

Interleukin-17A potentiates interleukin-13-induced eotaxin-3 production by human nasal epithelial cells from patients with allergic rhinitis

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Background: Interleukin (IL)-17A is involved in the pathogenesis of allergic rhinitis (AR). Increased expression of IL-17A is correlated with disease severity and nasal eosinophilia. However, the molecular mechanisms by which IL-17A contributes to T-helper 2 cytokine IL-13-driven pathology in AR remain unclear. We sought to obtain mechanistic insight into how IL-17A and IL-13 regulate the epithelial production of eotaxin-3 representing eosinophilic inflammation in AR.

Methods: Human nasal epithelial cells (HNECs) from AR patients were cultured and stimulated with IL-17A, IL-13, or IL-17A and IL-13. Phosphorylated signal transducer activator of transcription 6 (p-STAT6) and suppressor of cytokine signaling 1 (SOCS1) in HNECs were assayed using Western blotting. Immunocytochemistry was used to determine p-STAT6-positive expression in the cells. Eotaxin-3 expression in the cells and culture supernatants was evaluated using real-time polymerase chain reaction and enzyme-linked immunosorbent assays.

Results: Stimulation with IL-13 alone induced STAT6 phosphorylation and promoted p-STAT6 nuclear translocation, leading to eotaxin-3 production by HNECs. These effects

were further enhanced by cotreatment with IL-13 and IL-17A, whereas IL-17A alone had no impact on STAT6 or eotaxin-3 expression. Incubation with IL-17A or IL-13 increased the level of SOCS1 protein in the cells, whereas the addition of IL-17A attenuated IL-13-induced SOCS1 expression.

Conclusion: IL-17A potentiated IL-13-driven STAT6 activation through the downregulation of SOCS1 expression, leading to enhancement of eotaxin-3 production by HNECs. These factors contributed to eosinophilic inflammation in AR. © 2019 ARS-AAOA, LLC.

Key Words:

allergic rhinitis; interleukin-17A; interleukin-13; eotaxin-3; signal transducer activator of transcription 6; suppressor of cytokine signaling 1

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Allergic rhinitis (AR) is a chronic inflammatory disease with a prevalence of 10%-40%.¹ CD4⁺ T-helper (Th) cells play a vital role in local mucosal immune responses of allergic airway diseases.² AR has long been hypothesized as the result of dysregulation of the balance between Th1 and

Th2 cells, leading to the activation of excess Th2 cells that produce Th2 cytokines, including interleukin (IL)-4, -5, and -13.^{2,3} AR is characterized by Th2-skewed inflammation with the infiltration of mucosal eosinophils.⁴ Eosinophils are thought of as the main effector cells contributing to airway inflammation.⁵ Nasal eosinophils display the best correlation with all clinical and immunologic parameters in AR.⁶

The recruitment of eosinophils arises from the combined action of a number of cellular and molecular signals, including chemoattractants.⁷ The CC chemokines eotaxin-1, -2, and -3 are potent chemoattractants for eosinophils. The airway epithelium is known to be one of the main sources of eotaxins.⁸ The eotaxins recruit eosinophils into airways via binding to CC chemokine receptor 3 (CCR3) on eosinophils.⁹ Eotaxin-3 was shown to be the most effective chemokine for inducing eosinophil recruitment among CC chemokines.⁸ Th2 cytokines, particularly

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IL-13, have been shown to induce eotaxin-3 production by airway epithelial cells.^{8,9} Increased eotaxin-3 is found in the nasal mucosa and lavages of AR patients,^{10,11} and was shown to be associated with mucosal eosinophilia and disease severity.¹² Eotaxin-3 is considered a potential target for treating patients with eosinophilic diseases.¹³

Recently, the classic paradigm of Th1/Th2-mediated immunity has evolved to include another Th-cell lineage distinct from Th1 and Th2 cells, namely Th17 cells, which play a major role in inflammation during allergic conditions and immune-related tissue injury.^{2,14,15} Th17 cells are characterized by the predominant production of IL-17A.¹⁶ Levels of IL-17A are increased in nasal biopsies, sputum, and serum of AR patients.¹⁴ Local expression of IL-17A is correlated with disease severity and nasal eosinophilia of AR.¹⁷ In a mouse model of AR, IL-17A deficiency was shown to reduce mucosal eosinophils and serum immunoglobulin E levels, with improvement of nasal symptoms.^{18,19} IL-17A appears to be involved in the pathogenesis of AR.²⁰ However, the studies also raised new questions about how IL-17A and Th2 cytokines cooperate in the pathogenesis of allergic diseases.

The knockout of the IL-17A gene has been shown to attenuate eosinophil accumulation in a mouse model of AR,^{19,21} which highlights the major role of IL-17A in eosinophilic inflammation. However, the mechanism by which IL-17A regulates eosinophil recruitment mediated by eotaxins remains unknown. Regulation of eotaxins has been studied extensively in airway epithelial cells exposed to IL-13.^{9,13,22} Thus, in this study, human nasal epithelial cells (HNECs) from AR patients as an in-vitro model were stimulated with IL-17A, IL-13, or IL-17A and IL-13. We investigated the molecular mechanisms by which IL-17A and IL-13 cooperated in regulating eotaxin-3 production by HNECs. A complete understanding of the mechanisms involved would lead to therapeutic strategies to improve eosinophilic inflammation associated with IL-17A in the management of AR.

Patients and methods

Culture and stimulation of HNECs

This study was approved by the institutional review board of Huzhou University. Written informed consent was obtained from the patients with regard to the surgery and use of resected specimens for research. Fifty-six patients, between 18 and 45 years old, were diagnosed with persistent AR complicated with turbinate hypertrophy according to criteria of the international guidelines,²³ and were recruited based on medical history and skin-prick testing in the Department of Head and Neck Surgery, Huzhou Central Hospital. They had not received treatment with glucocorticoids and antibiotics for at least 4 weeks before surgery. The clinical characteristics of these patients are summarized in Table 1.

TABLE 1. Clinical characteristics of patients in this study

Characteristics	AR patients (n = 56)
Age (years)	32.64 ± 7.88
Sex (male/female)	43/13
Smoking, n (%)	3 (5.36%)
Asthma, n (%)	1 (1.79%)
Rhinosinusitis, n (%)	0 (0)

AR = acute rhinosinusitis.

The mucosal tissues on the inferior turbinate from these patients were obtained and digested with 0.01% collagenase I (4A Biotech Co, Ltd, Beijing, PR China), and epithelial cells were harvested and cultured as described elsewhere.²⁴ The confluent cells were stimulated with recombinant human IL-13 (100 ng/mL; Beyotime, Haimen, Jiangsu, PR China) and/or recombinant human IL-17A (100 ng/mL; Beyotime) for 24 hours. Control cells were only treated with normal saline (NS). Optimal concentrations of the cytokines were chosen based on a previous study.²⁵ The cells and culture supernatants were collected.

Western blotting

The protein from HNECs was extracted using radio-immunoprecipitation assay buffer (Beyotime). After denaturation, equal amounts of protein from each sample were prepared for 12% sodium dodecylsulfate gel electrophoresis. Protein was transferred after electrophoresis to polyvinylidene fluoride membranes (4A Biotech). The membranes were blocked with blocking buffer, and then were incubated with a 1:300 dilution of rabbit anti-p-STAT6 (Tyr641) antibody (4A Biotech), 1:250 dilution of rabbit suppressor of cytokine signaling 1 (SOCS1) antibody (4A Biotech), or a 1:1000 dilution of mouse anti-β-actin antibody (Beyotime). The membranes were then incubated with secondary antibody (Beyotime). Antibody binding was detected with Beyond ECL Plus (Beyotime). The density of the bands was analyzed using Quantity One software (Silk Scientific Corp, Orem, UT).

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde solution. Intrinsic peroxidase activity was blocked with H₂O₂, and then non-specific antibody binding was blocked with nonimmune serum (Beyotime). The cells were incubated overnight at 4°C with a 1:200 dilution of rabbit anti-p-STAT6 (Tyr641) antibody (4A Biotech) before incubation with the secondary antibody (Beyotime). Finally, cells were incubated with diaminobenzidine (Beyotime). The results were photographed with a digital camera at a magnification of ×200. The number of p-STAT6-positive cells in cell nucleus was measured.

Real-time polymerase chain reaction

Total RNA from HNECs was isolated by TRIzol reagent (Invitrogen Corporation, Carlsbad, CA). One microgram of total RNA was reverse transcribed to cDNA using the ReverAid™ First Strand cDNA Synthesis Kit (Fermentas, Inc, Hanover, MD). Real-time polymerase chain reaction (PCR) was performed using a Platinum® SYBR® Green qPCR Super Mix-UDG kit (Invitrogen) and a real-time thermal cycler (ABI7500; Applied Biosystems, Foster, CA). The primers were as follows: eotaxin-3, 5'-GGAAGTCC-ACACGTGGGAGTGAC-3', 5'-CTCTGGGAGGAAACACCCTCTCC-3'; and β -actin, 5'-ACCGTGAAA-AGATGACCCAGAT-3', 5'-AGCTGTGGTGGTG AAGCTGTAG-3'. The amplification reaction was at 50°C for 2 minutes and 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, and then terminated by a cooling step at 4°C. The mean cycle threshold (Ct) from the control group was used as the calibrator. The equation used was: ratio (sample to calibrator) = $2(-\Delta\Delta CT)$, where $\Delta CT = CT(\text{goal gene}) - CT(\text{reference gene})$ and $\Delta\Delta CT = \Delta CT(\text{sample}) - \Delta CT(\text{calibrator})$.

Enzyme-linked immunosorbent assays

The concentration of eotaxin-3 protein in culture supernatants was analyzed using an enzyme-linked immunoassay (ELISA) kit (Westang Bio-Tech Co, Ltd, Shanghai, China). The procedures were performed according to the manufacturer's instructions. Optical density values were measured at 450 nm by an ELISA plate reader (Bio-Rad, Hercules, California).

Statistical analysis

Statistical software (SPSS version 13.0; SPSS, Inc, Chicago, IL) was used for analysis. Statistical significance was evaluated using analysis of variance, followed by the Dunnett test. $p < 0.05$ was considered statistically significant. Each datapoint was expressed as mean $\pm$ standard deviation (SD).

Results

To investigate the effects of IL-17A on STAT6 activation in HNECs, we assayed the level of STAT6 phosphorylation using Western blotting. As shown in Figure 1, no p-STAT6 (Tyr641) was detected in control cells and IL-17A-stimulated cells, whereas p-STAT6 protein was induced by stimulation with IL-13 alone or IL-13 and IL-17A. Expression of p-STAT6 protein was increased by 45.99% after treatment with IL-13 and IL-17A when compared with IL-13 alone ($p < 0.05$). Thus, IL-17A enhanced IL-13-induced STAT6 phosphorylation in HNECs.

To determine the regulation of p-STAT6 nuclear translocation by IL-17A, the cell nuclei positive for p-STAT6 were detected using immunocytochemistry. As shown in Figure 2, p-STAT6-positive cells were only observed

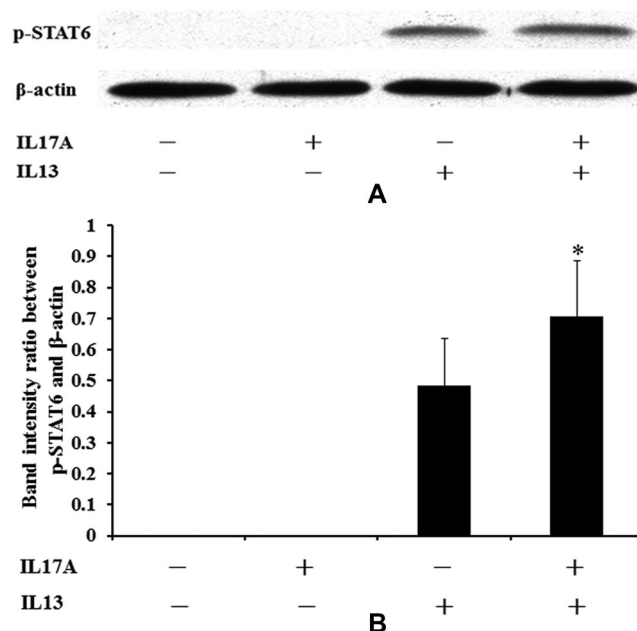


FIGURE 1. Western blotting for p-STAT6 (Tyr641) protein in HNECs from patients with AR. β -Actin was used as loading control, and the intensity of p-STAT6 (Tyr641) bands was normalized to the intensity of β -actin bands. The band intensity ratio between p-STAT6 and β -actin was calculated, and the data are presented as mean ($n = 7$). * $p < 0.05$ vs stimulation with IL-13. AR = allergic rhinosinusitis; HNECs = human nasal epithelial cells; IL-13 = interleukin-13; p-STAT6 = phosphorylated signal transducer activator of transcription 6; Tyr = tyrosine.

after treatment with IL-13 alone or IL-13 and IL-17A, and p-STAT6 protein was located in the cell cytoplasm and nucleus of the cells. The number of cells positive for p-STAT6 nuclear translocation induced by IL-13 and IL-17A was increased by 29.35% when compared with IL-13 alone ($p < 0.05$), indicating that IL-17A acted synergistically with IL-13 to potentiate p-STAT6 nuclear translocation in HNECs.

To investigate the transcriptional regulation of eotaxin-3 by IL-17A, we used real-time PCR to detect levels of eotaxin-3 messenger RNA (mRNA) in HNECs. No difference was observed in level of eotaxin-3 mRNA between control cells and IL-17A-stimulated cells (Fig. 3), whereas expression in IL-13-stimulated cells was 1.68 times that of control cells ($p < 0.05$). A 0.68- and 1.48-fold increase in eotaxin-3 mRNA level was induced by IL-13 alone and IL-13 plus IL-17A, respectively, when compared with IL-17A alone ($p < 0.05$). IL-13 and IL-17A stimulated a 47.72% increase in eotaxin-3 mRNA expression compared with IL-13 alone ($p < 0.05$). These results show that IL-17A enhanced IL-13-induced eotaxin-3 gene transcription.

To further investigate the effect of IL-17A on the secretion of HNECs, the concentration of eotaxin-3 in culture supernatants was assayed by using ELISA. As shown in Figure 4, there was no difference, although the supernatant level of eotaxin-3 in IL-17A-treated cells increased by 8.55% compared with control cells. Eotaxin-3 concentration in IL-13-treated cells increased by 66.22%

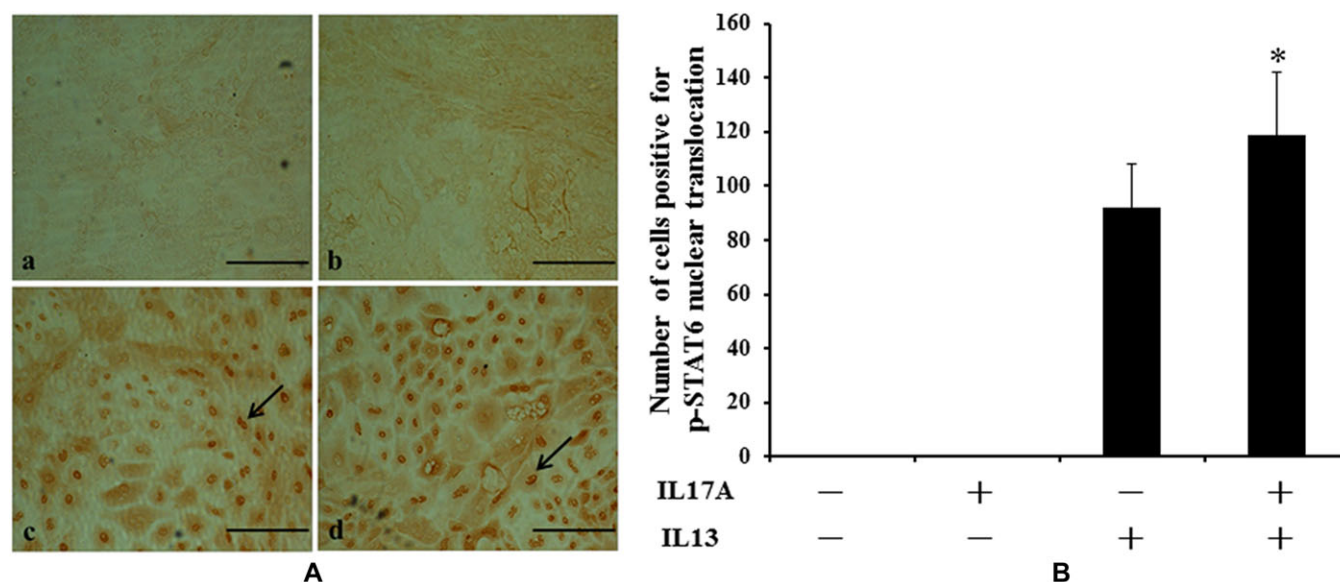


FIGURE 2. Nuclear translocation of p-STAT6 (Tyr641) protein in HNECs from patients with AR using immunocytochemistry: a = after treatment with NS; b = after treatment with IL-17A; c = after treatment with IL-13; d = after treatment with IL-13 and IL-17A. Black arrows represents positive expression of p-STAT6 in HNECs (scale bars = 200 μ m). The number of p-STAT6-positive cells was measured by labeling of the cell nucleus. Data are presented as mean ($n = 7$). * $p < 0.05$ vs stimulation with IL-13. AR = allergic rhinosinusitis; HNECs = human nasal epithelial cells; IL-13 = interleukin-13; NS = normal saline; p-STAT6 = phosphorylated signal transducer activator of transcription 6; Tyr = tyrosine.

compared with control cells ($p < 0.05$). Stimulation with IL-13 alone or IL-13 with IL-17A upregulated eotaxin-3 expression by 0.53- and 1.16-fold when compared with IL-17A alone ($p < 0.05$). Stimulation with IL-13 and IL-17A resulted in a 40.92% increase in eotaxin-3 production compared with IL-13 alone ($p < 0.05$). Our data show that IL-17A promoted IL-13-induced eotaxin-3 secretion by HNECs.

To determine the effects of IL-17A on the SOCS1 signaling pathway, we detected SOCS1 protein in HNECs using

Western blotting. SOCS1 expression in IL-17A-stimulated cells was increased by 73.19% compared with control cells ($p < 0.05$) (Fig. 5). A 2.58-fold increase in SOCS1 level was induced in IL-13-stimulated cells compared with control cells ($p < 0.05$). Stimulation with IL-13 alone or IL-17A and IL-13 upregulated SOCS1 expression by 1.07- and 0.46-fold, respectively, when compared with IL-17A alone ($p < 0.05$). However, expression induced by IL-13 and IL-17A was reduced by 29.28% compared with IL-13 alone ($p < 0.05$). IL-17A attenuated IL-13-induced SOCS1 expression in HNECs.

Discussion

Th2 cytokines orchestrate recruitment and activation of the primary effector cells in allergic response.^{26,27} Among these cytokines, IL-13 is thought to be particularly critical in the immunopathology of allergic airway diseases.²⁸ IL-13-mediated inflammation is a hallmark of asthma and AR.²⁹ IL-13 signaling has been shown to drive epithelial responses, including barrier dysfunction, mucus hypersecretion, and chemokine production.^{9,28,30}

STAT6 is a critical transcription factor activated in response to IL-13.³¹ Targeted expression of STAT6 in airway epithelial cells is sufficient for IL-13-induced airway hyperreactivity and mucus production.^{28,31} IL-13 initially binds to IL-13 receptor $\alpha 1$ (IL-13 $\alpha 1$), and dimerizes with IL-4 receptor α (IL-4 α).³² The dimerized receptors recruit and activate Janus tyrosine kinases (JAKs), which phosphorylate tyrosine residues on IL-4R, providing a docking site for STAT6 recruitment. STAT6 itself becomes phosphorylated at its conserved tyrosine residue Y641, and dimerizes

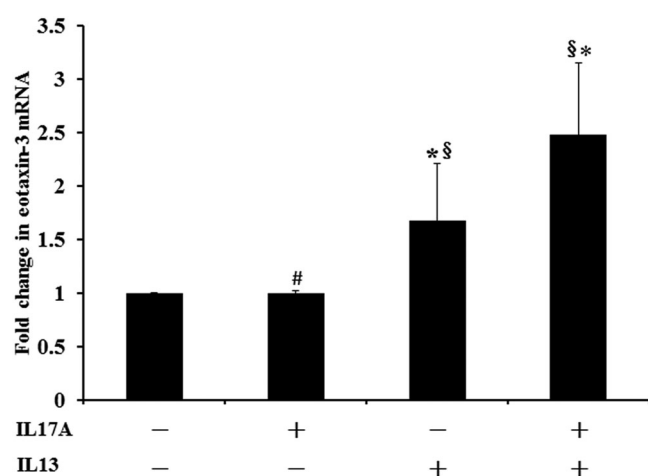


FIGURE 3. Real-time polymerase chain reaction for eotaxin-3 messenger RNA in HNECs from patients with AR. The fold change in mRNA of the eotaxin-3 gene was normalized to β -actin. Data are presented as mean ($n = 7$). # $p > 0.05$ vs stimulation with NS; * $p < 0.05$ vs stimulation with NS; § $p < 0.05$ vs stimulation with IL-17A; * $p < 0.05$ vs stimulation with IL-13. HNEC = human nasal epithelial cell; IL = interleukin; NS = normal saline.

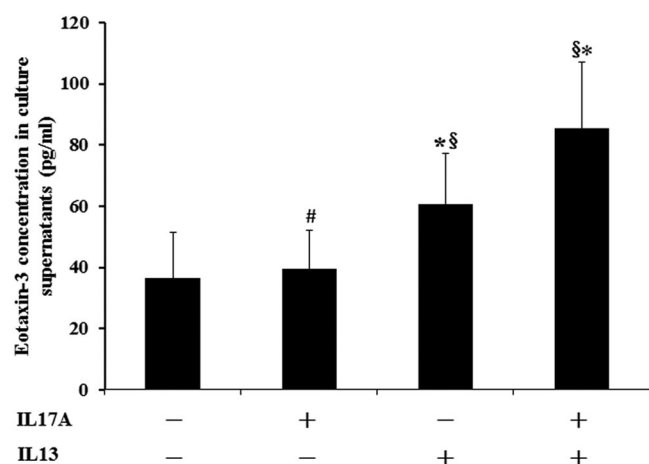


FIGURE 4. ELISA assay for eotaxin-3 protein in culture supernatants. Concentration of eotaxin-3 protein released by HNECs was measured in the culture supernatants. Data are presented as mean ($n = 7$). # $p > 0.05$ vs stimulation with NS; * $p < 0.05$ vs stimulation with NS; § $p < 0.05$ vs stimulation with IL-17A; * $p < 0.05$ vs stimulation with IL-13. ELISA = enzyme-linked immunoassay; HNEC = human nasal epithelial cell; IL = interleukin; NS = normal saline.

and translocates into the nucleus, where it induces the transcription of target genes.³³

IL-13-mediated signaling was found to be associated with eosinophil recruitment via the induction of eosinophil-specific chemokines.³⁴ In human epithelial cells from airway, oesophagus and intestine, the regulation of eotaxin-3 expression by IL-13 has been shown to require STAT6 activation.^{35–37} Knockout analysis and gene promoter studies have also indicated that STAT6 is a master regulator of eotaxin-3 expression in multiple epithelial cells.^{36–38} In the present study, IL-13 promoted STAT6 phosphorylation and nuclear translocation, and strongly induced eotaxin-3 production by HNECs. The data presented herein are consistent with those of previous reports.^{31,35}

Recent progress in characterizing IL-17A have advanced our understanding of the mechanisms underlying allergic inflammation.^{3,30} In this study, we observed that IL-17A alone had no effect on eotaxin-3 and p-STAT6 expression. However, the addition of IL-17A enhanced IL-13-induced eotaxin-3 production by HNECs. We further investigated whether STAT6 phosphorylation was sufficient to explain IL-17A and IL-13-induced synergism in eotaxin-3 enhancement, and found that IL-17A potentiated IL-13-driven STAT6 phosphorylation and nuclear translocation. Recent studies demonstrated that IL-17A acts synergistically with IL-13 to potentiate the STAT6 pathway, although IL-17A alone resulted in no or very low STAT6 activation.^{25,39} Our data indicate that IL-17A enhanced IL-13-induced eotaxin-3 production through potentiating STAT6 activation. However, the molecular mechanisms underlying IL-17A-mediated enhancement of IL-13-induced STAT6 activation remain to be fully elucidated.

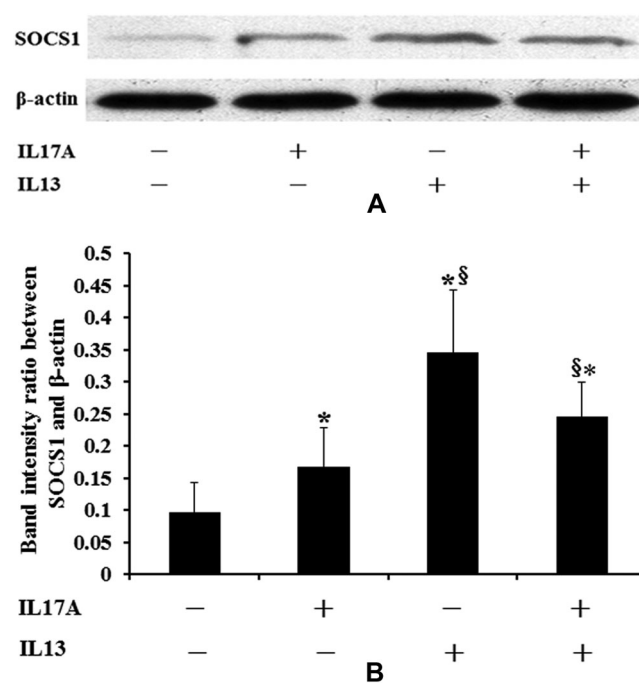


FIGURE 5. Western blotting for SOCS1 protein in HNECs from patients with AR. β-Actin was used as loading control, and the intensity of SOCS1 bands was normalized to the intensity of β-actin bands. The band intensity ratio between SOCS1 and β-actin was calculated, and data are presented as mean ($n = 7$). * $p < 0.05$ vs stimulation with NS; § $p < 0.05$ vs stimulation with IL-17A; * $p < 0.05$ vs stimulation with IL-13. AR = acute rhinosinusitis; HNEC = human nasal epithelial cell; NS = normal saline; SOCS1 = suppressor of cytokine signaling 1.

SOCS1 is known to regulate Th2-cytokine signaling in both in-vitro cell cultures and in-vivo mouse models. It blocks the phosphorylation of STAT6 through inhibiting JAK activity, and has been identified as a negative feedback regulator of the JAK/STAT6 pathway.^{40–42} Mucosal SOCS1 is increased in patients with AR and asthma compared with healthy controls.^{43–45} The present study results proved consistent with published data showing that IL-13 or IL-17A stimulation upregulated SOCS1 expression in airway epithelial cells.^{40,46} Recently, reduced expression of airway epithelial SOCS1 was found in patients with severe asthma with persistent airway eosinophilia and Th2 inflammation as compared to those with mild or moderate asthma.⁴⁰ Further data have shown increased IL-17A in patients with severe asthma and AR.^{47,48} We speculated that IL-17A downregulates SOCS1 expression under Th2 inflammatory conditions. To test this hypothesis, we evaluated SOCS1 expression in the cotreatment with IL-13 and IL-17A, showing that addition of IL-17A attenuated IL-13-induced SOCS1 expression. Decreased SOCS1 demonstrated reduced inhibitory effects on IL-13-mediated STAT6 activation, leading to higher STAT6 phosphorylation. Although no direct experimentation was undertaken in this study to test this suggestion, our findings do imply that decreased SOCS1 caused higher STAT6 phosphorylation. Therefore, we concluded that IL-17A enhanced

IL-13–driven STAT6 activation through downregulation of SOCS1 expression.

In spite of these findings, the role of IL-17 remains controversial, as seen in the mouse model of allergic asthma, with data indicating either an inhibitory or an enhancing action.^{49,50} In the future we plan to evaluate whether our in-vitro results could be transferred into an in-vivo situation of experimental AR. In addition, the binding sites of nuclear factor-kappaB (NF- κ B) are found in the promoters of IL-17A-enhanced IL-13-induced genes. STAT6 and NF- κ B bind cooperatively to the DNA sequences containing both sites, and the presence of a complex formed by their cooperative binding correlates with the synergistic activation of the promoter by STAT6 and NF- κ B.^{25,51–53} Our study data are limited because we have not yet determined whether this mechanism is also involved in the reciprocal coregulation of eotaxin-3 expression by IL-13 and IL-17A. Therefore, we plan to investigate the effects of synergistic coregulation mediated by NF- κ B and STAT6 activation on eotaxin-3 production.

Conclusion

IL-17A potentiated IL-13–driven STAT6 activation through the downregulation of SOCS1 expression, leading to the enhancement of eotaxin-3 production by HNECs. These factors contribute to the recruitment of eosinophils into the nasal mucosa in AR. We found that IL-17A is an important mediator that acts synergistically with Th2 cytokines to enhance eosinophilic inflammation in AR. A complete description would provide mechanistic insight into therapeutic strategies to improve eosinophilic inflammation associated with IL-17A in the management of AR. 

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