

Thymic stromal lymphopoietin activity is increased in nasal polyps of patients with chronic rhinosinusitis

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Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is associated with T_H2-dominant inflammation. Thymic stromal lymphopoietin (TSLP) is a cytokine that triggers dendritic cell-mediated T_H2 inflammatory responses and that enhances IL-1-dependent T_H2 cytokine production in mast cells. Although increased TSLP mRNA levels have been found in nasal polyps (NPs), expression of TSLP protein and its function in patients with chronic rhinosinusitis (CRS) have not been fully explored.

Objectives: The objective of this study was to investigate the role of TSLP in patients with CRS.

Methods: We investigated the presence and stability of TSLP protein in NPs using ELISA and Western blotting and investigated the function of TSLP in nasal tissue extracts with a bioassay based on activation of human mast cells.

Results: Although TSLP mRNA levels were significantly increased in NP tissue from patients with CRSwNP compared with uncinatate tissue from patients with CRS or control subjects, TSLP protein was significantly decreased in NP tissue, as detected by using the commercial ELISA kit. We found that recombinant TSLP was time-dependently degraded by NP extracts, and this degradation was completely inhibited by a protease inhibitor cocktail, suggesting that TSLP is sensitive to tissue proteases. Interestingly, NP extract-treated TSLP had

higher activity in mast cells, although the amount of full-length TSLP was reduced up to 85%. NP extracts significantly enhanced IL-1 β -dependent IL-5 production in mast cells compared with uncinatate tissue homogenates, and responses were significantly inhibited by anti-TSLP, suggesting that NPs contain biologically relevant levels of TSLP activity.

Conclusion: TSLP and its metabolic products might play an important role in the inflammation seen in patients with CRSwNP. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: Chronic rhinosinusitis, nasal polyps, thymic stromal lymphopoietin, epithelial cells, mast cells, T_H2 cells, IL-5, proteases

Chronic rhinosinusitis (CRS) is a heterogeneous disease characterized by local inflammation of the upper airways that persists for at least 12 weeks. CRS is one of the most common chronic diseases in adults in the United States, affecting more than 10 million Americans, and has a severe effect on patients' quality of life.¹⁻⁴ CRS is frequently divided into 2 groups based on histology and physical examination: CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP). In general, CRSwNP is associated more closely with clinical complaints of nasal obstruction and olfactory loss and is characterized by eosinophilia and T_H2-related inflammation, especially in western countries.^{5,6} However, the mechanisms underlying the amplification of T_H2-related inflammation in patients with CRSwNP have not been identified.

Thymic stromal lymphopoietin (TSLP), an epithelial cell-derived cytokine, is now widely recognized as a master regulator of T_H2 inflammation. TSLP is an IL-7-like cytokine that stimulates dendritic cells (DCs) to induce naive CD4⁺ T-cell differentiation into T_H2 cells.⁷⁻¹³ Although TSLP alone is not sufficient to stimulate mast cells, TSLP synergizes with the inflammatory cytokine IL-1 to potentially activate mast cells to produce T_H2 cytokines, including IL-5 and IL-13.^{14,15} TSLP, which is produced mainly by epithelial cells, is also known to be produced by skin keratinocytes, stromal cells, smooth muscle cells, fibroblasts, and mast cells.^{7,9,13,16} The thymic stromal lymphopoietin receptor (TSLPR) is a heterodimeric receptor consisting of the IL-7 receptor α chain and a common γ -like TSLPR.¹¹⁻¹³ Considerable evidence now implicates TSLP in the pathogenesis of several T_H2-related inflammatory diseases, including atopic dermatitis, bronchial asthma, and eosinophilic esophagitis.^{9,17-20}

Although CRSwNP is a T_H2-related disease, there are limited studies investigating the role of TSLP in patients with CRS and in nasal polyps (NPs) in particular.^{21,22} In this study we investigated the presence and activity of TSLP in patients with CRS and found that TSLP activity was increased in NPs. Additionally,

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Abbreviations used

CRS:	Chronic rhinosinusitis
CRSsNP:	Chronic rhinosinusitis without nasal polyps
CRSwNP:	Chronic rhinosinusitis with nasal polyps
DC:	Dendritic cell
dsRNA:	Double-stranded RNA
ILC2:	Type 2 innate lymphoid cell
IMDM:	Iscove modified Dulbecco medium
mDC:	Myeloid dendritic cell
2-ME:	2-Mercaptoethanol
NHBE:	Normal human bronchial epithelial cell
NMFF:	Northwestern Medical Faculty Foundation
NP:	Nasal polyp
PIC:	Protease inhibitor cocktail
PNEC:	Primary nasal epithelial cell
SCF:	Stem cell factor
TLR:	Toll-like receptor
TSLP:	Thymic stromal lymphopoietin
TSLPR:	Thymic stromal lymphopoietin receptor
UT:	Uncinate tissue

we found that TSLP was processed by endogenous proteases in tissue, which can convert it to a more active form.

METHODS**Patients and biopsy specimens**

Patients with CRS were recruited from the Allergy-Immunology Clinic and the Otolaryngology Clinic of the Northwestern Medical Faculty Foundation (NMFF) and the Northwestern Sinus Center at NMFF. Sinonasal and NP tissues were obtained from routine functional endoscopic sinus surgery in patients with CRS. All subjects met the criteria for CRS, as defined by the American Academy of Otolaryngology–Head and Neck Surgery Chronic Rhinosinusitis Task Force.² Patients with an established immunodeficiency, pregnancy, coagulation disorder, Churg-Strauss syndrome, or diagnosis of classic allergic fungal sinusitis or cystic fibrosis were excluded from the study. Details of subjects' characteristics are included in Table 1 and in the Methods section in this article's Online Repository at www.jacionline.org. All subjects signed informed consent forms, and the protocol governing procedures for this study was approved by the Institutional Review Board of Northwestern University Feinberg School of Medicine.

Cell culture

Human primary nasal epithelial cells (PNECs) were collected from the uncinate tissue (UT) or NP tissue by means of curettage with a Rhinoprobe (Arlington Scientific, Springville, Utah), as described previously.²³ Human peripheral blood–derived mast cells were obtained, as described previously.²⁴ Recombinant TSLP was preincubated with BSA (control) or NP extracts for 24 hours, and then mast cells were stimulated with those mixtures in the presence of 20 ng/mL IL-1 β for 48 hours. Further details can be found in the Methods section in this article's Online Repository.

Real-time PCR

Real-time RT-PCR was performed with the TaqMan method, as described previously.²⁵ Primer and probe sets were purchased or synthesized from Applied Biosystems (Foster City, Calif). The mRNA expression levels were normalized to the median expression of the housekeeping genes β -glucuronidase (*in vivo*) and β -actin (*in vitro* experiments). Details can be found in the Methods section in this article's Online Repository.

ELISA, cytometric bead array, and Western blotting

The concentration of TSLP in cell-free supernatants was determined by using a commercial ELISA kit (R&D Systems, Minneapolis, Minn). The

minimal detection limit for this kit is 15.6 pg/mL. The concentration of TSLP in tissue homogenates was normalized to the concentration of total protein, as detected with the BCA protein assay kit (Thermo Scientific, Rockford, Ill). The concentration of IL-5 in cell-free supernatants was measured with a cytometric bead array flex set from BD Biosciences (San Jose, Calif). The limit of detection is 2.5 pg/mL. Western blot analysis was performed with 50 ng/mL biotinylated goat anti-human TSLP antibody (R&D Systems). Further details can be found in the Methods section in this article's Online Repository.

Statistics

All data are reported as medians (25% to 75% interquartile ranges) or as means $\pm$ SEMs. Differences between groups were analyzed by using 1-way ANOVA or the paired Student *t* test. Correlations were assessed by using Spearman rank correlation. A *P* value of less than .05 was considered significant.

RESULTS**TSLP expression in patients with CRS**

To examine whether nasal epithelial cells can produce TSLP, we collected epithelial scrapings and cultured PNECs. We found that double-stranded RNA (dsRNA) strongly induced the production of TSLP (73.8 ± 12.8 pg/mL, *n* = 5; see Fig E1, B, in this article's Online Repository at www.jacionline.org) and that IL-4 synergistically enhanced dsRNA-dependent TSLP production (149.7 ± 10.8 pg/mL, *n* = 3; see Fig E1, B) in PNECs (details can be found in the Results section in this article's Online Repository at www.jacionline.org). This suggests that nasal epithelial cells produce TSLP.

Because we found evidence that the resident epithelial cells in nasal mucosa can produce TSLP, we examined the relevance of TSLP in patients with CRS. To measure the expression of TSLP in patients with CRS, UTs, NP tissues, and epithelial scrapings were collected from patients with CRSsNP and patients with CRSwNP, as well as from control subjects. We found that TSLP mRNA levels were significantly increased in epithelial scrapings from NPs compared with UT from control subjects and patients with CRSsNP (Fig 1, A). We also examined the expression of TSLP and TSLPR in whole sinus tissue and found that mRNAs for TSLP and TSLPR were significantly increased in NP tissue from patients with CRSwNP in comparison with UT from either patients with CRS or control subjects (Fig 1, B and C). Interestingly, TSLP expression positively correlated with TSLPR levels in sinus tissue (Fig 1, D). However, we found no significant difference in TSLP levels between atopic and nonatopic patients or asthmatic and nonasthmatic patients (see Fig E2 in this article's Online Repository at www.jacionline.org). To examine this observation at the protein level, we prepared detergent extracts of UT and NP tissues and measured the concentration of TSLP using ELISA. Surprisingly, TSLP protein levels were significantly decreased in NP tissue from patients with CRSwNP (*n* = 35, 27.8 ± 6.4 pg/mg) compared with UT from patients with CRSsNP (*P* < .001, *n* = 25, 51.2 ± 6.2 pg/mg), patients with CRSwNP (*P* < .001, *n* = 24, 41.9 ± 5.0 pg/mg), and control subjects (*P* < .001, *n* = 17, 153.2 ± 31.0 pg/mg; Fig 1, E). Levels of TSLP protein in control UT were higher than in any other tissue set (*P* < .05).

Stability of TSLP in NPs

It is known that TSLP is susceptible to mast cell proteases and that mast cell numbers are increased in NPs.^{26,27} In

TABLE I. Subjects' characteristics

Total no. of subjects	Control subjects n = 47 (24 male)			Patients with CRSsNP n = 60 (22 male)			Patients with CRSwNP n = 86 (62 male)		
	Y	N	U	Y	N	U	Y	N	U
Age (y), median (range)	47 (16-78)			35 (18-67)			44 (26-73)		
Atopy	2	41	4	29	26	5	45	25	16
Asthma	0	46	1	11	47	2	39	47	0
Aspirin intolerance	0	47	0	0	60	0	1	85	0
Nasal steroid	0	47	0	6	53	1	10	73	3
Inhaled steroid	0	47	0	5	54	1	9	74	3
Oral steroid	2	45	0	2	57	1	17	66	3
Methodologies used	UT			UT			UT		
Epithelial scrapings	n = 21 (13 male)			n = 32 (12 male)			n = 22 (17 male)		
Tissue RNA	n = 16 (7 male)			n = 16 (5 male)			n = 17 (11 male)		
Tissue extracts	n = 17 (8 male)			n = 25 (9 male)			n = 24 (15 male)		
TSLP bioassay	n = 10 (5 male)			n = 11 (6 male)			n = 12 (7 male)		
							NP		
							n = 36 (28 male)		
							n = 29 (24 male)		
							n = 35 (24 male)		
							n = 26 (20 male)		

N, No; U, unknown; Y, yes.

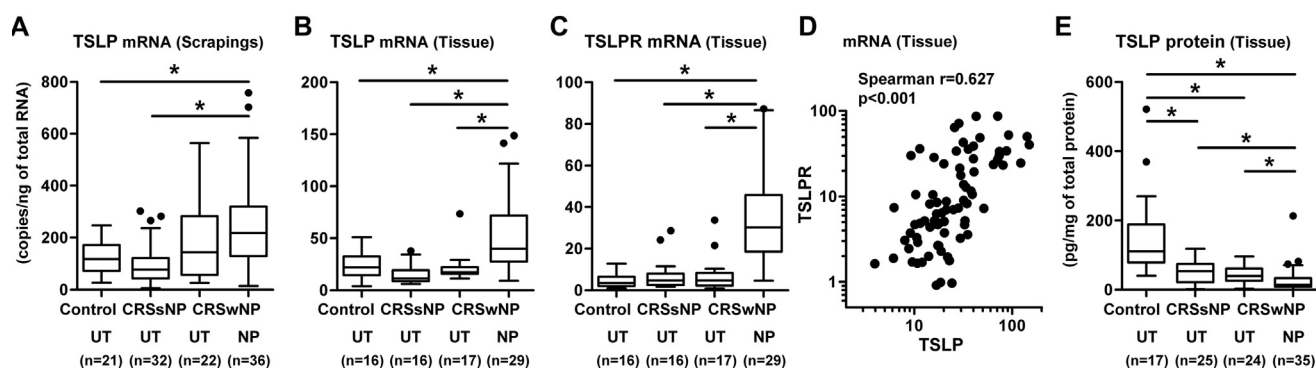


FIG 1. Increased expression of TSLP in NPs. **A-D**, Total RNA was extracted from epithelial scrapings (Fig 1, A) and whole tissue (Fig 1, B-D) from NPs and UT from control subjects and patients with CRSsNP and patients with CRSwNP. Expression of mRNAs for TSLP and TSLPR was analyzed by using real-time PCR (Fig 1, A-D). The correlations were assessed by using Spearman rank correlation (Fig 1, D). **E**, Expression of TSLP protein in tissue homogenates was measured by using ELISA. The TSLP protein concentration was normalized to the concentration of total protein. Results are shown as medians (25% to 75% interquartile ranges). * $P < .05$, 1-way ANOVA.

addition, numbers of inflammatory cells, including eosinophils, neutrophils, and macrophages, that contain various proteases are known to be increased in NPs,^{5,28} and NPs contain various tissue proteases.²⁹ To test whether the decreased TSLP protein detected in NP tissue reflects breakdown of TSLP, we incubated recombinant TSLP with 1 mg/mL NP extracts for 24 and 48 hours and determined the concentration of TSLP using ELISA. TSLP was also incubated with the equivalent concentration of BSA as a control to protect against the absorption of recombinant TSLP onto the incubation tube. We normally generate tissue extracts in the presence of a protease inhibitor cocktail (PIC) to prevent the degradation of molecules during homogenization. In this experiment we needed to assess the effect of protease activity in the tissue extracts. Therefore we prepared tissue extracts without protease inhibitors for use in the TSLP stability assay and TSLP cleavage assay. We found that immunoreactive TSLP detected in ELISAs was significantly reduced by exposure to NP extracts ($P < .001$, $75.2\% \pm 3.4\%$ reduction at 24 hours and $82.8\% \pm 2.6\%$ reduction at 48 hours, $n = 7$) but not by BSA controls (Fig 2, A). We next assessed whether the observed reduction in immunoreactivity to TSLP was due to

complete degradation of TSLP, truncation of TSLP, or both. Recombinant TSLP was incubated with 1 mg/mL NP tissue extracts or BSA for 24 hours, and the stability of TSLP was assessed by means of Western blotting. We found that TSLP was cleaved by NP extracts, although the extent of truncation and degradation varied among patient samples (Fig 2, B, and see Fig E3 in this article's Online Repository at www.jacionline.org). Importantly, we detected truncated TSLP in all samples, and the molecular weight was approximately 10 to 11 kDa (Fig 2, B). We evaluated the cleaved products using SDS-PAGE and Coomassie Blue staining and found that the major truncated product was a 10-kDa protein and that minor products were approximately 5-kDa and 3.5-kDa proteins ($n = 3$, data not shown). Interestingly, a cleaved 10-kDa protein was time-dependently generated by NP extracts, whereas full-length TSLP protein was time-dependently reduced ($n = 3$, Fig 2, C, and see Fig E3, B). When we incubated TSLP and NP extracts in the presence of a PIC or dimethyl sulfoxide (vehicle control), we found that PIC completely blocked NP-dependent degradation of TSLP, suggesting that TSLP was truncated by endogenous tissue proteases ($n = 3$, Fig 2, D).

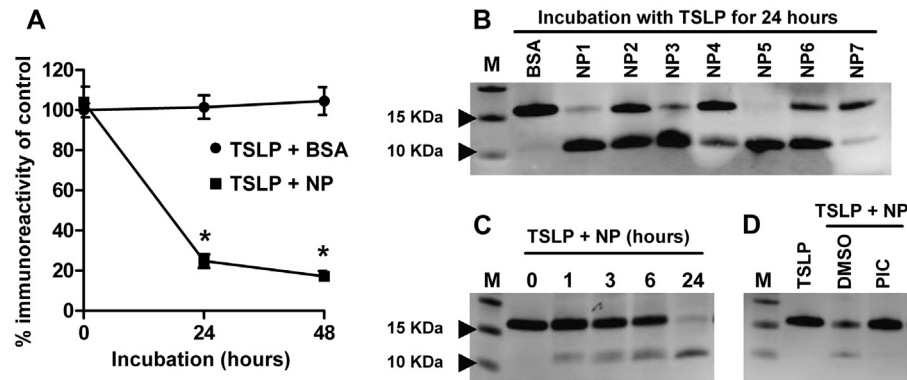


FIG 2. TSLP protein was cleaved by NP extracts. **A**, Recombinant TSLP was incubated with 1 mg/mL NP tissue extracts or 1 mg/mL BSA (control) for 24 to 48 hours, and the stability of TSLP was determined by using ELISA ($n = 5$). **B**, TSLP was incubated with 1 mg/mL BSA or 1 mg/mL NP tissue extracts from donors 1 to 7 (NP1-7) for 24 hours, and cleavage of TSLP was determined by means of Western blotting. **C**, TSLP was incubated with 1 mg/mL NP extracts for 0 to 24 hours. **D**, TSLP was incubated with NP extracts in the presence of 1% PIC or 1% dimethyl sulfoxide (vehicle control) for 24 hours. The results are representative of 3 separate experiments with separate donors (Fig 2, B-D). M, Marker. * $P < .05$, paired Student t test.

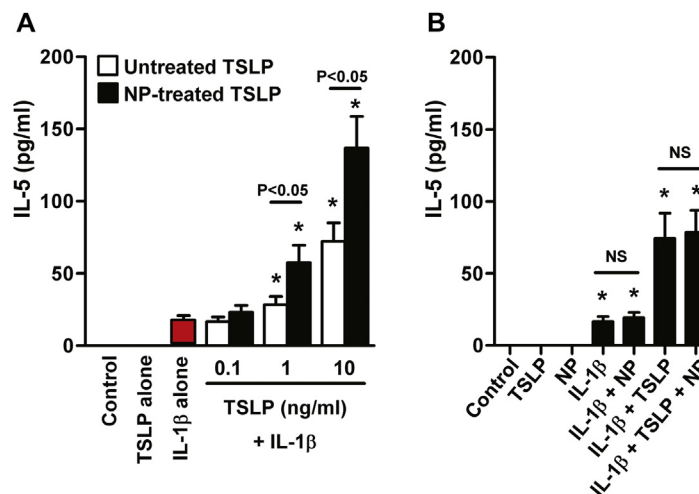


FIG 3. NP extract-treated TSLP had higher activity in mast cells. TSLP was preincubated with NP extract for 24 hours. **A**, Mast cells were then stimulated with NP-treated TSLP and untreated TSLP in the presence of 20 ng/mL IL-1 β for 48 hours ($n = 8$). **B**, Mast cells were stimulated with 4 μ g/mL NP extracts, 20 ng/mL IL-1 β , 10 ng/mL TSLP, or their combination but without preincubation for 48 hours ($n = 5$). The concentration of IL-5 was measured by using the cytometric bead array. * $P < .05$ compared with IL-1 β alone (Fig 3, A, red bar) or medium control (Fig 3, B). NS, Not significant.

Endogenous proteases are involved in the inactivation of proteins by means of degradation and participate in processing of inactive proproteins into their biologically active forms. Therefore we examined whether NP-treated TSLP possessed biological activity. Allakhverdi et al¹⁴ have reported that TSLP is a key enhancer of IL-1-dependent T_H2 cytokine production in mast cells, and we have recently confirmed their findings.¹⁵ Because this response can be inhibited by anti-TSLP antibody,¹⁵ we used human mast cells in a TSLP activity bioassay. To test for TSLP activity, mast cells were stimulated with NP-treated and untreated TSLP in the presence of 20 ng/mL IL-1 β . TSLP dose-dependently enhanced IL-1 β -dependent IL-5 production in mast cells (Fig 3, A). Importantly, this enhancement of potency was time-dependently increased by preincubation with NP extracts (see Fig E4 in this article's Online Repository at www.jacionline.org), and potency was significantly higher in 24 hour NP-treated

TSLP (136.8 ± 22.1 pg/mL at 10 ng/mL TSLP, $n = 8$, $P < .05$) than untreated TSLP (72.2 ± 12.9 pg/mL at 10 ng/mL TSLP, $n = 8$; Fig 3, A), even though immunoreactivity in the TSLP ELISA kit was reduced up to 85% (Fig 2, A). To exclude the possibility that NP products directly enhanced TSLP or IL-1 β activity, mast cells were stimulated with IL-1 β and TSLP in the presence of the same concentration of NP extracts (4 μ g/mL) but without the preincubation. We found that NP extract did not directly enhance IL-1 β - and IL-1 β /TSLP-dependent IL-5 production in mast cells ($n = 6$, Fig 3, B). These results suggest that enhanced activity of TSLP requires preincubation with NP extracts and that cleaved TSLP might have higher activity than the full-length molecule.

TSLP activity in NPs

Our results suggest that TSLP synthesis might be upregulated in NPs and that the TSLP produced was truncated by proteases to

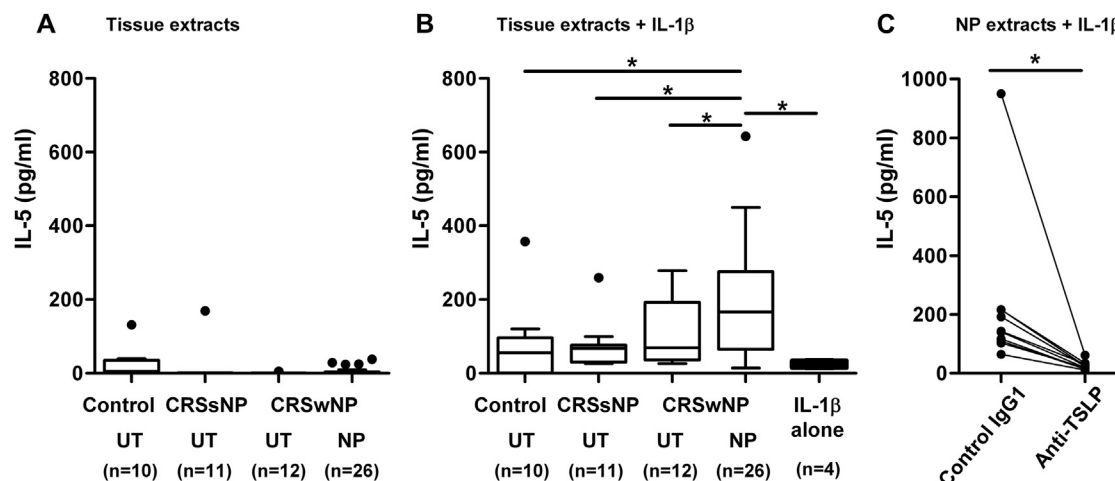


FIG 4. TSLP activity was increased in NPs. **A** and **B**, Mast cells were stimulated with 300 μ g/mL UT extracts or 300 μ g/mL NP extracts in the presence (Fig 4, B) or absence (Fig 4, A) of 20 ng/mL IL-1 β for 48 hours. **C**, Mast cells were stimulated with 300 μ g/mL NP extracts and 20 ng/mL IL-1 β in the presence of control mouse IgG₁ or anti-TSLP ($n = 10$). The concentration of IL-5 was measured by using the cytometric bead array. The concentration of IL-5 in tissue extracts was subtracted. The results are shown as medians (25% to 75% interquartile range). * $P < .05$, 1-way ANOVA (Fig 4, A and B) and paired Student t test (Fig 4, C).

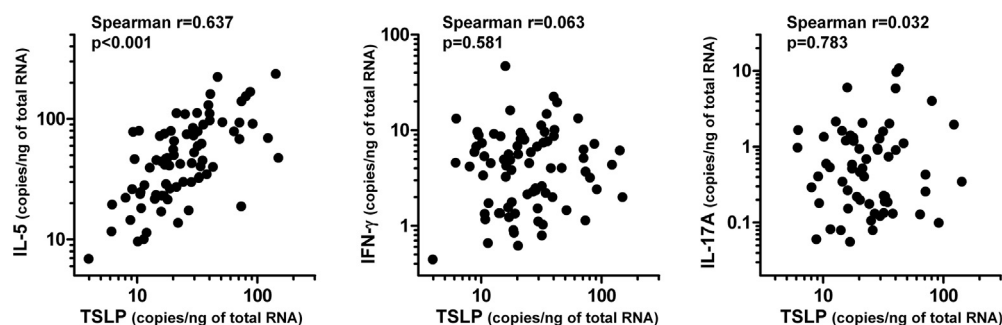


FIG 5. Correlation of TSLP and markers of TH1/TH2/TH17 inflammation in sinus tissue. mRNA for TSLP, IL-5, IFN γ , and IL-17A in sinus tissue that was used in Fig 1, B, was assessed by using real-time PCR ($n = 78$). The correlations were assessed by using Spearman rank correlation.

create more active forms. We therefore examined whether endogenous TSLP activity was increased in NPs, even though TSLP concentrations determined by means of ELISA were reduced. We generated tissue extracts from UTs and NPs and then stimulated mast cells with 300 μ g/mL tissue extracts in the presence of IL-1 β . We found that NP extracts significantly enhanced IL-1 β -dependent IL-5 production in mast cells (191.5 ± 28.8 pg/mL, $n = 26$, $P < .05$) compared with UT from control subjects (76.5 ± 34.3 pg/mL, $n = 10$), patients with CRSsNP (76.0 ± 19.8 pg/mL, $n = 11$), and patients with CRSwNP (106.0 ± 26.8 pg/mL, $n = 12$) and compared with IL-1 β alone (24.9 ± 5.2 pg/mL, $n = 4$; Fig 4, B) or tissue extracts alone (see Fig E5 in this article's Online Repository at www.jacionline.org). In contrast, tissue extracts alone did not stimulate mast cells (Fig 4, A). Furthermore, we found that NP extract-dependent IL-5 production was significantly inhibited by neutralization with an anti-TSLP antibody ($n = 10$, $P < .05$; Fig 4, C). These results suggest that TSLP activity is upregulated in NPs, possibly reflecting a cleavage process leading to a product that has gained bioactivity.

Because TSLP activity was increased in NPs, we examined whether expression of TSLP correlated with TH2 inflammation

in the sinus mucosa. TSLP expression positively correlated with markers of TH2 inflammation, including IL-5 ($r = 0.637$, $P < .001$), IL-13 ($r = 0.532$, $P < .001$), and CCL17 (thymus and activation-regulated chemokine; $r = 0.619$, $P < .001$), and with markers of eosinophilia, including eosinophil cationic protein (ECP; $r = 0.326$, $P = .005$) and Charcot-Leyden crystal protein ($r = 0.614$, $P < .001$), but not with markers of TH1 (IFN γ ; $r = 0.063$, $P = .581$) or TH17 (IL-17A; $r = 0.032$, $P = .783$) inflammation in the sinus mucosa ($n = 78$, Fig 5 and see Fig E6 in this article's Online Repository at www.jacionline.org).

DISCUSSION

CRSwNP is characterized by TH2-mediated inflammation and eosinophilia.^{5,6} However the role of TSLP in patients with CRSwNP is not well understood. In the current study we demonstrated that TSLP mRNA levels were significantly increased in NPs and significantly correlated with markers of TH2 inflammation in the nasal mucosa (Figs 1 and 5). Although TSLP protein levels were reduced in NPs, as detected by using commercial

ELISA, TSLP activity was significantly increased in NPs (Figs 1 and 4). We also found that TSLP protein was truncated by putative endogenous proteases in NPs and that the cleavage product might have more potent activity than its full-length form (Figs 2 and 3).

Although we found that TSLP mRNA levels were increased in NPs, the TSLP-producing cells in NPs are not clearly defined. We therefore initiated immunohistochemistry studies using anti-human TSLP antibody. We found that anti-TSLP antibody did not stain epithelial cells but did stain some inflammatory cells ($n = 4$; see Fig E7, A, in this article's Online Repository at www.jacionline.org), even though increased expression of TSLP mRNA was observed in epithelial cells from NPs (Fig 1, A). Therefore we examined whether epithelial cells stored TSLP or released it immediately after stimulation. Primary normal human bronchial epithelial cells (NHBEs) were stimulated with dsRNA or dsRNA plus IL-4 for 24 hours, and subsequently, supernatants and cell lysates were collected. We found that TSLP protein was detected in supernatants after stimulation with dsRNA (49.1 ± 4.8 pg/mL) or dsRNA plus IL-4 (186.4 ± 30.2 pg/mL) but not in cell lysates ($n = 3$; see Fig E7, B). These results suggest that more than 95% of the TSLP protein synthesized was released from epithelial cells within 24 hours, possibly explaining the absence of TSLP protein detected by using immunohistochemistry.

Glucocorticoids are widely used in the therapeutic management of inflammatory airway diseases and are known as one of the mainstay drugs in the treatment of CRS. In addition, we previously found that a corticosteroid (fluticasone propionate) inhibited dsRNA-dependent TSLP production in bronchial epithelial cells.⁷ When we evaluated whether overexpression of TSLP in patients with CRSwNP was associated with glucocorticoid treatment, we found no significant difference in TSLP levels based on the status of steroid treatment, although it was slightly reduced in treated subjects (see Fig E2, C). Because patients receiving steroids tend to have more severe disease and because we did not have large enough subgroups of patients to make firm conclusions about the effect of steroid treatment ($n = 5$), future studies will be required to test this question prospectively in a larger cohort.

We found that TSLP mRNA expression was increased but TSLP protein levels were decreased in NP tissues, indicating that TSLP protein might not be stable in tissues. Moreover, we found that TSLP protein was time-dependently degraded by NP extracts in the absence of inhibitors of endogenous proteases. To address the discrepancy between TSLP mRNA and protein levels in NPs, we initially hypothesized that TSLP was truncated only by NP extracts and not by UT extracts, the antibodies used in the ELISA did not recognize truncated TSLP, or both. However, we found that UT extracts also degraded recombinant TSLP (see Fig E8, A, in this article's Online Repository at www.jacionline.org). In addition, both capture and detection antibodies in the ELISA kit detected truncated 10-kDa TSLP by means of Western blotting, although antibodies in the ELISA kit had weaker binding to truncated TSLP than full-length TSLP when compared by using SDS-PAGE (Fig E8, B, and data not shown). Although we cannot make a firm conclusion, this apparent reduced affinity of the ELISA antibodies for cleaved TSLP might explain in part why the ELISA shows lower TSLP protein concentration in NPs. We also hypothesized that the TSLP ELISA kit might have more nonspecific reactivity to UT proteins than to NP proteins. We found that nonspecific reactivity appears to be higher in control UT than

NP tissue, although we did not find a relationship between the concentration detected using ELISA and staining intensity determined by means of Western blotting (Fig E8, B, and not shown). These results indicate that the discrepancy in TSLP mRNA and protein levels might be due to reduced sensitivity for truncated TSLP and nonspecific reactivity detected by using the commercial ELISA. However, we cannot make firm conclusions because Western blotting is not a quantitative assay system. Future studies with definitive assays, such as gas chromatography-mass spectrometry or liquid chromatography-mass spectrometry, that are highly specific and sensitive for TSLP protein and TSLP degradation products will be required. The important point in this regard is that both TSLP mRNA and TSLP activity are highly increased in NP tissue.

Several groups have reported that human TSLP is upregulated in patients with T_H2 -related diseases; however, most groups have shown the increase in TSLP mRNA levels using PCR and *in situ* hybridization or the overexpression of TSLP protein using immunohistochemistry.^{9,17,19,20} Immunohistochemistry is not widely considered a sensitive or reliable quantitative assay, making it challenging to determine the levels of TSLP protein in tissues. However, to date, no investigators have reported successful detection or quantitation of TSLP protein in tissues of patients with asthma or atopic dermatitis. These results suggest that TSLP protein might not be stable in tissues of patients with other T_H2 -related diseases and/or current TSLP quantification systems might not have sufficient sensitivity and specificity to detect TSLP in tissue, thus underscoring the challenge of reporting the increase in TSLP protein levels in tissues by using quantitative methods, including ELISA.

We provide here the first evidence that TSLP can be posttranslationally modified by proteases in tissue to generate more active forms. Interestingly, we found that a cleaved 10-kDa protein was also time-dependently generated by NP extracts (Fig 2). Comeau and Ziegler¹³ identified a putative furin cleavage site in the C-terminus of human TSLP protein that was not present in any rodent sequence identified to date.³⁰⁻³² Future studies will be required to identify the sequence of cleaved TSLP and to test whether furin is a key enzyme for this truncation.

We also examined whether we can detect endogenous TSLP in tissue extracts using Western blotting. We detected 25-kDa and 20-kDa proteins using Western blotting with anti-TSLP, and those bands were stronger in NP extracts than in control UT extracts (Fig E8, B). Reche et al³³ reported that human TSLP had putative N-glycosylation sites and that human TSLP adenovirus vector-infected human 293T cells produced a 23-kDa TSLP protein. Future studies will be required to examine whether those protein bands are TSLP.

We recently investigated the presence of mast cells in patients with CRS and found that mast cell numbers were increased only within the NP epithelium in patients with CRSwNP.²⁷ Furthermore, we reported that airway epithelial cells directly promoted T_H2 cytokine production in mast cells during viral infection through the production of IL-1 and TSLP and that this occurred only in a T_H2 microenvironment.¹⁵ It is conceivable that active forms of TSLP protein in NP tissue can act in concert with inflammatory cytokines to stimulate T_H2 cytokine production in mast cells. To this effect, we found NP extracts had significantly enhanced IL-1-dependent IL-5 production in mast cells compared with UT extracts from control subjects and patients with CRS,

and this was significantly blocked by anti-TSLP, suggesting that TSLP activity was upregulated in NPs (Fig 4). In contrast, Fig 3, B, showed that NP extracts did not directly enhance IL-1 β -dependent IL-5 production in mast cells. This discrepancy can be explained by the fact that we used 75-fold lower concentrations of NP extracts (4 μ g/mL) in Fig 3 compared with Fig 4 (300 μ g/mL). Indeed, we found that NP extracts dose-dependently enhanced IL-1-dependent IL-5 production and that 4 μ g/mL NP extract was not adequate to significantly enhance this reaction (see Fig E9, A, in this article's Online Repository at www.jacionline.org). In addition, 4 μ g/mL NP extracts could not induce truncation of TSLP (see Fig E9, B). It will be important to test whether TSLP activity is increased and whether cleavage products can be detected in affected tissue in patients with other T_H2-related diseases.

We also found upregulation of TSLPR mRNA in NPs (Fig 1). TSLPR is known to be highly expressed on myeloid dendritic cells (mDCs), mast cells, and type 2 innate lymphoid cells (ILC2).^{11,13,34} Although accumulation of mast cells in NPs has been clearly reported,²⁷ the presence of mDCs in NPs is not well understood. Recently, a small study in a Chinese cohort by Liu et al²² reported that populations of OX40 ligand-expressing CD11c⁺ DCs were higher in NPs than in control tissue (n = 3 in each group). It will be important to examine whether mDC numbers are increased in NPs and whether they are activated by TSLP because TSLP-stimulated mDCs are known to promote T_H2 differentiation.^{11,13} Finally, Mjosberg et al³⁵ recently reported that numbers of ILC2s, another potential source for T_H2 cytokines, were also increased in NPs. Importantly, they recently discovered that TSLP can directly activate human ILC2s to produce T_H2 cytokines.³⁴ Thus future study will be required to identify the major T_H2 cytokine-producing cells in NPs.

In summary, we report here that TSLP protein is posttranslationally modified by endogenous proteases in NP tissue and that patients with CRSwNP have increased levels of TSLP activity in polypoid tissue. We also found that TSLP levels correlated with markers of T_H2 inflammation in sinus tissue. Our findings indicate that the overproduction and cleavage of TSLP in NPs might contribute to the pathogenesis of CRSwNP and suggest that a similar process could occur in patients with similar T_H2-mediated diseases.

Clinical implications: Overexpression of TSLP in NPs might have a pathogenic role in CRSwNP.

REFERENCES

- Bhattacharyya N. Incremental health care utilization and expenditures for chronic rhinosinusitis in the United States. *Ann Otol Rhinol Laryngol* 2011;120:423-7.
- Meltzer EO, Hamilos DL, Hadley JA, Lanza DC, Marple BF, Nicklas RA, et al. Rhinosinusitis: establishing definitions for clinical research and patient care. *J Allergy Clin Immunol* 2004;114:155-212.
- Kern RC, Conley DB, Walsh W, Chandra R, Kato A, Tripathi-Peters A, et al. Perspectives on the etiology of chronic rhinosinusitis: an immune barrier hypothesis. *Am J Rhinol* 2008;22:549-59.
- Schleimer RP, Kato A, Peters A, Conley D, Kim J, Liu MC, et al. Epithelium, inflammation, and immunity in the upper airways of humans: studies in chronic rhinosinusitis. *Proc Am Thorac Soc* 2009;6:288-94.
- Bachert C, Van Bruene N, Toskala E, Zhang N, Olze H, Scadding G, et al. Important research questions in allergy and related diseases: 3-chronic rhinosinusitis and nasal polyposis—a GALEN study. *Allergy* 2009;64:520-33.
- Van Zele T, Claeys S, Gevaert P, Van Maele G, Holtappels G, Van Cauwenberge P, et al. Differentiation of chronic sinus diseases by measurement of inflammatory mediators. *Allergy* 2006;61:1280-9.
- Kato A, Favoreto S Jr, Avila PC, Schleimer RP. TLR3- and Th2 cytokine-dependent production of thymic stromal lymphopoietin in human airway epithelial cells. *J Immunol* 2007;179:1080-7.
- Kato A, Schleimer RP. Beyond inflammation: airway epithelial cells are at the interface of innate and adaptive immunity. *Curr Opin Immunol* 2007;19:711-20.
- Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol* 2002;3:673-80.
- Ito T, Wang YH, Duramad O, Hori T, Delespesse GJ, Watanabe N, et al. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J Exp Med* 2005;202:1213-23.
- Liu YJ. Thymic stromal lymphopoietin: master switch for allergic inflammation. *J Exp Med* 2006;203:269-73.
- Liu YJ, Soumelis V, Watanabe N, Ito T, Wang YH, Malefyt Rde W, et al. TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. *Annu Rev Immunol* 2007;25:193-219.
- Comeau MR, Ziegler SF. The influence of TSLP on the allergic response. *Mucosal Immunol* 2010;3:138-47.
- Allakhverdi Z, Comeau MR, Jessup HK, Yoon BR, Brewer A, Chartier S, et al. Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potentially activates mast cells. *J Exp Med* 2007;204:253-8.
- Nagarkar DR, Poposki JA, Comeau MR, Biyasheva A, Avila PC, Schleimer RP, et al. Airway epithelial cells activate T(H)2 cytokine production in mast cells through IL-1 and thymic stromal lymphopoietin. *J Allergy Clin Immunol* 2012;130:225-32.e4.
- Bogiatzi SI, Fernandez I, Bichet JC, Marloie-Provost MA, Volpe E, Sastre X, et al. Cutting edge: proinflammatory and Th2 cytokines synergize to induce thymic stromal lymphopoietin production by human skin keratinocytes. *J Immunol* 2007;178:3373-7.
- Ying S, O'Connor B, Ratoff J, Meng Q, Mallett K, Cousins D, et al. Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity. *J Immunol* 2005;174:8183-90.
- Ying S, O'Connor B, Ratoff J, Meng Q, Fang C, Cousins D, et al. Expression and cellular provenance of thymic stromal lymphopoietin and chemokines in patients with severe asthma and chronic obstructive pulmonary disease. *J Immunol* 2008;181:2790-8.
- Rothenberg ME, Spergel JM, Sherrill JD, Annaiah K, Martin LJ, Cianferoni A, et al. Common variants at 5q22 associate with pediatric eosinophilic esophagitis. *Nat Genet* 2010;42:289-91.
- Shikotra A, Choy DF, Ohri CM, Doran E, Butler C, Hargadon B, et al. Increased expression of immunoreactive thymic stromal lymphopoietin in patients with severe asthma. *J Allergy Clin Immunol* 2012;129:104-11, e1-9.
- Kimura S, Pawankar R, Mori S, Nonaka M, Masuno S, Yagi T, et al. Increased expression and role of thymic stromal lymphopoietin in nasal polyposis. *Allergy Asthma Immunol Res* 2011;3:186-93.
- Liu T, Li TL, Zhao F, Xie C, Liu AM, Chen X, et al. Role of thymic stromal lymphopoietin in the pathogenesis of nasal polyposis. *Am J Med Sci* 2011;341:40-7.
- Kato A, Peters A, Suh L, Carter R, Harris KE, Chandra R, et al. Evidence of a role for B cell-activating factor of the TNF family in the pathogenesis of chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol* 2008;121:1385-92, e1-2.
- Kato A, Chustz RT, Ogasawara T, Kulka M, Saito H, Schleimer RP, et al. Dexamethasone and FK506 inhibit expression of distinct subsets of chemokines in human mast cells. *J Immunol* 2009;182:7233-43.
- Kato A, Truong-Tran AQ, Scott AL, Matsumoto K, Schleimer RP. Airway epithelial cells produce B cell-activating factor of TNF family by an IFN-beta-dependent mechanism. *J Immunol* 2006;177:7164-72.
- Okayama Y, Okumura S, Sagara H, Yuki K, Sasaki T, Watanabe N, et al. FcepsilonRI-mediated thymic stromal lymphopoietin production by interleukin-4-primed human mast cells. *Eur Respir J* 2009;34:425-35.
- Takabayashi T, Kato A, Peters AT, Suh LA, Carter R, Norton J, et al. Glandular mast cells with distinct phenotype are highly elevated in chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol* 2012;130:410-20.e5.
- Peterson S, Poposki JA, Nagarkar DR, Chustz RT, Peters AT, Suh LA, et al. Increased expression of CC chemokine ligand 18 in patients with chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol* 2012;129:119-27.e9.
- Van Bruene N, Bachert C. Tissue remodeling in chronic rhinosinusitis. *Curr Opin Allergy Clin Immunol* 2011;11:8-11.
- Quentmeier H, Drexler HG, Fleckenstein D, Zaborski M, Armstrong A, Sims JE, et al. Cloning of human thymic stromal lymphopoietin (TSLP) and signaling mechanisms leading to proliferation. *Leukemia* 2001;15:1286-92.

31. Thomas G. Furin at the cutting edge: from protein traffic to embryogenesis and disease. *Nat Rev Mol Cell Biol* 2002;3:753-66.
32. Seidah NG, Prat A. The biology and therapeutic targeting of the proprotein convertases. *Nat Rev Drug Discov* 2012;11:367-83.
33. Reche PA, Soumelis V, Gorman DM, Clifford T, Liu M, Travis M, et al. Human thymic stromal lymphopoietin preferentially stimulates myeloid cells. *J Immunol* 2001;167:336-43.
34. Mjosberg J, Bernink J, Golebski K, Karrich JJ, Peters CP, Blom B, et al. The transcription factor GATA3 is essential for the function of human type 2 innate lymphoid cells. *Immunity* 2012;37:649-59.
35. Mjosberg JM, Trifari S, Crellin NK, Peters CP, van Drunen CM, Piet B, et al. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nat Immunol* 2011;12:1055-62.

METHODS

Patients and biopsy specimens

Patients with CRS were recruited from the Allergy-Immunology Clinic and the Otolaryngology Clinic of the NMFF and the Northwestern Sinus Center at NMFF. Sinonasal and NP tissues were obtained from routine functional endoscopic sinus surgery in patients with CRS. All subjects met the criteria for CRS, as defined by the American Academy of Otolaryngology–Head and Neck Surgery Chronic Rhinosinusitis Task Force. The presence of sinusitis or bilateral NPs was confirmed by using office endoscopy and computed tomographic imaging. All patients scheduled for surgery had previously failed adequate trials of conservative medical therapy (prolonged antibiotic regimens, nasal steroid sprays, oral steroids, saline irrigations, and decongestants) for control of symptoms. Patients with an established immunodeficiency, pregnancy, coagulation disorder, Churg–Strauss syndrome, or diagnosis of classic allergic fungal sinusitis or cystic fibrosis were excluded from the study. NPs in this study were characterized by T_H2 -related inflammation (IL-4, IL-5, and IL-13) but not by T_H1 - or T_H17 -related inflammation (IFN- γ or IL-17A, Fig E10). Details of subjects' characteristics are included in Table I. Sinus tissues from disease-free control subjects were obtained during endoscopic skull base brain tumor excisions, as well as intranasal procedures for obstructive sleep apnea and facial fracture repairs on patients without a history of CRS, nasal inflammation, or asthma recruited from the Otolaryngology Clinic at NMFF. Subjects underwent skin testing to pollens, dust mites, pets, molds, and cockroach by using Hollister-Stier (Spokane, Wash) extracts. Patients were skin tested to environmental allergens, including pollen, dust mites, molds, and pets, as routinely performed in evaluation of rhinosinusitis at our institution and elsewhere in the United States. Histamine was used as a positive control. Patients who were tested by allergists outside our institution were considered to be atopic if they had positive test results previously. Repeat testing was not conducted unless the diagnosis of atopy was questionable. Skin test results were considered positive if the wheal was 3 mm or greater than that elicited by the negative control; this is used by the majority of the practicing allergists in the United States and indicates the presence of clinically significant specific IgE.^{E1} Patients were taking a variety of medications, including nasal corticosteroids, antihistamines, decongestants, and short- or long-acting β -agonists, among others. All subjects signed informed consent forms, and the protocol governing procedures for this study has been approved by the Institutional Review Board of Northwestern University Feinberg School of Medicine.

Reagents

Recombinant human TSLP, TNF, IL-4, IL-13, IL-17A, and IFN- γ were purchased from R&D Systems. Recombinant human IL-3, IL-6, and stem cell factor (SCF) were purchased from Peprotech (Rocky Hill, NJ). Peptidoglycan, dsRNA (poly[I:C]), and LPS were purchased from InvivoGen (San Diego, Calif). Mouse anti-human TSLP mAb was a kind gift from Amgen (Seattle, Wash).^{E2}

Epithelial cell culture

Human PNECs were collected from the inferior turbinate tissue or UT by using curettage with a Rhinoprobe (Arlington Scientific, Springville, Utah) under a Northwestern University Feinberg School of Medicine Institutional Review Board–approved human subject research protocol. In past studies the purity of epithelial cells collected with this method has been determined to be generally more than 95% based on cytokeratin staining. PNECs for *in vitro* cell culture experiments were collected from patients with CRS or control subjects and expanded in 6-well culture plates. The purity of PNECs for *in vitro* experiments has previously been found to exceed 99%. Three separate lots of primary NHBs were purchased from Lonza (Walkersville, Md). PNECs and NHBs were maintained in serum-free bronchial epithelial cell growth medium (Lonza). PNECs and NHBs were plated in 24-well (PNECs) or 12-well (NHBs) culture plates coated with collagen (PureCol; Advanced BioMatrix, Poway, Calif). Before stimulation, PNECs and NHBs were cultured in bronchial epithelial cell growth medium without

hydrocortisone for at least 36 hours. PNECs were stimulated with 10 μ g/mL peptidoglycan, 25 μ g/mL dsRNA, 1 μ g/mL LPS, 100 ng/mL TNF, 100 ng/mL IL-4, 100 ng/mL IL-6, 100 ng/mL IL-13, 100 ng/mL IL-17A, and 10 ng/mL IFN- γ for 24 hours. NHBs were stimulated with 20 ng/mL IL-4, 5 μ g/mL dsRNA alone, or in combination for 24 hours. After collecting the supernatants, cells were washed with 2 mL of Dulbecco's PBS and harvested in 200 μ L of cell lysis buffer (Cell Signaling Technology, Beverly, Mass) supplemented with a PIC.

Real-time PCR

Nasal epithelial scrapings were collected from the UT or NPs by means of curettage with a Rhinoprobe and were transferred in RNeasy Protect (Qiagen, Valencia, Calif) and stored at -20°C . A portion of nasal tissues for isolation of RNA was transferred in RNeasy Lysis Buffer (Qiagen, Valencia, Calif) and stored at -20°C . Total RNA from sinus tissue was extracted with QIAzol (Qiagen) and cleaned and treated with DNase I by using RNeasy, according to the manufacturer's instructions. Total RNA from epithelial scrapings was extracted with NucleoSpin XS (Clontech Laboratories, Mountain View, Calif) and cleaned and treated with DNase I according to the manufacturer's instructions. The quality of total RNA from sinus tissue was assessed by using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, Calif) with an RNA 6000 Nano LabChip (Agilent Technologies). Single-stranded cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, Calif) and random primers. Semiquantitative real-time RT-PCR was performed with a TaqMan method by using an Applied Biosystems 7500 Sequence Detection System (Applied Biosystems) in 15- μ L reactions (7.5 μ L of $2\times$ TaqMan Gene Expression Master mix, [Applied Biosystems], 400 nmol/L of each primer, and 200 nmol/L of TaqMan probe plus cDNA). Primer and probe sets for TSLP (sense, 5'-CCCAGGCTATTCGGAACTCA-3'; antisense, 5'-ACGCCA-CAATCCTTGTAATTGTG-3'; and FAM/MGB probe, 5'-AAGGAAAGT-CACAACCAATAA-3'), TSLPR (sense, 5'-TGGTGTCTCTCTCTCTTCTGTCTTTA-3'; antisense, 5'-TGGCACGCTGGGAATGAG-3'; and FAM/MGB probe, 5'-AAATTATGGAGAGTGAAGAAGTT-3'), IL-5 (sense, 5'-AGCTGCCACGTGTATGCCA-3'; antisense, 5'-GTGCCAAGGTC TCTTTCACCA-3'; and FAM/BHQ1 probe, 5'-CCCCACAGAAATTCCTCA-CAAGTGCA-3'), IFNG (Hs00989291_m1), IL-4 (Hs00174122_m1), IL-17A (Hs00936345_m1), IL-13 (sense, 5'-AAGGTCTCAGCTGGG-CAGTTTA-3'; antisense, 5'-AACTGGGCCACCTCGATT-3'; and FAM/BHQ1 probe, 5'-CCAGCTTGCATGTCCGAGACACCA-3'), CCL17 (sense, 5'-CTGCAAAGCCTTGAGAGGTCTT-3'; antisense, 5'-ACGGTG-GAGGTCCCAGGA-3'; and FAM/BHQ1 probe, 5'-CCTCCTCACCCCA-GACTCCTGACTGTC-3'), ECP (Hs01923184_s1), Charcot-Leyden crystal protein (CLC; Hs00171342_m1), β -actin (sense, 5'-CTGGCCGGGACCT-GACT-3'; antisense, 5'-GCAGCCGTGGCCATCTC-3'; and FAM/MGB probe, 5'-CACCAACACGGCCGA-3'), and β -glucuronidase (human β -glucuronidase endogenous control, PN; 4326320E) were purchased from Applied Biosystems or Integrated DNA Technologies (Coralville, Iowa). To determine the exact copy number of the target genes, quantified aliquots of purified PCR fragments of the target genes were serially diluted and used as standards in each experiment. Aliquots of cDNA equivalent to 10 ng of total RNA were used for real-time PCR. The mRNA expression levels were normalized to the median expression of the housekeeping genes β -glucuronidase or β -actin (only *in vitro* study). Expression of β -glucuronidase was not significantly different among the 4 groups (data not shown).

Tissue homogenates

Freshly obtained tissue specimens were weighed, and 1 mL of PBS supplemented with 0.05% Tween 20 (Sigma-Aldrich, St Louis, Mo) and 1% PIC (PN; P8340, Sigma-Aldrich) was added for every 100 mg of tissue. The tissue was then homogenized with a Bullet Blender Blue (Next Advance, Averill Park, NY) at setting 7 for 8 minutes at 4°C . After homogenization, the suspension was centrifuged at 4000 rpm for 20 minutes at 4°C , and the supernatants were stored at -80°C until analysis. For the TSLP stability and cleavage assay, we prepared tissue homogenates in PIC-free and Tween 20-free conditions.

TSLP cleavage assay and Western blot analysis

Recombinant TSLP (Peprotech) was preincubated with 1 mg/mL tissue extracts or 1 mg/mL BSA (control) for 1 to 24 hours. Mixtures were dissolved in LDL sample buffer (Invitrogen). To detect TSLP, reduced samples were resolved on a 4–12% NuPAGE Bis-Tris Gel (Invitrogen) in an MES buffer system, and then proteins were transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, Calif). The membranes were blocked in blocking buffer for fluorescent Western blotting (Rockland, Gilbertsville, Pa) for 2 hours and subsequently incubated overnight at 4°C with 50 ng/mL biotinylated goat anti-human TSLP polyclonal antibody (R&D Systems). After primary antibody incubation, the membranes were washed repeatedly in PBS/0.1% Tween 20 and then labeled by 45 minutes of incubation at room temperature with IRDye 700DX–conjugated streptavidin (1:50,000, Rockland Immunochemicals). Protein was detected on an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, Neb).

Reduced control UT or NP tissue extracts (18 µg) were resolved on a 4–12% NuPAGE Bis-Tris Gel in an MES buffer system, and then proteins were transferred onto a polyvinylidene difluoride membrane. The membranes were blocked in blocking buffer for fluorescent Western blotting for 2 hours and subsequently incubated overnight at 4°C with 1 µg of capture antibody or 100 ng/mL detection antibody from the TSLP ELISA Kit (R&D Systems). After primary antibody incubation, the membranes were washed repeatedly in PBS/0.1% Tween 20 and then labeled by 45 minutes of incubation at room temperature with 2 µg/mL 700-conjugated Rabbit anti-Sheep IgG IRDye (Rockland Immunochemicals) or IRDye 700DX–conjugated streptavidin (1:50,000).

Mast cell TSLP bioassay

Buffy coats from human peripheral blood were obtained from the American Red Cross (St Paul, Minn). CD34⁺ hematopoietic progenitor cells were separated from human PBMCs by using the MACS system (Miltenyi Biotec, Auburn, Calif) with anti-CD34 MicroBeads (Miltenyi Biotec), according to the manufacturer's instructions. The cells were suspended in serum-free Iscove methylcellulose medium (MethoCult SFBIT H4236; STEMCELL Technologies, Vancouver, British Columbia, Canada) containing 20 ng/mL SCF, 50 ng/mL IL-6, 5 ng/mL IL-3, 11 µmol/L 2-mercaptoethanol (2-ME; Invitrogen), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen) and then incubated at 37°C in 5% CO₂. At 2 weeks of culture, fresh methylcellulose medium containing 200 ng/mL SCF, 50 ng/mL IL-6, 11 µmol/L 2-ME, 100 U/mL penicillin, and 100 µg/mL streptomycin was layered over the methylcellulose cultures. At 4 weeks, a 1-mL aliquot of Iscove modified Dulbecco medium (IMDM) supplemented with 200 ng/mL SCF, 50 ng/mL IL-6, insulin-transferrin-selenium (Invitrogen), 55 µmol/L 2-ME, 100 U/mL penicillin, and 100 µg/mL streptomycin was layered over the methylcellulose cultures. At 6 weeks, all cells were retrieved after dissolving the methylcellulose medium with PBS. The cells were then suspended and cultured in IMDM supplemented with 100 ng/mL SCF, 50 ng/mL IL-6, 0.1% BSA, insulin-transferrin-selenium, 55 µmol/L 2-ME, 100 U/mL penicillin, and 100 µg/mL streptomycin, and the culture medium was replaced a week later. After an additional week of culture, the culture medium was switched to IMDM supplemented with 100 ng/mL SCF, 50 ng/mL IL-6, 5% FBS (Invitrogen), 55 µmol/L 2-ME, 100 U/mL penicillin, and 100 µg/mL streptomycin. The culture medium was changed weekly thereafter, and the cells were incubated for an additional 5 to 10 weeks. We generated human mast cells from more than 8 donors. The final purity of the mast cells was determined by measuring FcεRI expression with flow cytometry after 12 weeks of culture and after 48 hours of stimulation with 1 µg/mL myeloma IgE, and the expression always exceeded 95%.

TSLP (2.5 µg/mL, R&D Systems) was preincubated with 1 mg/mL NP tissue extracts for 24 hours. The mast cells were then stimulated with 0.1 to 10 ng/mL untreated or NP-treated TSLP, which contained 0.04 to 4 µg/mL NP extracts in the presence of 20 ng/mL IL-1β (R&D Systems) at 37°C for

48 hours. In select experiments mast cells were stimulated with 300 µg/mL NP extracts and 20 ng/mL IL-1β in the presence of 16 µg/mL mouse anti-TSLP or 16 µg/mL mouse control IgG₁ (clone P3; eBiosciences, San Diego, Calif) for 48 hours.

Immunofluorescence assay

Nasal tissue was dehydrated, infiltrated, and embedded with paraffin and sectioned at 3 µm with a Leica RM2240 Cryostat. Rehydrated tissue sections were trypsinized for 10 minutes at 37°C to induce antigen retrieval. After washing, tissue sections were blocked for nonspecific binding with 5% goat serum/0.3% Tween-20/PBS. Tissue sections were then incubated with 17 µg/mL mouse anti-TSLP mAb and 0.1 µg/mL mouse anti-human ECP mAb (clone EG2, IgG₁; Diagnostics Development, Uppsala, Sweden) in blocking buffer at 4°C overnight. In control experiments sections were incubated with the same concentrations of control mouse IgG₁ (clone P3, eBiosciences) and control mouse IgG_{2b} (clone eBMG2b, eBiosciences). After washing, sections were incubated with 4 µg/mL Alexa Fluor 488–conjugated goat anti-mouse IgG_{2b} (Invitrogen) and 4 µg/mL Alexa Fluor 568–conjugated goat anti-mouse IgG₁ (Invitrogen) for 1 hour at room temperature in the dark. Sections were then washed and incubated with 0.1% Sudan Black solution for 10 minutes in the dark. After a final wash, cover slips were mounted onto slides by using SlowFade Gold antifade reagent with 4',6-diamidino-2-phenylindole (Invitrogen). Images from immunofluorescence slides were obtained with an Olympus IX71 inverted research microscope using a ×40 objective lens and collected with SlideBook software (Olympus, Center Valley, Pa).

RESULTS

TSLP expression in patients with CRS

We have previously shown that TSLP is synergistically induced in primary human bronchial epithelial cells through the combination of the T_H2 cytokine IL-4 and either dsRNA, a Toll-like receptor (TLR) 3 ligand and a viral mimic, or rhinovirus infection in a TLR3- and STAT6-dependent manner.^{E3} To examine whether the same mechanism occurs in the nasal epithelium, we collected epithelial scrapings and cultured PNECs. We stimulated PNECs with cytokines and TLR ligands and subsequently examined the expression of TSLP mRNA by using real-time PCR. We found that dsRNA was the strongest stimulus for TSLP expression in PNECs (Fig E1, A). Although the T_H2 cytokines IL-4 and IL-13 weakly but significantly induced expression of TSLP, IL-4 synergistically enhanced dsRNA-dependent TSLP expression (Fig E1, A). We confirmed this result at the protein level, showing that dsRNA strongly induced the production of TSLP (73.8 ± 12.8 pg/mL, n = 5; Fig E1, B). IL-4 alone could not induce TSLP protein; however, IL-4 synergistically enhanced dsRNA-dependent TSLP production (149.7 ± 10.8 pg/mL, n = 3; Fig E1, B). This suggests that nasal epithelial cells produce TSLP and that the regulation of TSLP is similar to that seen in bronchial epithelial cells.

REFERENCES

- Bernstein IL, Li JT, Bernstein DI, Hamilton R, Spector SL, Tan R, et al. Allergy diagnostic testing: an updated practice parameter. *Ann Allergy Asthma Immunol* 2008;100(suppl):S1-148.
- Nagarkar DR, Poposki JA, Comeau MR, Biyasheva A, Avila PC, Schleimer RP, et al. Airway epithelial cells activate T(H)2 cytokine production in mast cells through IL-1 and thymic stromal lymphopoietin. *J Allergy Clin Immunol* 2012; 130:225-32.e4.
- Kato A, Favoreto S Jr, Avila PC, Schleimer RP. TLR3- and Th2 cytokine-dependent production of thymic stromal lymphopoietin in human airway epithelial cells. *J Immunol* 2007;179:1080-7.

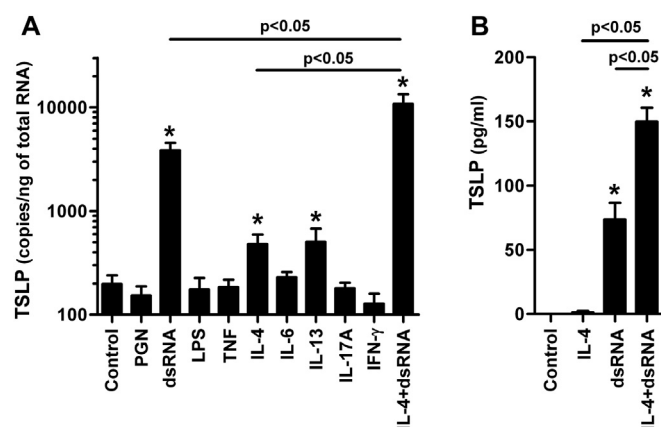


FIG E1. Expression of TSLP in nasal epithelial cells. PNECs were incubated for 6 (**A**) or 24 (**B**) hours with 10 μ g/mL peptidoglycan (PGN; $n = 4$), 25 μ g/mL dsRNA ($n = 7$), 1 μ g/mL LPS ($n = 4$), 100 ng/mL TNF ($n = 4$), 100 ng/mL IL-4 ($n = 7$), 100 ng/mL IL-6 ($n = 3$), 100 ng/mL IL-13 ($n = 3$), 100 ng/mL IL-17A ($n = 3$), and 10 ng/mL IFN- γ ($n = 7$), as indicated, and then expression of mRNA for TSLP was analyzed by using real-time PCR (Fig E1, A) and detection of TSLP protein by using ELISA ($n = 5$; Fig E1, B). Results shown are means $\pm$ SEMs of 3 to 7 independent donors. * $P < .05$ compared with medium control.

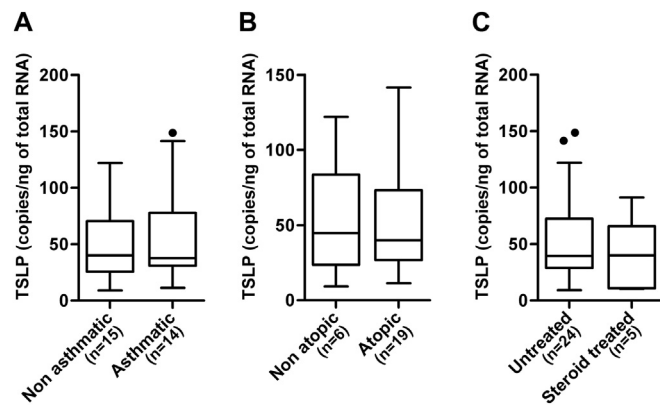


FIG E2. Asthmatic or atopic status or steroid treatment did not affect TSLP levels in NPs. Total RNA was extracted from whole tissue from NPs, and expression of mRNA for TSLP was analyzed by using real-time PCR (**A-C**). The results are shown as medians (25% to 75% interquartile ranges).

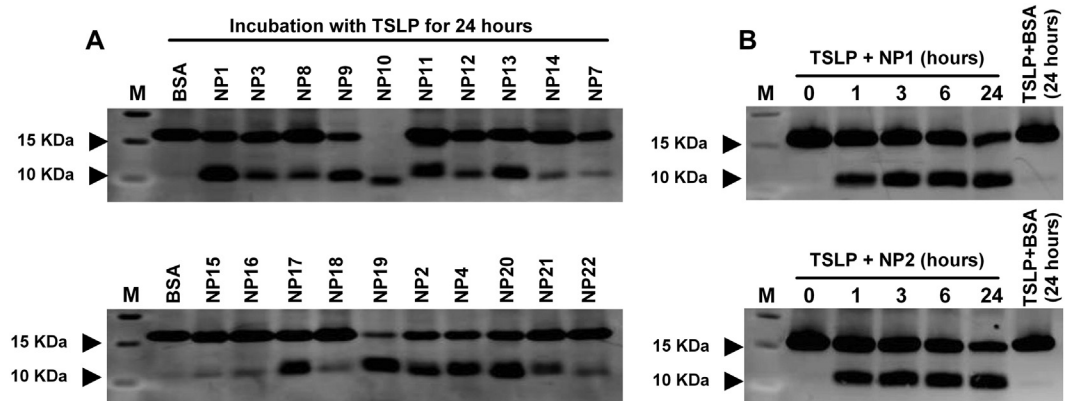


FIG E3. TSLP protein was cleaved by NP extracts. Recombinant TSLP was incubated with 1 mg/mL BSA or 1 mg/mL NP tissue extracts from donors 1 to 22 (NP1-22) for 24 (A) or 0 to 24 (B) hours, and cleavage of TSLP was determined by means of Western blotting. *M*, Marker.

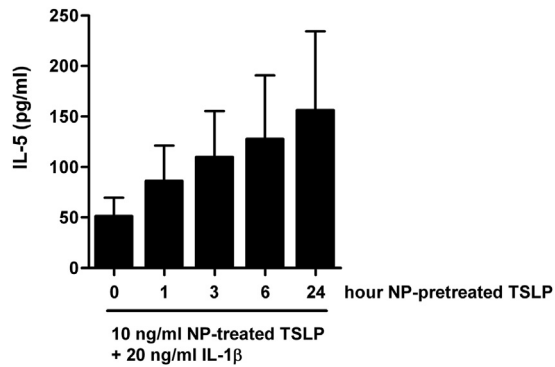


FIG E4. Time dependency of pretreatment of TSLP by NP extracts on the function of mast cells. TSLP (2.5 μ g/mL) was preincubated with 1 mg/mL NP extracts for 0 to 24 hours. Mast cells were then stimulated with 10 ng/mL NP-pretreated TSLP in the presence of 20 ng/mL IL-1 β for 48 hours ($n = 4$). The concentration of IL-5 was measured by using the cytometric bead array.

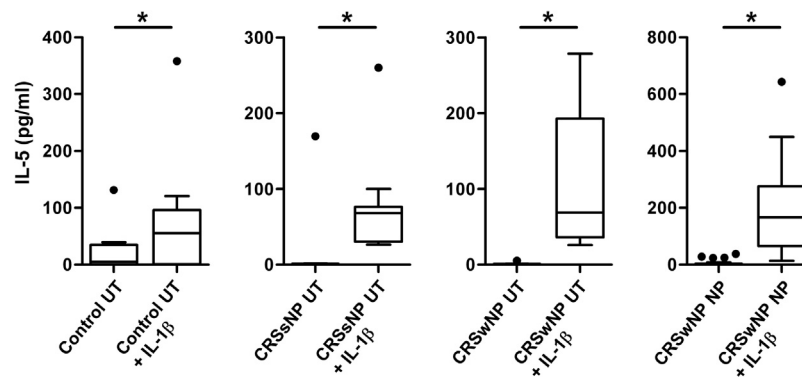


FIG E5. Mast cells were stimulated with 300 μ g/mL UT extracts or 300 μ g/mL NP extracts in the presence or absence of 20 ng/mL IL-1 β for 48 hours. The results are shown as medians (25% to 75% interquartile ranges). * $P < .05$, paired Student t test.

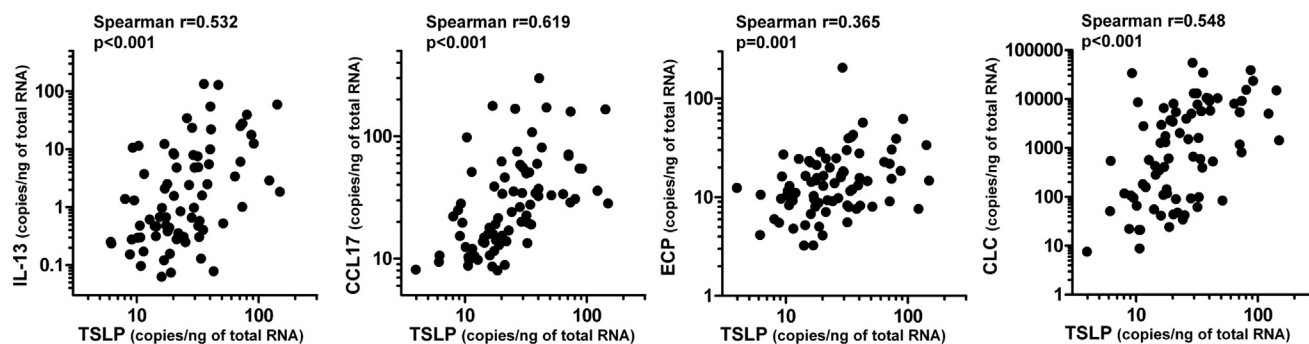


FIG E6. Correlation of TSLP and markers of T_H2 inflammation and eosinophilia in sinus tissue. mRNA for TSLP, IL-13, CCL17, ECP, and Charcot-Leyden crystal protein (*CLC*) was assessed by using real-time PCR ($n = 78$). The correlations were assessed by using Spearman rank correlation.

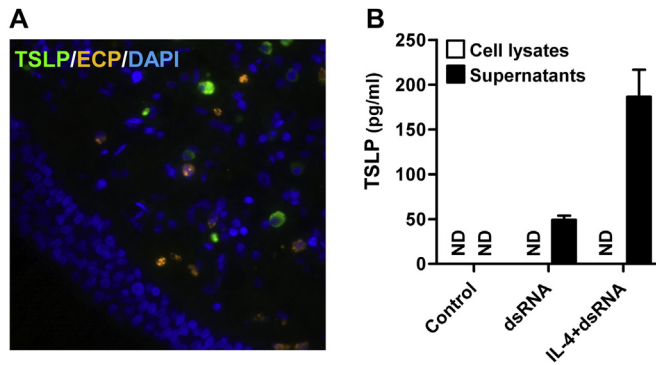


FIG E7. Expression of TSLP in NPs. **A**, Immunofluorescence assay was performed by using anti-TSLP mAb (*green* fluorescence) and anti-ECP mAb (for eosinophils, *orange* fluorescence). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; *blue* fluorescence). Results are representative of 4 separate patients. **B**, NHBEs were stimulated with medium control, 5 μ g/mL dsRNA alone, or 5 μ g/mL dsRNA plus 20 ng/mL IL-4 for 24 hours. Protein expression of TSLP in supernatants and cell lysates was analyzed by using ELISA. Results shown are means $\pm$ SEMs of 3 independent experiments with independent NHBE donors. ND, Not detected.

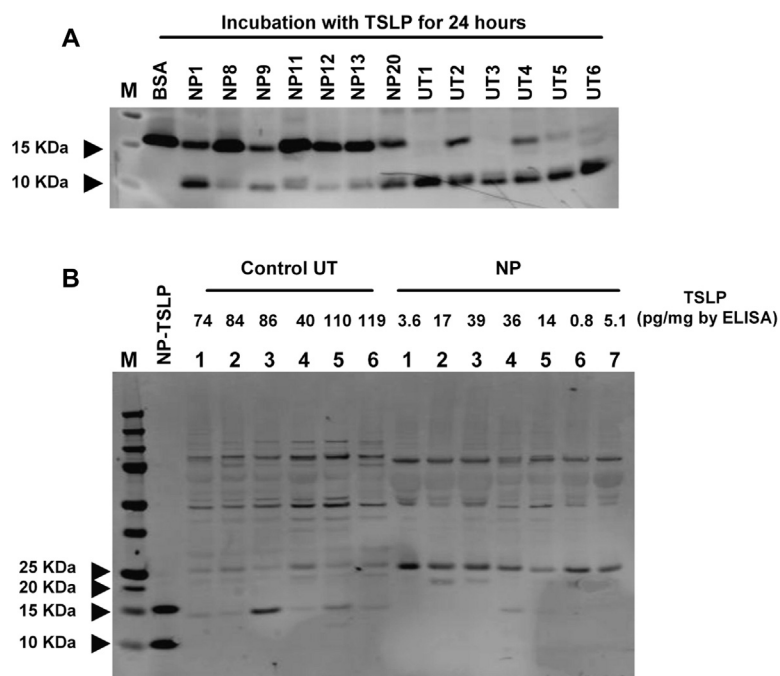


FIG E8. A, Recombinant TSLP was incubated with 1 mg/mL BSA, 1 mg/mL NP tissue extracts, or 1 mg/mL UT extracts from control subjects for 24 hours, and cleavage of TSLP was determined by means of Western blotting. **B,** Reduced control UT or NP tissue extracts (18 μ g) were resolved by means of SDS-PAGE. After transferring proteins onto a membrane, TSLP levels were determined by 0.1 μ g of detection antibody derived from the TSLP ELISA kit. We used NP extract 24-hour pretreated recombinant TSLP as a positive control. Results are representative of 2 separate experiments. *M*, Marker.

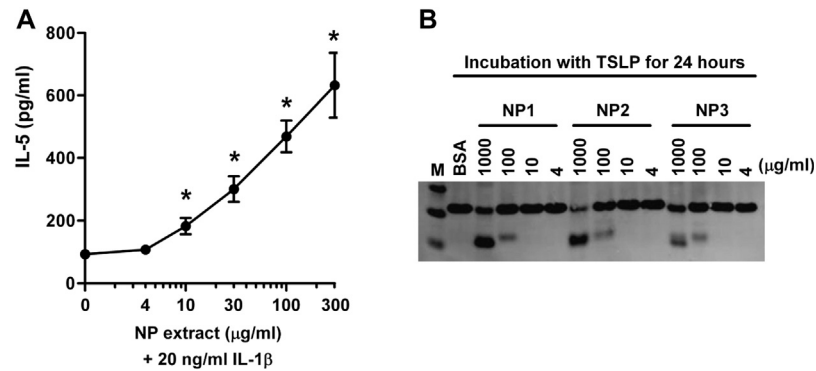


FIG E9. Effect of NP extracts on the activation of mast cells. Mast cells were stimulated with 4 to 300 $\mu\text{g/mL}$ NP extracts in the presence of 20 ng/mL IL-1 β for 48 hours. **A**, The expression of IL-5 protein in supernatants was analyzed by using the cytometric bead array. Results shown are means $\pm$ SEMs of 5 separate patients with NPs. **B**, The cleavage of TSLP was determined by means of Western blotting. * $P < .05$ compared with IL-1 β alone. M, Marker.

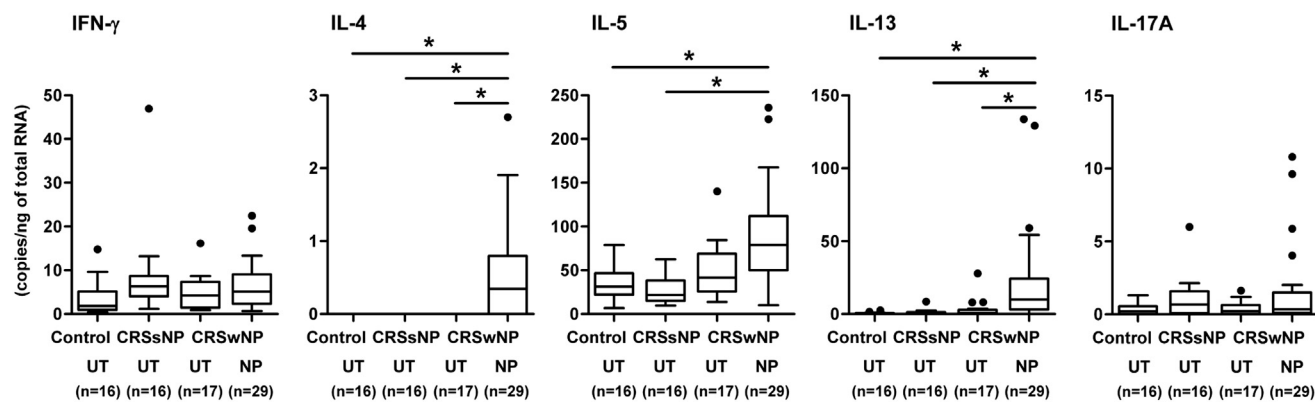


FIG E10. Increased expression of T_H2-related cytokines in NPs. Total RNA was extracted from whole tissue from NPs and UT from control subjects and patients with CRSsNP and CRSwNP. Expression of mRNAs for IFN γ , IL-4, IL-5, IL-13, and IL-17A was analyzed by using real-time PCR. Results are shown as medians (25% to 75% interquartile ranges). * $P < .05$, 1-way ANOVA.