TSLP regulates eotaxin-1 production by nasal epithelial cells from patients with eosinophilic CRSwNP*

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Background: Eosinophilic chronic rhinosinusitis with nasal polyps (eCRSwNP) is characterized by Th2-skewed inflammation with eosinophilic infiltration. Thymic stromal lymphopoietin (TSLP) promotes the development of allergic inflammation. Although increased TSLP is found in eCRSwNP, little is known about whether TSLP regulates eotaxin-1 production, a potent eosinophil chemoattractant that recruits and activates eosinophils.

Objective: The aim of this study was to investigate the effects and mechanisms of TSLP in eotaxin-1 production in the eosinophilic inflammation of eCRSwNP.

Methods: Human nasal epithelial cells (HNECs) from eCRSwNP patients were stimulated with recombinant human TSLP in the presence or absence of CYT387 (Janus kinase 1/2 inhibitor). Phosphorylated signal transducer activator of transcription 3 (p-STAT3) was measured by using immunocytochemistry. Eotaxin-1 expression was determined by using real-time PCR. Western blotting was used to detect the levels of p-STAT3 and eotaxin-1 protein.

Results: The treatment with TSLP induced STAT3 phosphorylation in HNECs, and promoted p-STAT3 nuclear translocation, leading to a time-dependent increase of eotaxin-1 expression. However, these effects were attenuated by CYT387 pretreatment.

Conclusion: TSLP regulated eotaxin-1 production in HNECs via JAK1/2-STAT3 signaling, which might contribute to the eosinophilic inflammation of eCRSwNP.

Key words: eosinophilic chronic rhinosinusitis with nasal polyps, thymic stromal lymphopoietin, eotaxin-1, signal transducer activator of transcription 3, Janus kinase 1/2

Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a common inflammatory disorder with still unclear pathogenesis. In China, CRSwNP is divided into two subgroups with distinct immunopathologic profiles, including eosinophilic CRSwNP (eCRSwNP) and non eosinophilic CRSwNP (non-eCRSwNP) [1]. Although it is not directly linked to allergy, eCRSwNP often displays Th2-skewed inflammation and eosinophil infiltration [2, 3]. Recently, it has been recognized that inflammation rather than chronic infection plays a crucial role in the pathogenesis of eCRS. The application of antibiotics in eCRS treatment without anti-inflammatory properties has limited efficacy [4]. Therefore, understanding the underlying mechanisms in chronic inflammation may provide valuable insights for the therapeutic strategies of eCRSwNP. Eosinophil accumulation is a cardinal feature of eCRSwNP, resulting in the eosinophilic inflammation in the nasal and sinus mucosa [5, 6]. The presence of eosinophils also increases the risk of eCRSwNP recurrence [7]. Eosinophil recruitment is generally regulated by Th2 cytokines and eotaxins. Th2 cytokines induce epithelial cells to produce eotaxins, recruiting eosinophils into the airway via binding to chemokine (C-C motif) receptor 3 on eosinophils [8, 9]. Eotaxin-1 is a potent chemoattractant for eosinophils [10], and is associated with allergic diseases such as asthma and allergic rhinintis (AR) [11, 12]. Increased eotaxin-1 has recently been found in eCRSwNP patients [13]. Moreover, eotaxin levels in eCRSwNP are intrinsically higher than those in non-

eCRSwNP or healthy controls [14, 15]. Hence, eotaxin-1 plays an important role in the eosinophilic inflammation of eCRSwNP. Although elevated Th2 cytokines and eotaxin-1 facilitate eosinophil recruitment, the upstream molecules that regulate eotaxin-1 in eCRSwNP remain obscure.

The recent concept that the epithelial barrier is fundamental to the onset of Th2 responses has been supported by the discovery of thymic stromal lymphopoietin (TSLP) [16]. TSLP is an interleukin (IL)-7-like cytokine, and is mainly produced by epithelial cells. It activates dendritic cells (DCs) that prime the differentiation of naïve T cells into Th2 cells [17]. TSLP is associated with Th2-mediated diseases such as asthma and AR [18, 19]. The treatment with an anti-TSLP antibody in recent clinical trials has attenuated airway inflammation of asthmatic patients [20]. Hence, TSLP is thought of as a master switch for initiating allergic inflammation [21]. Compared with non-eCRSwNP or healthy controls, TSLP expression is significantly increased in eCRSwNP, predominantly in epithelial cells [22]. It facilitates Th2-skewed inflammation in the nasal mucosa and polyps of eCRSwNP patients [22, 23]. Although compelling evidence supports the pivotal role of TSLP in allergic inflammation, little is known about whether TSLP regulates eotaxin-1 that reflects the eosinophilic inflammation of eCRSwNP. Signal transducer activator of transcription 3 (STAT3) has recently been implicated as a critical molecule for allergic inflammation [24], and epithelial STAT3 disruption reduced the number of airway eosinophils in asthmatic mice [25]. Janus kinase 1 and 2 (JAK1/2) are the upstream molecules of STAT3 activation. The blockade of the JAK/STAT3 pathway inhibited Th2 responses mediated by TSLP in AR [26]. Based on the evidence above, we hypothesized that TSLP might regulated the eotaxin-1 expression in eCRSwNP via JAK1/2/STAT3 signaling. To test this hypothesis, the nasal epithelial cells from eCRSwNP patients were cultured and stimulated with recombinant human TSLP in the presence or absence of CYT387 (JAK1/2 inhibitor) pretreatment. We investigated the effects and mechanisms of TSLP in eotaxin-1 production.

Materials and methods

Cell culture and stimulation

Nasal polyp samples were obtained from the middle meatus of 15 eCRSwNP patients during endoscopic sinus surgery in the Head and Neck Surgery Department of Huzhou Central Hospital. Diagnostic criteria of CRSwNP were consistent with the relevant literature [27]. Histologically, when≥10% of the total inflammatory cells were eosinophils, a diagnosis of eCRSwNP was concluded as previously described [28]. All patients had no history of allergy, asthma, smoking, cystic fibrosis or aspirin intolerance, and had not received treatment with glucocorticoids or antibiotics for at least four weeks before surgery. The demographic and clinical characteristics of these patients including sex and age are summarized in Table 1. The tissues were placed in sterile saline after surgery, and were processed for the culture of nasal epithelial cells (HNECs) as previously described [29]. After two passages, the cells were seeded in twelve-well cell culture plates. When the cells reached 80% confluence, they were stimulated with recombinant human TSLP (10ng/ml; R&D Systems, Minneapolis, MN, USA) for 24h and 48h in the presence or absence of CYT387 (100nM; Lab-Bio, Beijing, China) pretreatment one hour before TSLP stimulation, and control cells were treated with only the medium without CYT387 and TSLP. Based on prior studies, the optimal concentration and time for TSLP and CYT387 were chosen [26, 30]. This study was approved by the Institutional Review Board of Huzhou University, and was conducted from December 2014 to June 2016. The study design used in this study is shown in Figure 1.

Table 1. The demographic and clinical characteristics of patients in this study.

		eCRSwNP patients (n=15)
Demographic characteristic	Sex (Male/Female)	11/4
	Age	35.67±9.18
Clinical characteristic	Medical history, n (%)	13(86.67%)
	Surgical history, n (%)	3(20%)
	Allergic history, n (%)	0(0)
	Asthma, n (%)	0(0)
	Smoking, n (%)	0(0)
	Cystic fibrosis, n (%)	0(0)
	Aspirin intolerance	0(0)

Western blotting

Nucleoprotein and cytoplasmic protein were isolated using a nuclear-cytosol Extraction Reagent Kit (Pierce, Rockford, IL, USA). Nuclear components were used to detect phosphorylated STAT3 (p-STAT3), and cytoplasmic components were used to detect eotaxin-1. After denaturation, equal amounts of protein from each sample were prepared for 12% SDS gel electrophoresis. Proteins were transferred after electrophoresis to PVDF membranes (Beyotime). The membranes were blocked with blocking buffer (Beyotime) and were then incubated overnight at 4°C with a 1:300 dilution of rabbit anti-p-STAT3 (Y705) antibody (BOSTER, Wuhan, Hubei, China), a 1:500 dilution of rabbit anti-eotaxin-1 antibody (BOSTER), a 1:1000 dilution of mouse anti-β-actin antibody (Beyotime), or a 1:300 dilution of anti-histone H2A-1 antibody (Abcam, Cambridge, MA, USA). The membranes were incubated with the corresponding secondary antibody (Beyotime), and antibody binding was detected with Beyond ECL Plus. The density of the bands in the Western blot was analyzed using Quantity One software (Silk Scientific Corporation, Orem, UT, USA).

Immunocytochemistry

Cells were fixed in a 4% paraformaldehyde solution. Intrinsic per-

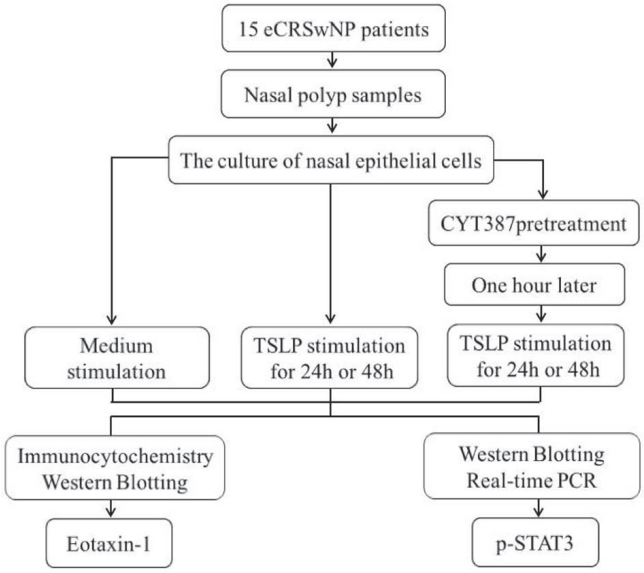

Figure 1. The study design.

oxidase activity was blocked with H_2O_2, and then non-specific antibody binding was blocked with non-immune serum. The cells were incubated overnight at 4°C with a 1:300 dilution of rabbit anti-p-STAT3 (Y705) antibody (BOSTER) before incubation with the secondary antibody (Beyotime). Finally, they were incubated with diaminobenzidine. The results were photographed with a digital camera at a magnification of 200×. The number of p-STAT3 positive cells was measured by labeling of the cell nucleus.

Real-time PCR

Total RNA from HNECs was isolated by TRIzol Reagent (Beyotime). One microgram of total RNA was reverse transcribed to cDNA using the ReverAid™ First Strand cDNA Synthesis Kit (Fermentas Inc, Hanover, MD, USA). Real-time PCR was performed using the Platinum® SYBR® Green qPCR Super Mix-UDG kit (Invitrogen Corporation, Carlsbad, CA, USA) and a real-time thermal cycler (ABI7500, Applied Biosystems, Foster, CA, USA). The primers are as follows: eotaxin-1 5'-GAGCAGGAAAG-AACAGGGAAGACT-3', 5'-ATGGGCACAGGATTA GAGACAGC-3'; β-actin 5'-ACCGTGAAAAGATGACCCAGAT-3', 5'-AGCTGTGGTGG-TGAAGCTGTAG-3'.

The amplification reaction was at 50°C for 2 min and 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min, and terminated by a cooling step at 4°C. The mean cycle threshold (CT) derived from the control group was used as the calibrator. The equation used was:

$$\text{Ratio (sample to calibrator)} = 2^{(-\Delta\Delta CT)}$$

Where ΔCT=CT (Goal Gene) - CT (Reference gene), and ΔΔCT = ΔCT (sample) - ΔCT (calibrator).

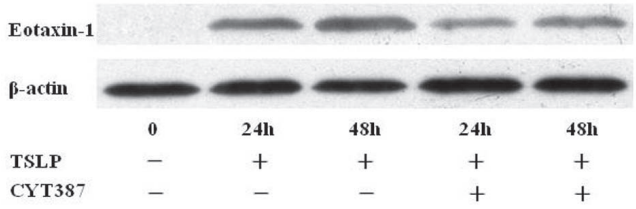

Figure 2. The expression of eotaxin-1 protein in HNECs. The cells were stimulated with TSLP for 24h and 48h in the presence or absence of CYT387 pretreatment, respectively, and control cells were treated with only the medium. β-actin was used as loading control. The intensity of eotaxin-1 bands was normalized to the intensity of β-actin bands.

Statistical analysis

SPSSS 13.0 software was used for statistical analysis. Statistical significance was evaluated using ANOVA followed by a Dunnet's test. Experiments were repeated ten times for each group. A p value < 0.05 was considered to indicate statistical significance. Each data point is expressed as the mean ± S.D.

Results

The expression of Eotaxin-1 protein in HNECs

To investigate whether TSLP affected the synthetic function of HNECs, eotaxin-1 protein were evaluated by using Western blotting. As seen in Figure 2, eotaxin-1 protein was not detected in control cells. After the cells were stimulated with 10ng/ml TSLP for 24h and 48h, the expression of eotaxin-1 protein at 48h time point were increased by 50.85% compared with 24h time point (Table 2). TSLP stimulation induced a time-dependent increase of eotaxin-1 expression. However, when the cells were stimulated with TSLP (10ng/ml) after CYT387 (100nM) pretreatment, the eotaxin-1 expression at 24h time point was decreased by 33.62% compared with induction by TSLP alone. The expression induced by TSLP in the presence of CYT387 pretreatment at 48h was lower by 35.69% than that induced by TSLP alone. The pretreatment with the JAK1/2 inhibitor inhibited the eotaxin-1 expression induced by TSLP.

The expression of Eotaxin-1 mRNA in HNECs

To further investigate the transcriptional regulation of TSLP in eotaxin-1 expression, real time PCR was used to detect eotaxin-1 mRNA. As shown in Table 2, a 2.26-fold or 3.04-fold increase of eotaxin-1 expression was observed following the treatment with TSLP at 24h and 48h time points compared with control conditions, and the levels of eotaxin-1 mRNA at 48h time point were increased by 34.41% compared with 24h time point. But TSLP stimulation after CYT387 pretreatment resulted in a 0.26-fold decrease of the eotaxin-1 expression compared with TSLP alone at 24h time points, and a 0.25-fold decrease compared with TSLP alone at 48h time points. The JAK1/2 inhibitor attenuated

Table 2. The expression of eotaxin-1 protein and mRNA in HNECs.

			Western Blotting		Real-time PCR	
CYT387	TSLP		Mean ± SD	p value	Mean ± SD	p value
-	-	0	0		1	
-	+	24h	0.702±0.216		2.260±0.678	
--	+	48h	1.059±0.329	0.0103[&]	3.038±0.792[&]	0.0289[&]
+	+	24h	0.466±0.227[&]	0.0285[&]	1.679±0.532[&]	0.0469[&]
+	+	48h	0.681±0.275[§]	0.0122[&]	2.291±0.649[§]	0.0332[§]

The cells were, respectively, stimulated by TSLP for 24h and 48h in the presence or absence of CYT387 pretreatment, and control cells were treated with only the medium. [&]Versus induction with TSLP alone for 24h, and [§]versus induction with TSLP alone for 48h.

eotaxin-1 gene transcription induced by TSLP.

The protein expression by immunocytochemical staining P-STAT3 in HNECs

To determine the mechanisms by which TSLP regulated eotaxin-1 expression, immunocytochemistry was used to detect the p-STAT3 protein. Immunohistochemical results show in Figure 3 that p-STAT3 expression was almost not seen in control cells. But TSLP stimulation significantly induced STAT3 phosphorylation at tyrosine residue 705 (Y705) in the cell cytoplasm, and promoted p-STAT3 (Y705) translocation into the nucleus. Table 3 shows that the number of p-STAT3 (Y705) positive cells in the cell nucleus at 48h time point was increased by 23.09% compared with 24h time point. However, the expression induced by TSLP stimulation with CYT387 pretreatment resulted in a 20.72% decrease of p-STAT3 expression compared with TSLP alone at 24h time point, and a 20.55% decrease compared with TSLP alone at 48h time point. The pretreatment with the JAK1/2 inhibitor down-regulated STAT3 phosphorylation and nuclear translocation mediated by TSLP.

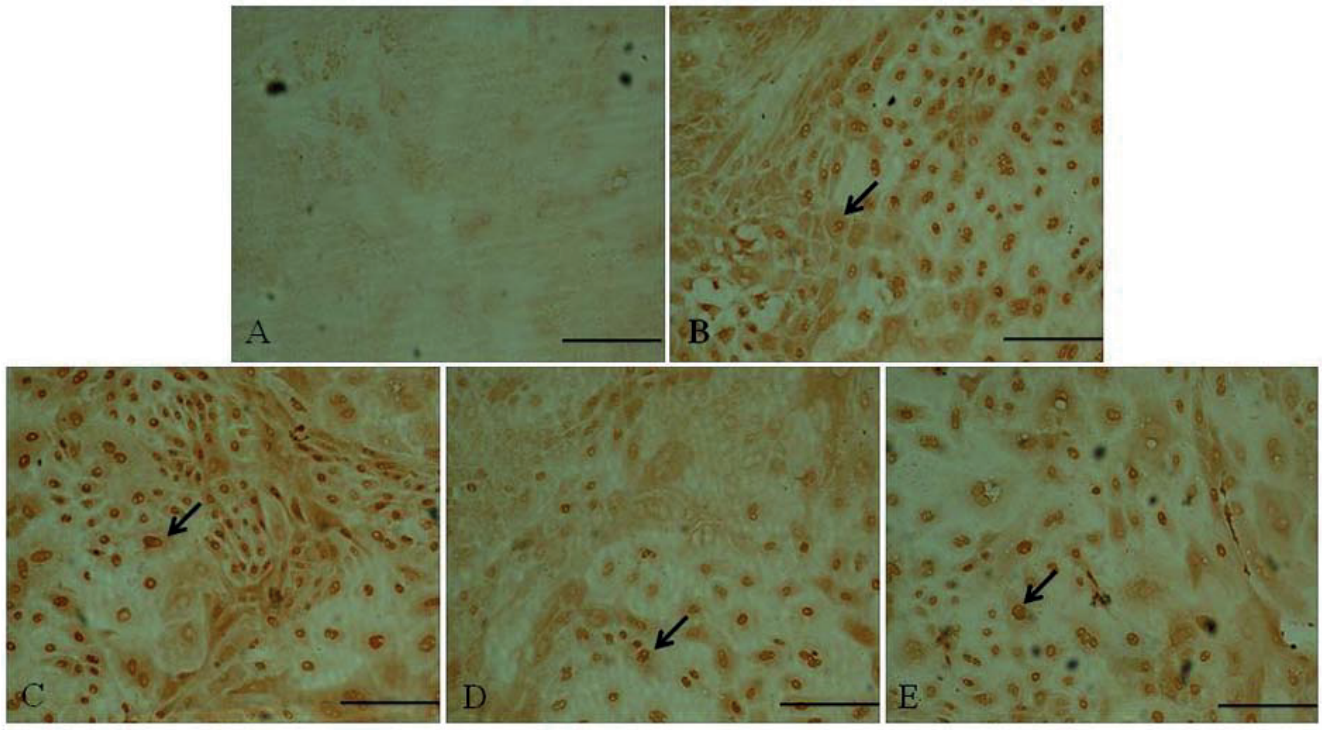

Figure 3. Immunocytochemical staining for p-STAT3 protein in HNECs. A) Control, only medium stimulation, B) TSLP stimulation alone for 24h, C) TSLP stimulation alone for 48h, D) TSLP stimulation for 24h with CYT387 pretreatment, and E) TSLP stimulation for 48h with CYT387 pretreatment. Black arrows represents the positive expression of p-STAT3 in HNECs (scale bars=200μm).

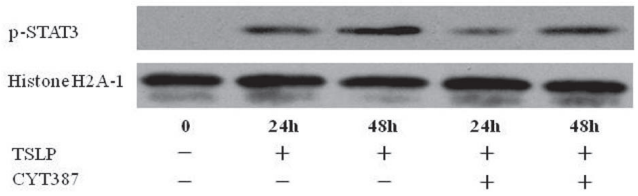

Figure 4. The expression of nuclear p-STAT3 protein in HNECs. Cells were respectively stimulated with TSLP for 24h and 48h in the presence or absence of CYT387 pretreatment, and control cells were treated with only the medium. Histone H2A-1 was used as loading control. The intensity of p-STAT3 bands was normalized to the intensity of histone H2A-1 bands.

The protein levels of nuclear p-STAT3 in HNECs

To further investigate the effect of TSLP on STAT3 nuclear translocation, western blotting was used to detect p-STAT3 (Y705) levels in the nucleoprotein fraction. Figure 4 shows that there was no the expression of p-STAT3 protein in cell nuclei of control cells. After HNECs were treated with TSLP, a 0.53-fold increase of p-STAT3 protein in cell nuclei was observed at 48h time point compared with 24h time point (Table 3). But when HNECs were stimulated with TSLP after CYT387 pretreatment, the protein levels were decreased by 36.52% at 24h time point compared with the cells that were only treated with TSLP, and were decreased by 37.54% at 48h time point compared with the cells that were only treated with TSLP. The JAK1/2 inhibitor inhibited the levels of p-STAT3 protein in the nucleus induced by TSLP stimulation.

Discussion

TSLP has recently been implicated as a key molecule for promoting the development of allergic airway inflammation [31, 32]. TSLP signals through a heterodimeric receptor complex consisting of a unique TSLP receptor (TSLPR) and an interleukin-7 receptor α chain. Cellular targets of TSLP include DCs, $CD4^+$ and $CD8^+$ T cells, B cells, mast cells, and regulatory T cells [33]. Recent study has shown that TSLPR is constitutively and preferentially expressed in airway mucosa [34], and TSLP/TSLPR signaling is significantly activated in eCRSwNP patients, predominantly in epithelial cells [22], and is pivotal in the induction of respiratory immunity [35]. In the present study, the nasal epithelial cells from eCRSwNP patients were stimulated with recombinant human TSLP, and we observed that the levels of eotaxin-1 mRNA and protein were up-regulated in a time-dependent manner. Therefore, TSLP induced nasal epithelial cells to produce eotaxin-1 in eCRSwNP via TSLP/TSLPR signaling.

The TSLP/TSLPR axis initiates and activates multiple signaling transduction pathways, including JAK/STAT, mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3 kinase (PI-3kinase) [33, 36]. Aberrant TSLP/TSLPR signaling is associated with a variety of human diseases such as asthma, atopic dermatitis and nasal polyposis [33]. Recently, signaling transduction mediated by human TSLPR has been found to depend on JAK function in atopic diseases [37]. The TSLP/TSLPR axis induces the phosphorylation and activation of JAKs, and activated JAK regulates the activity of STAT family, including STAT1, 3, 4, 5 and 6 [26]. Moreover, TSLP appears to initiate signaling transduction via STAT3 and STAT5 activation, inducing the transcription of STAT-responsive genes [26, 37]. STAT3 and STAT5 are the important downstream molecules of TSLP/TSLPR signaling. It has been demonstrated in current studies that signaling transduction mediated by TSLPR mainly activates DCs, Th2 cells and B cells via STAT5, and STAT5 is considered to be a signature signaling dock for TSLP in hematopoietic cells [38-41], but the signaling transduction via STAT3 acts on airway-derived cells such as airway epithelial cells, airway smooth muscle cells and airway fibroblasts [22, 36, 42, 43]. Human TSLP/TSLPR signaling requires the function of JAK1 and JAK2 for STAT activation [37], and inhibiting STAT3 activation via blocking JAK1 or/and JAK2 attenuates the immune responses mediated by TSLP [37, 44].

STAT3, known as a transcription factor as well as a signal transducer, plays a pivotal role in regulating immune and inflammatory responses. STAT3 protein is a latent protein residing in the cytoplasm [45]. In response to cytokines, STAT3 is recruited from the cytosol to associate with the activated receptors through its phospho-Tyr-SH2 domain, and is phosphorylated at its tyrosine residues by the receptor-associated JAK kinases [46]. Once it is phosphorylated, STAT3 protein dimerizes and translocates into the nucleus, where it induces the transcription of target genes by binding to the specific DNA motifs [36]. Our current study showed that TSLP stimulation induced STAT3 phosphorylation at the carboxy-terminal tyrosine (Tyr705), and promoted the nuclear translocation of p-STAT3 protein. But these effects were inhibited by CYT387 pretreatment. Our data suggested that TSLP activated JAK 1/2/STAT3 signaling in nasal epithelial cells. Emerging evidence suggests that STAT3 is critical for cytokine signaling, which has been implicated in promoting allergic inflammation. In particular, a predominant role of STAT3 was shown in a study where the knockout of STAT3 gene in airway epithelium resulted in a significant decrease of airway eosinophils in asthmatic mice [25, 47]. Binding sites for STAT3 were found in the promoter of the eotaxin-1 gene. STAT3 binding to the eotaxin-1 promoter in airway smooth muscle cells was involved in IL-17A-induced eotaxin-1 transcription [45]. In the present study, we investigated whether TSLP regulated eotaxin-1 production by nasal epithelial cells via JAK1/2/STAT3 signaling. Our data showed that TSLP stimulation led to a time-dependent up-regulation of eotaxin-1 protein and mRNA, but CYT387 pretreatment attenuated these effects. TSLP induced eotaxin-1 production via STAT3 activation in bronchial epithelial cells from asthmatic patients and airway smooth muscle cells [30, 36, 47]. Our results were consistent with these reports. We concluded that TSLP induced

Table 3. The expression of p-STAT3 positive cells and protein in cell nucleus in HNECs.

			Immunocytochemistry		Western Blotting	
CYT387	TSLP		Mean ± SD	p value	Mean ± SD	p value
-	-	0	0		0	
-	+	24h	126.9±23.662		0.597±0.218	
-	+	48h	156.2±26.670[&]	0.0181[&]	0.911±0.319[&]	0.0192[&]
+	+	24h	100.6±24.994[&]	0.0262[&]	0.379±0.209[&]	0.0349[&]
+	+	48h	124.1±28.838[§]	0.0187[&]	0.569±0.242[§]	0.0146[&]

The cells were, respectively, stimulated with TSLP for 24h and 48h in the presence or absence of CYT387 pretreatment, and control cells were treated with only the medium. [&]Versus induction with TSLP alone for 24h, and [§]versus induction with TSLP alone for 48h.

eotaxin-1 expression in nasal epithelial cells via JAK 1/2/STAT3 signaling. Moreover, TSLP over-expression in eCRSwNP promoted Th2-cell responses characterized by production of Th2 cytokines, and then Th2 cytokines activated macrophages, B cells, and epithelial cells to promote the recruitment of eosinophils into the airway of CRSwNP patients [23]. These factors contributed to the eosinophilic inflammation of eCRSwNP together.

Conclusion

In conclusion, we demonstrated that TSLP regulated eotaxin-1 production by the nasal epithelial cells from eCRSwNP patients via JAK1/2/STAT3 signaling, which is summarized in Figure 5. We hypothesized that TSLP directly regulated the eosinophilic inflammation of eCRSwNP. Therefore, Targeting TSLP as a therapeutic strategy could improve the eosinophilic inflammation of eCRSwNP patients. Although our current data showed that eotaxin-1 expression was regulated by JAK1/2/STAT3 signaling, it has been emphasized in recent studies that eotaxin-3 plays a critical role in the eosinophilic inflammation, and eotaxin-3 is thought to be more effective for eosinophil recruitment than eotaxin-1 or eotaxin-2 (9). STAT6, which is also an intracellular downstream molecule of JAK1/2-mediated signaling (48), regulates the expression of eotaxins such as eotaxin-1, 2 and 3 (49). Further investigation is to evaluate whether TSLP induces eotaxin-3 production via JAK1/2/STAT3 signaling, and demonstrate whether STAT6 activation is involved in eotaxin-1 expression mediated by TSLP.

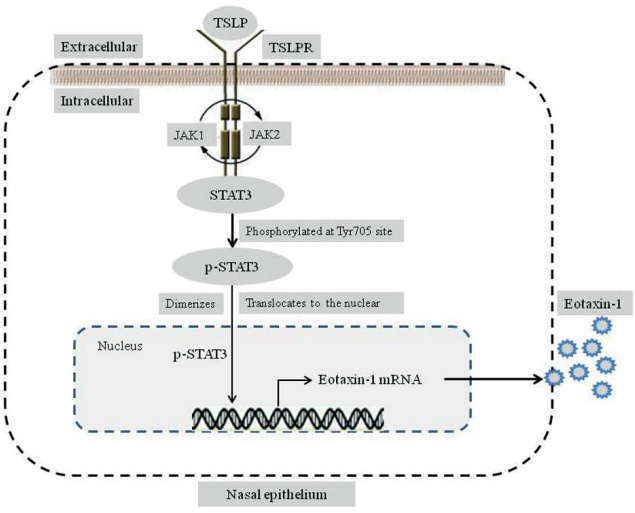

Figure 5. Diagram summarizing the conclusion of this study.

Acknowledgements

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Authorship contribution

WWW: Data Analysis; DML: The conception and design of the experiments; MZ: The writing of the paper; JGZ: The revision of the paper; BZ: The acquisition of the data

Conflict of interest

The authors declare no conflict of interests regarding the publication of this paper.

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