR, Leikauf GD, et al. Antimicrobial activity of PLUNC protects against *Pseudomonas aeruginosa* infection. *J Immunol* 2011;187:382-90.
 16. Gally F, Di YP, Smith SK, Minor MN, Liu Y, Bratton DL, et al. SPLUNC1 promotes lung innate defense against *Mycoplasma pneumoniae* infection in mice. *Am J Pathol* 2011;178:2159-67.
 17. Tsou YA, Chen CM, Lin TC, Hu FW, Tai CJ, Chen HC, et al. Decreased SPLUNC1 expression is associated with pseudomonas infection in surgically treated chronic rhinosinusitis patients who may require repeated sinus surgery. *Laryngoscope* 2013;123:845-51.
 18. Thaikoottathil JV, Martin RJ, Di PY, Minor M, Case S, Zhang B, et al. SPLUNC1 deficiency enhances airway eosinophilic inflammation in mice. *Am J Respir Cell Mol Biol* 2012;47:253-60.
 19. Chu HW, Thaikoottathil J, Rino JG, Zhang G, Wu Q, Moss T, et al. Function and regulation of SPLUNC1 protein in *Mycoplasma* infection and allergic inflammation. *J Immunol* 2007;179:3995-4002.
 20. Seshadri S, Lin DC, Rosati M, Carter RG, Norton JE, Suh L, et al. Reduced expression of antimicrobial PLUNC proteins in nasal polyp tissues of patients with chronic rhinosinusitis. *Allergy* 2012;67:920-8.
 21. Ghafouri B, Kihlström E, Tagesson C, Lindahl M. PLUNC in human nasal lavage fluid: multiple isoforms that bind to lipopolysaccharide. *Biochim Biophys Acta* 2004;1699:57-63.
 22. Bingle L, Barnes FA, Cross SS, Rassl D, Wallace WA, Campos MA, et al. Differential epithelial expression of the putative innate immune molecule SPLUNC1 in cystic fibrosis. *Respir Res* 2007;8:79.
 23. Wang H, Bai J, Ding M, Liu W, Xu R, Zhang J, et al. Interleukin-17A contributes to the expression of serum amyloid A in chronic rhinosinusitis with nasal polyps. *Eur Arch Otorhinolaryngol* 2013;270:1867-72.
 24. Zhang N, Truong-Tran QA, Tancowny B, Harris KE, Schleimer RP. Glucocorticoids enhance or spare innate immunity: effects in airway epithelium are mediated by CCAAT/enhancer binding proteins. *J Immunol* 2007;179:578-89.
 25. Akdis CA, Bachert C, Cingi C, Dykewicz MS, Hellings PW, Naclerio RM, et al. Endotypes and phenotypes of chronic rhinosinusitis: a PRACTALL document of the European Academy of Allergy and Clinical Immunology and the American Academy of Allergy, Asthma & Immunology. *J Allergy Clin Immunol* 2013;131:1479-90.
 26. Chin D, Harvey RJ. Nasal polyposis: an inflammatory condition requiring effective anti-inflammatory treatment. *Curr Opin Otolaryngol Head Neck Surg* 2013;21:23-30.
 27. Ramanathan M Jr, Lee WK, Spannhake EW, Lane AP. Th2 cytokines associated with chronic rhinosinusitis with polyps down-regulate the antimicrobial immune function of human sinonasal epithelial cells. *Am J Rhinol* 2008;22:115-21.
 28. Lane AP, Truong-Tran QA, Myers A, Bickel C, Schleimer RP. Serum amyloid A, properdin, complement 3, and toll-like receptors are expressed locally in human sinonasal tissue. *Am J Rhinol* 2006;20:117-23.
 29. Ramanathan M Jr, Lee WK, Dubin MG, Lin S, Spannhake EW, Lane AP. Sinonasal epithelial cell expression of toll-like receptor 9 is decreased in chronic rhinosinusitis with polyps. *Am J Rhinol* 2007;21:110-6.
 30. Claeys S, Van Hoecke H, Holtappels G, Gevaert P, De Belder T, Verhasselt B, et al. Nasal polyps in patients with and without cystic fibrosis: a differentiation by innate markers and inflammatory mediators. *Clin Exp Allergy* 2005;35:467-72.
 31. Garcia-Caballero A, Rasmussen JE, Gaillard E, Watson MJ, Olsen JC, Donaldson SH, et al. SPLUNC1 regulates airway surface liquid volume by protecting ENaC from proteolytic cleavage. *Proc Natl Acad Sci U S A* 2009;106:11412-7.
 32. Liu Y, Bartlett JA, Di ME, Bomberger JM, Chan YR, Gakhar L, et al. SPLUNC1/BPIFA1 contributes to pulmonary host defense against *Klebsiella pneumoniae* respiratory infection. *Am J Pathol* 2013;182:1519-31.
 33. Payne SC, Early SB, Huyett P, Han JK, Borish L, Steinke JW. Evidence for distinct histologic profile of nasal polyps with and without eosinophilia. *Laryngoscope* 2011;121:2262-7.
 34. Shi LL, Xiong P, Zhang L, Cao PP, Liao B, Lu X, et al. Features of airway remodeling in different types of Chinese chronic rhinosinusitis are associated with inflammation patterns. *Allergy* 2013;68:101-9.
 35. Berger G, Kattan A, Bernheim J, Ophir D. Polypoid mucosa with eosinophilia and glandular hyperplasia in chronic sinusitis: a histopathological and immunohistochemical study. *Laryngoscope* 2002;112:738-45.
 36. Chu HW, Gally F, Thaikoottathil J, Janssen-Heininger YM, Wu Q, Zhang G, et al. SPLUNC1 regulation in airway epithelial cells: role of Toll-like receptor 2 signaling. *Respir Res* 2010;11:155.
 37. Schleimer RP. Glucocorticoids suppress inflammation but spare innate immune responses in airway epithelium. *Proc Am Thorac Soc* 2004;1:222-30.

METHODS

Histologic staining

Paraffin sections (4 μ m) were used for histology. H&E, PAS, and Masson trichrome staining was performed, according to the manufacturer's instructions. In this study H&E staining was used to evaluate tissue edema in 10 randomly selected high-power fields (hpfs; $\times 400$ magnification) under light microscopy. The degree of tissue edema was semiquantitatively scored on a scale of 0 to 3 (0, none; 1, mild; 2, medium; and 3, severe) and then averaged. PAS staining was used to evaluate the mean number of submucosal glands in 10 randomly selected hpfs ($\times 400$ magnification). Masson trichrome staining was used to evaluate tissue collagen deposition, and the percentage of the positive area was determined in 10 randomly selected hpfs ($\times 400$ magnification) by using Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, Md) and then averaged.

Immunohistochemical staining was performed by using the peroxidase-labeled streptavidin-biotin technique. Briefly, 4- μ m-thick sections were deparaffinized by means of serial treatment. After blocking endogenous peroxidase with 3% hydrogen peroxide and 1% BSA, the sections were incubated overnight at 4°C in the presence of the primary antibodies listed in Table E1. Thereafter, each section was incubated with a secondary antibody and then with a horseradish peroxidase-labeled streptavidin complex (Zhongshanjinqiao, Beijing, China). The distribution of peroxidase was visualized by incubating the sections in a solution containing 3% 3,3'-diaminobenzidine tetrahydrochloride before counterstaining with hematoxylin and covering the section with a cover slip. Negative control staining was obtained by replacing the primary antibodies with normal IgG at the appropriate concentrations.

Similarly, glandular and SPLUNC1 staining intensity on histologic sections was assessed by measuring color intensity in a blinded manner. Eosinophil numbers were determined by counting the number of MBP-positive cells, as we described elsewhere.^{E1} Eosinophilic and noneosinophilic phenotypes were determined by assessing typical clinical features and computed tomographic scans^{E2} in addition to histologic criteria for the isolated tissues, as we previously established.^{E1}

qRT-PCR

The mRNA expression levels of SPLUNC1, LPLUNC2, BPI, TLR2, TLR3, and TLR4 were evaluated by using qRT-PCR analysis. Briefly, total RNA was extracted with the TRIzol reagent (Invitrogen, Carlsbad, Calif), according to the manufacturer's instructions. Reverse transcription was performed, and cDNA was synthesized from 2 μ g of total RNA by using an oligo(dT)18 primer and M-MLV reverse transcriptase (TAKARA, Syuzou, Shiga, Japan). RNA integrity and the success of the reverse transcription reaction were monitored by using PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts. mRNA expression was determined by using an ABI PRISM 7500 Detection System (Applied Biosystems, Foster City, Calif) and SYBR Premix Taq (TAKARA). The primer sequences for each gene used in the study are listed in Table E2. qRT-PCR samples contained 1 $\times$ SYBR Green Master Mix, 1.5 μ L of 5 μ mol/L primers, and 25 ng synthesized cDNA in a 25- μ L volume. Reactions were initially heated to 95°C for 10 minutes, followed by 40 cycles of a denaturation step at 95°C for 10 seconds and an annealing/extension step at 60°C for 60 seconds. Melting curve analysis was performed to control for amplification specificity. The mean threshold cycle (Ct) values were normalized to GAPDH, and the relative mRNA levels of the target genes were analyzed by using the $2^{-\Delta\Delta C_t}$ method. Experiments were performed in triplicate for each data point.

Western blot analysis

SPLUNC1, TLR2, TLR3, and TLR4 protein levels were determined by using Western blot analysis. Briefly, the tissues or cells were collected, and total cellular protein was extracted in 100 μ L of RIPA lysis buffer at 4°C for 30 minutes. Protein concentrations were determined by using the Bradford

method. Samples containing 10 μ g of protein were boiled, subjected to SDS-PAGE in 10% Tris-glycine gels, and electrophoretically transferred to polyvinylidene fluoride membranes. The membranes were incubated with 5% fat-free skim milk in Tris-buffered saline solution containing 0.05% Tween-20 for 1 hour at room temperature and then incubated with the primary antibodies listed in Table E1 overnight at 4°C. The membranes were then incubated with a horseradish peroxidase-linked secondary antibody and finally processed by using an ECL chemiluminescence reaction kit (Cell Signaling, Danvers, Mass), followed by exposure on medical film. The band density of the target protein relative to GAPDH was quantified by using Bio-Rad Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Hercules, Calif).

ELISA

SPLUNC1 protein levels in nasal tissue extracts, nasal secretions, and supernatants of stimulated epithelial cells were measured by means of ELISA, as we described elsewhere.^{E2} The SPLUNC1 sandwich ELISA was developed in our laboratory with anti-SPLUNC1 antibodies (R&D Systems, Minneapolis, Minn). The detection limit for SPLUNC1 was 10 pg/mL.

Cell culture and stimulation

Primary PECs were randomly collected from 4 patients with CRSwNP by using enzymatic digestion to establish the *in vitro* cell cultures, as we described elsewhere.^{E3} Briefly, fresh polyp tissues were treated with Protease (1.4 mg/mL) and DNase (1:100) in Dulbecco modified Eagle medium and Ham nutrient F12 supplemented with penicillin G sodium (50 IU/mL) and streptomycin sulfate (50 mg/mL) and placed in a 37°C/5% CO₂ incubator for 1 hour. After repeated centrifugation, the cells were rinsed in 5 mL of Dulbecco modified Eagle medium/F12, transferred into BEGM medium (Lonza, Walkersville, Md), poured into a plastic flask, and placed into a 37°C/5% CO₂ incubator overnight. PECs were collected 5 to 7 days later.

Collected PECs and A549 cells (purchased from American Type Culture Collection) were cultured in submersion cultures in BEGM medium (Lonza) until passaged. When 80% to 90% confluence was reached, the epithelial cells were washed with PBS (37°C, pH 7.4), and fresh media without hydrocortisone was added in the presence of the following stimulators or control PBS for varying lengths of time: the recombinant cytokines human IFN- γ (100 ng/mL), IL-4 (100 ng/mL), IL-13 (100 ng/mL), IL-17A (100 ng/mL), TGF- β (10 ng/mL), and IL-1 α (100 ng/mL; all purchased from R&D Systems); the TLR agonists Pam₃CSK₄ (500 ng/mL), poly I:C (500 ng/mL), LPS (500 ng/mL, from *Escherichia coli* serotype 0111:B4; purchased from Sigma, St Louis, Mo), flagellin (100 ng/mL), and human CpG ODN (5 μ mol/L; purchased from InvivoGen, San Diego, Calif); and the glucocorticoids dexamethasone (10 μ g/mL), budesonide (50 ng/mL), and fluticasone (50 ng/mL, all purchased from Sigma). After stimulation, cell pellets and supernatants were collected for qRT-PCR, Western blot analysis, and ELISA in accordance with the aforementioned protocols.

Assay to determine SPLUNC1 levels in nasal secretions after intranasal glucocorticoid treatment

Seven patients with eosinophilic CRSwNP and sustained nasal symptoms after sinus surgery were included after signing the written informed consent form. Eosinophilic phenotypes were determined by assessing typical clinical features, computed tomographic scans, and tissue histology, as described above; these characteristics are listed in Table E3. After a 1-week run-in period without any medication, the patients were administered fluticasone propionate nasal spray (Flixonase) at 200 μ g/d alone for 2 consecutive weeks. The total nasal symptom scores were evaluated on a 0- to 10-mm visual analog scale score (0, no complaints; 10, the worst possible symptoms) before and after treatment. Nasal secretions were collected before and after intranasal glucocorticoid treatment, as described elsewhere,^{E4} and the concentration of SPLUNC1 in nasal secretions was determined by using ELISA.

REFERENCES

- E1. Wen W, Liu W, Zhang L, Bai J, Fan Y, Xia W, et al. Increased neutrophilia in nasal polyps reduces the response to oral corticosteroid therapy. *J Allergy Clin Immunol* 2012;129:1522-8.e5.
- E2. Sakuma Y, Ishitoya J, Komatsu M, Shiono O, Hiramata M, Yamashita Y, et al. New clinical diagnostic criteria for eosinophilic chronic rhinosinusitis. *Auris Nasus Larynx* 2011;38:583-8.
- E3. Wang H, Bai J, Ding M, Liu W, Xu R, Zhang J, et al. Interleukin-17A contributes to the expression of serum amyloid A in chronic rhinosinusitis with nasal polyps. *Eur Arch Otorhinolaryngol* 2013;270:1867-72.
- E4. Luo Q, Chen F, Liu W, Li Z, Xu R, Fan Y, et al. Evaluation of long-term clarithromycin treatment in adult Chinese Patients with chronic rhinosinusitis without nasal polyps. *ORL J Otorhinolaryngol Relat Spec* 2011;73:206-11.

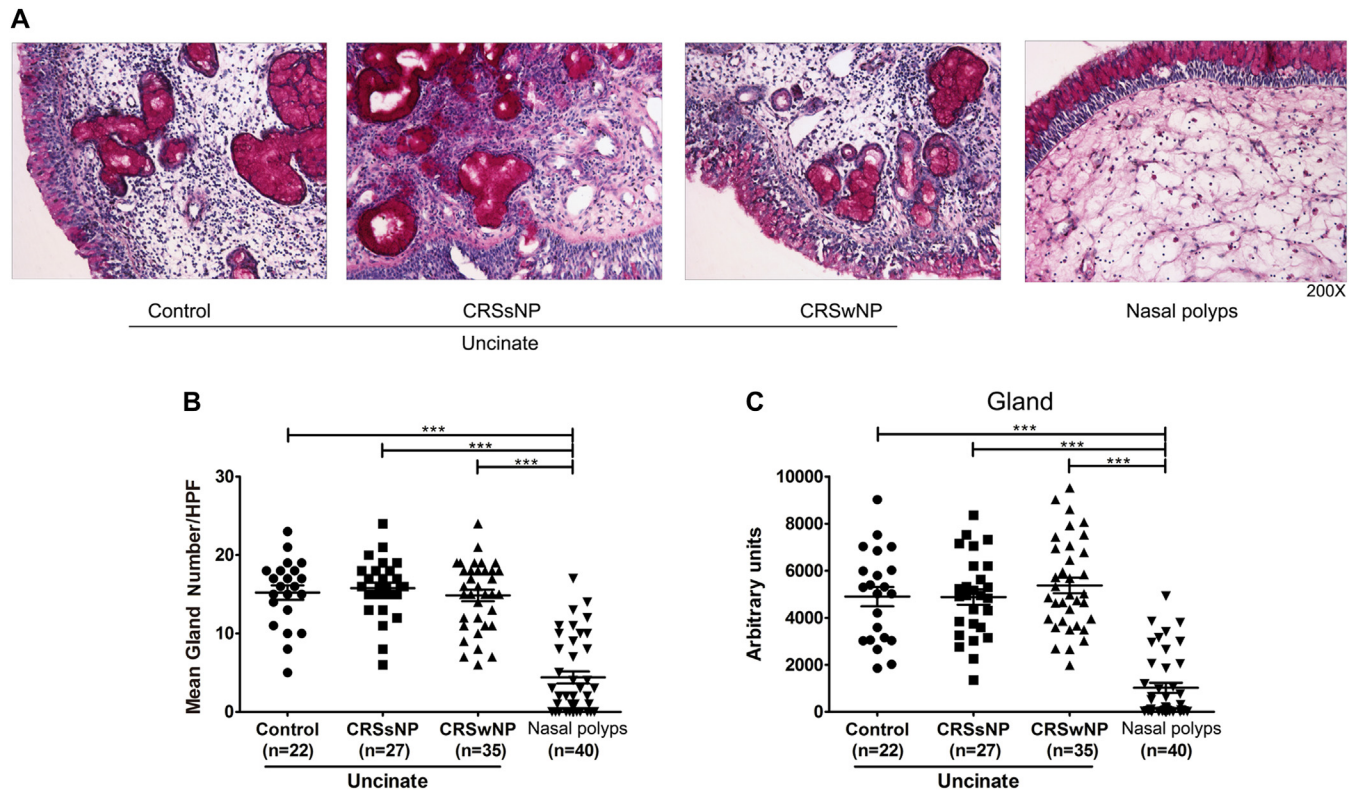


FIG E1. Submucosal gland number and staining intensity in nasal tissues. **A**, Representative results after PAS staining ($\times 200$ magnification). **B** and **C**, Mean number (Fig E1, **B**) and glandular staining intensity (Fig E1, **C**) of submucosal glands. Results are expressed as medians (interquartile ranges). *** $P < .001$.

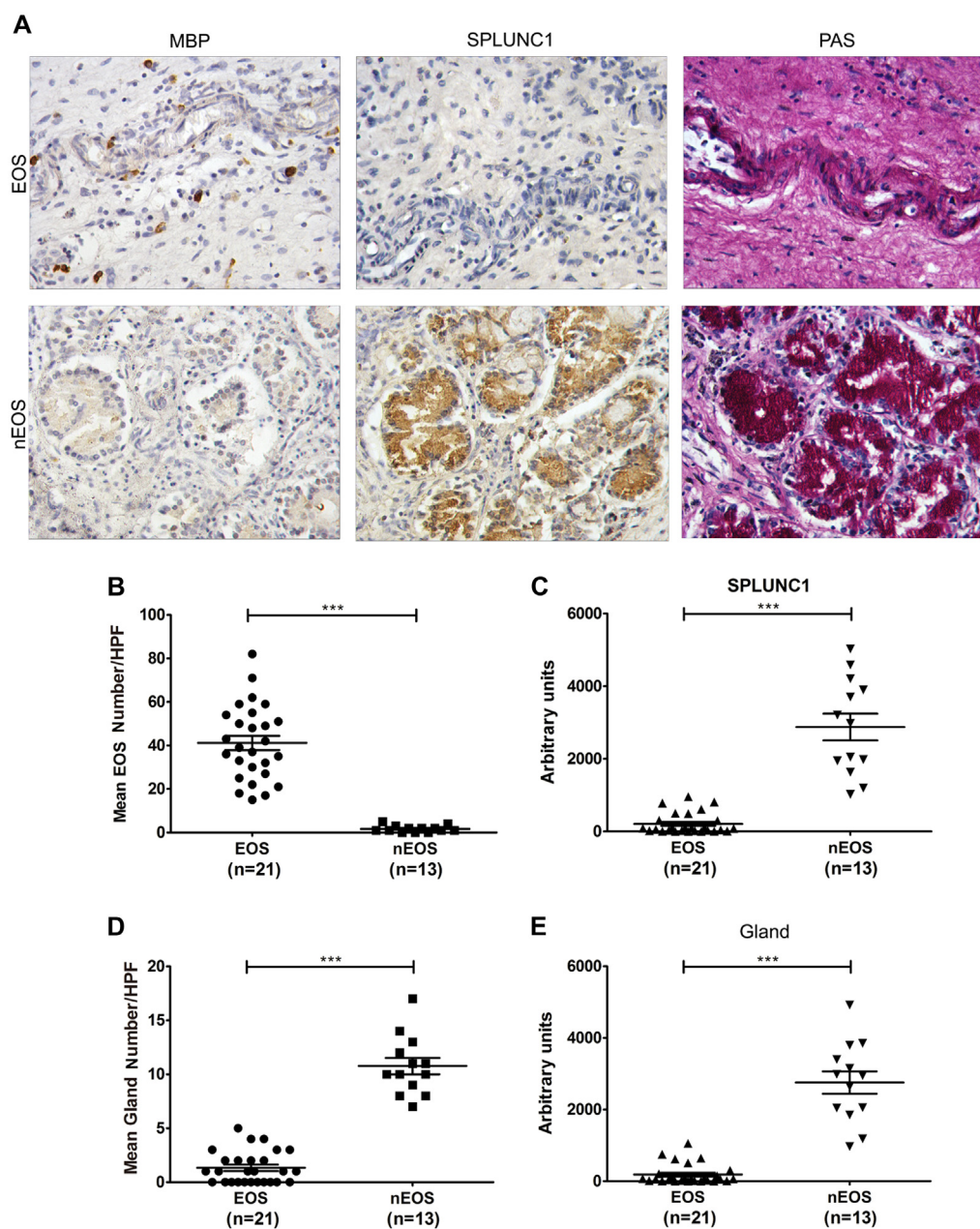


FIG E2. SPLUNC1 expression and submucosal glandular staining in tissue from patients with eosinophilic (EOS) and noneosinophilic (nEOS) CRSwNP. **A**, Representative results of MBP, SPLUNC1, and PAS staining ($\times 200$ magnification). **B**, Number of tissue eosinophils (MBP-positive cells per high-power field [HPF], $\times 200$ magnification). **C**, SPLUNC1 immunostaining intensity. **D** and **E**, Mean number (Fig E2, **D**) and glandular staining intensity (Fig E2, **E**) of submucosal glands. Data are expressed as medians (interquartile ranges). *** $P < .001$.

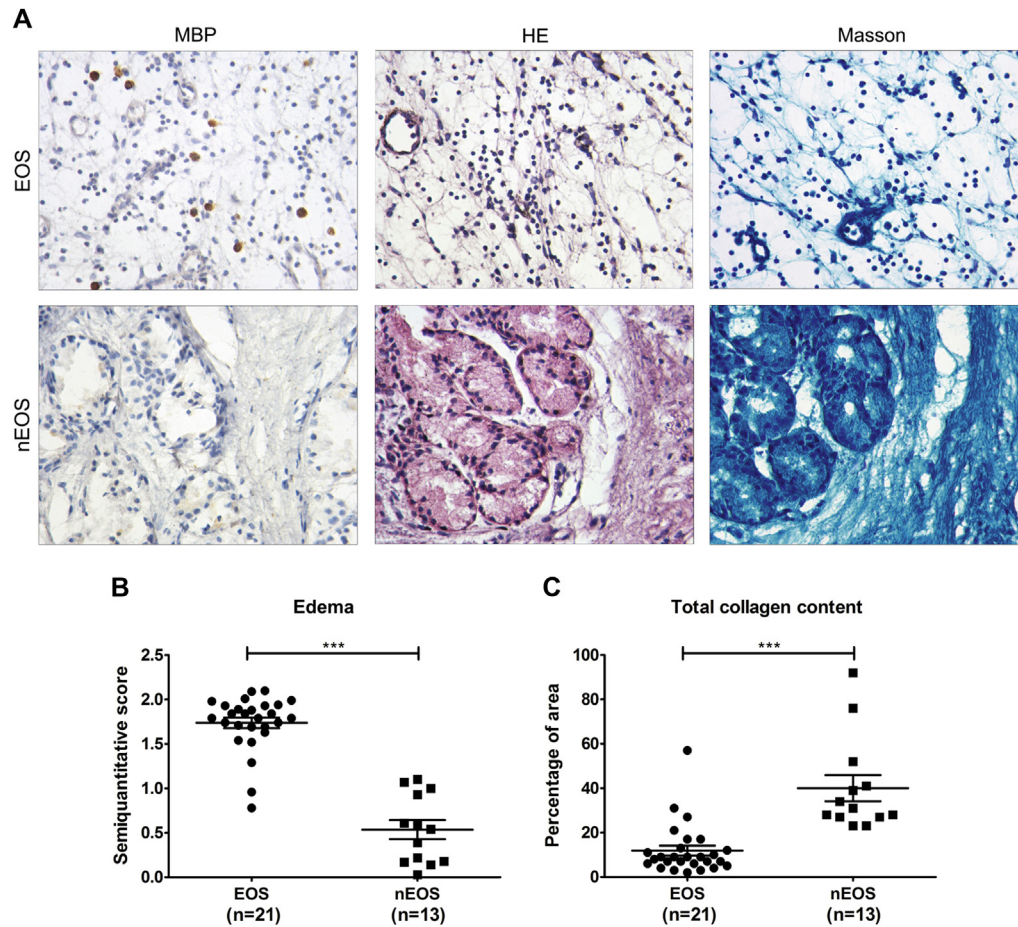


FIG E3. Histologic profiles of tissues from patients with eosinophilic (*EOS*) and noneosinophilic (*nEOS*) CRSwNP. **A**, Representative results of MBP, H&E, and Masson trichrome staining ($\times 200$ magnification). **B**, Semiquantitative score of tissue edema. **C**, Percentage of area covered by collagen deposition. Data are expressed as medians (interquartile ranges). *** $P < .001$.

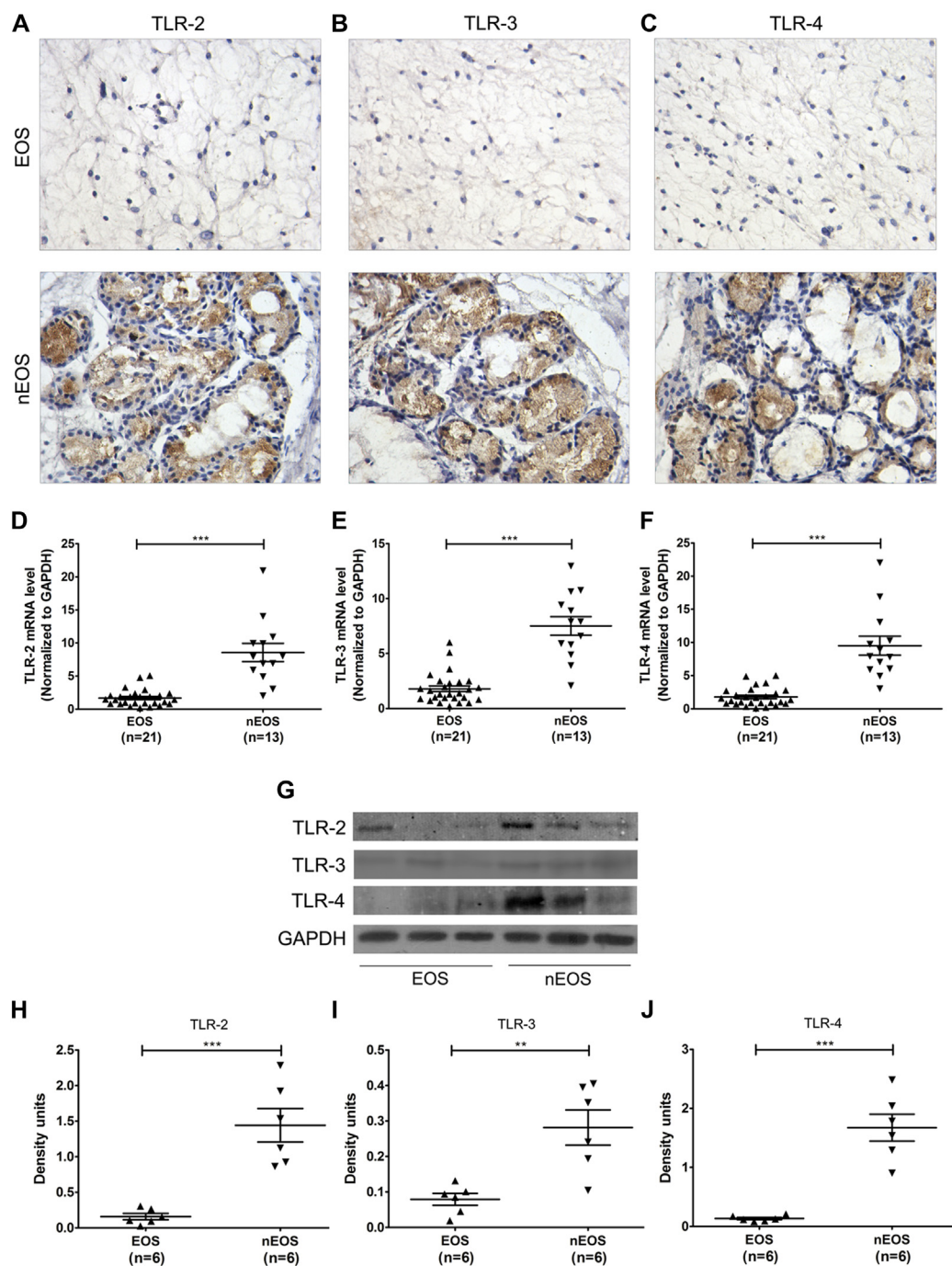


FIG E4. Tissue distribution, as well as mRNA and protein expression, of TLR2, TLR3, and TLR4 in tissue from patients with eosinophilic (*EOS*) and noneosinophilic (*nEOS*) CRSwNP. **A-C**, Representative TLR immunostaining in polyp tissues ($\times 200$ magnification). **D-F**, mRNA expression of TLR2, TLR3, and TLR4. **G-J**, Representative Western blot results (Fig E4, G) and densitometric analysis (Fig E4, H-J) of TLR2, TLR3, and TLR4 protein expression. $**P < .01$ and $***P < .001$.

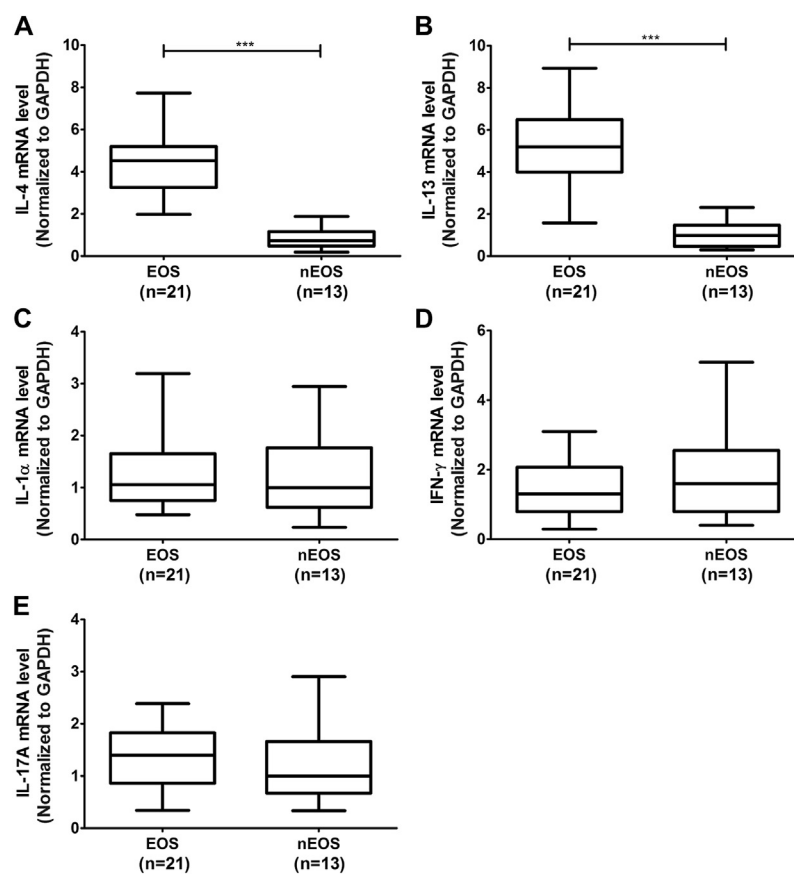


FIG E5. mRNA expression of the proinflammatory cytokines IL-4 (A), IL-13 (B), IL-1 α (C), IFN- γ (D), and IL-17A (E) in eosinophilic (EOS) and noneosinophilic (nEOS) polyp tissues. Data are expressed as medians (interquartile ranges). *** $P < .001$.

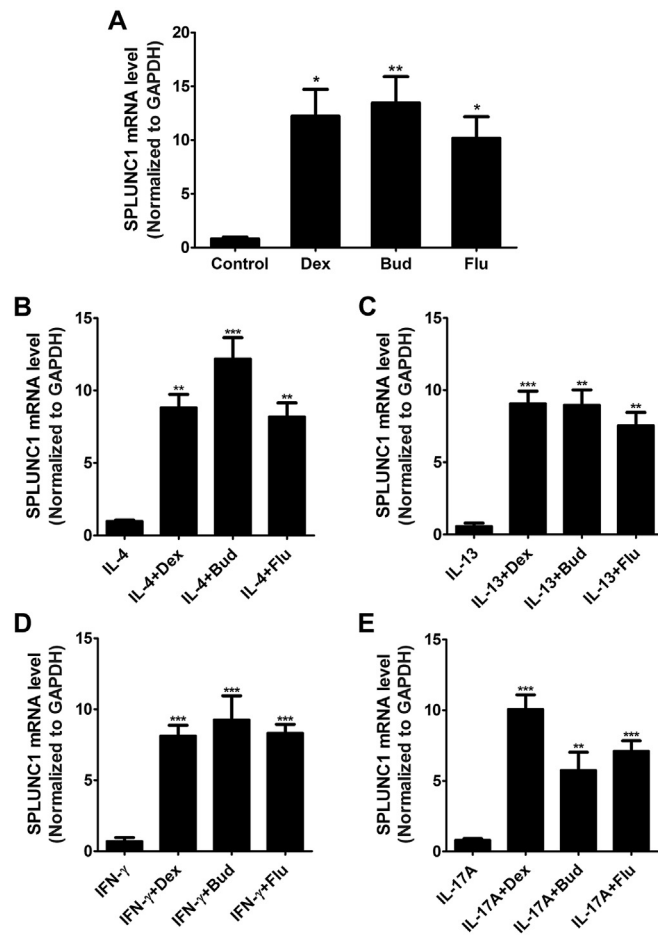


FIG E6. mRNA expression of SPLUNC1 in PECs in response to glucocorticoid treatment. SPLUNC1 mRNA levels were significantly upregulated after stimulation with glucocorticoid alone (**A**) or glucocorticoid in combination with the proinflammatory cytokines IL-4, IL-13, IFN- γ , and IL-17A (**B-E**). Results represent mean values from 3 independent experiments. Data are expressed as means (SEMs). *Bud*, Budesonide; *Dex*, dexamethasone; *Flu*, fluticasone. * $P < .05$, ** $P < .01$, and *** $P < .001$.

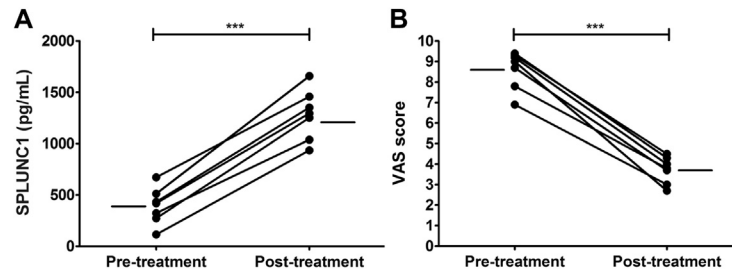


FIG E7. Protein levels of SPLUNC1 in nasal secretions from patients with CRSwNP. Increased SPLUNC1 in nasal secretions (**A**) and improved total nasal symptom scores (visual analog scale [VAS] score); (**B**) before and after patients received glucocorticoid treatment. *Solid bars* represent median values. *** $P < .01$.

TABLE E1. Antibodies used for immunohistochemical staining and WB analysis

Antibody	Company	Catalog no.	Source/host	Dilution of the primary antibody
SPLUNC1	R&D Systems	MAB1897	Mouse	1:100 (for IHC), 1:1000 (for WB)
MBP	AbD Serotec	MCA686S	Mouse	1:100 (for IHC)
TLR2	eBioscience	14-9922-82	Mouse	1:100 (for IHC), 1:1000 (for WB)
TLR3	eBioscience	14-9039-82	Mouse	1:100 (for IHC), 1:1000 (for WB)
TLR4	eBioscience	14-9917-82	Mouse	1:100 (for IHC), 1:1000 (for WB)

IHC, Immunohistochemistry; *WB*, Western blotting.

TABLE E2. Primer sequences for quantitative PCR analysis

Primer	Size (bp)	Sequence
SPLUNC1	102	Forward: 5'-CTTGGCCTTGTGCAGAGC-3' Reverse: 5'-CAACAGACTTGCACCGACC-3'
LPLUNC2	218	Forward: 5'-ATTGCTGGTTTCGGAGTGC-3' Reverse: 5'-GCGTGGGAGGTGCTGTTA-3'
BPI	101	Forward: 5'-TCAACTATGGTCTGGTGGCA-3' Reverse: 5'-GGGAGGTGGATTGTGGTG-3'
TLR2	100	Forward: 5'-GGTCCTGTGCCACCGTTTC-3' Reverse: 5'-GATGTTCTGCTGGGAGCTTT-3'
TLR3	201	Forward: 5'-TGATGCTCCGAAGGGTGG-3' Reverse: 5'-CAGGGTTTGCGTGTTTCC-3'
TLR4	100	Forward: 5'-GGATGAGGACTGGGTAAGGAATG-3' Reverse: 5'-CACACCGGGAATAAAGTCTCTGTA-3'
IL-4	169	Forward: 5'-TTGCTGCCTCCAAGAACACAAC-3' Reverse: 5'-TTCCTGTCGAGCCGTTTCAGGAAT-3'
IL-13	120	Forward: 5'-TGGTCAACATCACCCAGAACCAGA-3' Reverse: 5'-AGCCTGACACGTTGATCAGGGATT-3'
IL-17A	230	Forward: 5'-CAACCGATCCACCTCACC-3' Reverse: 5'-AGCCACGGACACCAGTA-3'
IL-1 α	112	Forward: 5'-TGACGACGCACTTGTAGC-3' Reverse: 5'-CGCCAATGAAATGACTCCCT-3'
IFN- γ	182	Forward: 5'-TGCAGGTCATTGATGTAGCGGA-3' Reverse: 5'-TGTCTTCCTTGATGGTCTCCACACTC-3'
GAPDH	226	Forward: 5'-GAAGGTGAAGGTCGGAGT-3' Reverse: 5'-GAAGATGGTGATGGGATTTC-3'

TABLE E3. Characteristics of 7 patients with eosinophilic CRSwNP

Subject	Eosinophilic CRSwNP
No. of patients	7
Sex, male/female	5/2
Age (y)	38 (26-55)
Duration (y)	4 (3-7)
Skin prick test response, positive/negative	2/5
Asthma history, yes/no	2/5
No. of endoscopic surgeries	2 (1-3)
Smoking, yes/no	3/4
Blood eosinophils ($10^9/L$)	0.65 (0.51-0.84)
Endoscopic score (0, no; 1, mild; 2, medium; 3, severe)	
Mucosal swelling	2 (1-3)
Nasal secretion	2 (1-3)