

Forkhead box protein 3 in human nasal polyp regulatory T cells is regulated by the protein suppressor of cytokine signaling 3

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Background: In patients with persistent upper airway inflammation, the number of forkhead box protein 3 (Foxp3)⁺ regulatory T (Treg) cells is reduced, but the regulation of Foxp3 expression in Treg cells is poorly understood.

Objective: We investigated the interaction between suppressor of cytokine signaling 3 (SOCS3) and Foxp3 expression in the airway mucosa.

Methods: Expression of SOCS3 and Foxp3 was measured in tissue from patients with chronic rhinosinusitis with nasal polyps (CRSwNP) and control tissue. Coexpression of SOCS3 and Foxp3 was evaluated in PBMCs and in tissue from patients with CRSwNP. We also switched off and overexpressed SOCS3 in tissue from patients with CRSwNP and in pancreatic carcinoma epithelial-like cell line (PANC-1) cells and examined the effect on Foxp3 expression.

Results: SOCS3 gene and protein expression was upregulated in inflammatory cells in airway mucosa, whereas Foxp3 gene and protein expression was downregulated. Mucosal Treg cells coexpressed both proteins. Switching off the expression of SOCS3 in human airway mucosa resulted in Foxp3 upregulation, whereas inducing it in PANC-1 cells led to Foxp3 downregulation. We also found that phosphorylation of signal transducer and activator of transcription (STAT) 3 was decreased in inflamed mucosa, and we hypothesized that SOCS3

was responsible. Phosphorylation of STAT3 increased on silencing SOCS3 expression in inflamed mucosa and decreased on SOCS3 plasmid transfection in PANC-1 cells.

Conclusion: For the first time, we demonstrate that SOCS3 and Foxp3 are coexpressed in Treg cells in human nasal mucosa and that SOCS3 negatively regulates Foxp3 expression in human airway mucosa, possibly through phosphorylation of STAT3. Hence SOCS3 could be a potential target for restoring Foxp3 expression in Treg cells in patients with persistent mucosal inflammation. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: Forkhead box protein 3, suppressor of cytokine signaling 3, signal transducer and activator of transcription 3, airway disease, nasal polyps

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a chronic inflammatory condition in the upper airways that is often accompanied by comorbid asthma. Two different inflammatory patterns have been described in patients with CRSwNP: either a T_H2 bias or a mixed T_H1 and T_H17 cell preponderance. Both patterns are accompanied by a deficit in forkhead box protein 3 (Foxp3)⁺ regulatory T (Treg) cells in European and Chinese patients.¹⁻⁴

Foxp3⁺ Treg cells play a critical role in immune regulation by maintaining peripheral self-tolerance, thereby preventing chronic inflammatory and autoimmune disease.⁵ A loss-of-function mutation in the Foxp3 gene in mouse and human models is associated with immunodysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome.⁵ Overexpression of Foxp3 in T_H cells imparts a suppressive phenotype.⁶ However, regulation of Foxp3 expression in Treg cells is still poorly understood. The role of suppressor of cytokine signaling 3 (SOCS3) protein in the development and function of Treg cells remains controversial. SOCS3 expression in antigen-presenting cells (APCs) indirectly suppresses the development of Treg cells.⁷ On the other hand, Foxp3 might interact with SOCS3 by binding to the promoter or to other regulatory sites.⁸ SOCS3 mediates the suppression of Treg cell proliferation and the suppression of Foxp3 and cytotoxic T lymphocyte-associated antigen 4 by reducing IL-2 levels.⁹ The inactivation of SOCS3 in CD4⁺ cells prevents the development of symptoms associated with asthma, such as airway eosinophilia in mouse models.¹⁰

The SOCS family of proteins is at present composed of 8 members characterized by the presence of an Src homology 2 domain and a C-terminal conserved domain called the SOCS box.¹¹ SOCS proteins negatively modulate the activation of signal transducer and activator of transcription (STAT) in the Janus kinase (JAK)–STAT signaling pathway, which mediates the

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Abbreviations used

APC:	Antigen-presenting cell
CRSwNP:	Chronic rhinosinusitis with nasal polyps
Foxp3:	Forkhead box protein 3
JAK:	Janus kinase
PANC-1:	Pancreatic carcinoma epithelial-like cell line
pSTAT:	Phosphorylated signal transducer and activator of transcription
siRNA:	Small interfering RNA
SOCS3:	Suppressor of cytokine signaling 3
STAT:	Signal transducer and activator of transcription
Treg:	Regulatory T

cytokine negative feedback plural loops.¹¹ The binding of cytokines, such as IL-6, IL-2, IL-23, IL-4, INF- γ , and growth hormones, to their specific receptors stimulates the cross-phosphorylation of STATs to induce SOCS genes. The SOCS proteins in turn bind to a cytokine-receptor complex, degrade the complex through the SOCS box, and interact with the JAK enzyme through their SH2 region. This results in a decrease in levels of phosphorylated signal transducers and activators of transcription (pSTATs).^{11,12} SOCS3 protein acts mainly on the phosphorylation of STAT3 and STAT5.¹³

In mouse models SOCS3 protein is a major regulator of the IL-23-mediated T_H17 response.¹⁴ SOCS3 has an important role in balancing T_H1/T_H2 toward a T_H2-type and not T_H1 differentiation.¹⁵ High SOCS3 levels in mouse T cells were also shown to cause T_H2 skewing and allergic responses.¹⁵

We analyzed whether SOCS3 is involved in the regulation of Foxp3 expression in human nasal mucosal tissue of patients with CRSwNP and studied the mechanisms of this interaction. We used human nasal mucosa as a model to investigate the regulatory role of SOCS3 in the deficit in Foxp3⁺ Treg cells.

METHODS**Patients**

Nasal tissue was obtained from 71 patients with CRSwNP and 12 control subjects at the Department of Oto-Rhino-Laryngology of the West China Hospital of Sichuan University, Chengdu, China. None of the patients had taken oral corticosteroids for at least 2 months and topical medication for at least 4 weeks before surgery. The diagnosis of nasal polyps was made according to the European Position Paper on Rhinosinusitis and Nasal Polyps 2007 guidelines.¹⁶ The inferior turbinates of patients with septal deviation were used as controls. Before surgery, the rhinosinusitis symptoms were evaluated by a physician on a 4-point scale of 0 to 3 (0, no symptoms; 1, mild symptoms; 2, moderate symptoms; and 3, severe symptoms), and polyps were graded by size and extent in both the left and right nasal fossa, according to the Davos classification (0–4 per side). The clinical data of all the patients are shown in Table E1 in this article's Online Repository at www.jacionline.org. This study was approved by the local Ethics Committee on Human Experimentation of the West China Hospital, Sichuan University, Sichuan, China. Informed consent was obtained from all patients and healthy donors before sample collection.

Immunohistochemistry and immunocytochemistry

The nasal tissues were fixed in 10% neutral buffered formalin and processed to paraffin. Sections of 4 to 5 μ m were incubated overnight at 4°C with primary antibody against SOCS3 (1:100; Cell Signaling Technology, Beverly, Mass). The secondary antibody was anti-goat (Zhongshanjingqiao, Beijing, China). Positive cells were visualized with the diaminobenzidine chromogenic reaction. Quantitative analysis of the histologic sections was

performed by 2 independent observers, who counted a total of 300 cells in 10 fields per tissue section from 12 control subjects and 12 patients with CRSwNP. Additionally, the sorted Treg cells from PBMCs of 3 healthy donors were fixed and incubated with anti-SOCS3 antibody (1:100). As a control, the specimens were incubated with isotype antibody IgG.

Quantitative RT-PCR

Quantitative RT-PCR was used to estimate the mRNA levels of SOCS3, Foxp3, STAT3, and STAT5A/B in 71 patients with CRSwNP and 12 control subjects. After extraction of total RNA with the RNeasy kit, according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany), cDNA was synthesized, starting with 40 ng of total RNA using the Prime Script RT Reagent Kit (Takara, Dalian, China). Total RNA was quantified by using RT-PCR with the iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, Calif) with the following primer sequences: SOCS3, 5'-ATG GTC ACC CAC AGC AAG TT-3' and 5'-ACT GAG CAG CAG GTT CG C-3'; Foxp3, 5'-GAA ACA GCA CAT TCC CAG AGT TC-3' and 5'-ATG GCC CAG CGG ATG AG-3'; STAT3, 5'-GCA CAT TCA TGC TAA GAT TCA G-3' and 5'-TGA TGC TTT GTG TAT GGT TCC-3'; STAT5A, 5'-CCA CAG ATG AAG CAA GTG GTC-3' and 5'-ATC GAG TAC ATG CTC ATG GTT C-3'; and STAT5B, 5'-GAT GCC TTT TAC CAC CAG AGA C-3' and 5'-AGT AGC AGA CTC GCA GGG AAC T-3'. PCR amplification consisted of 40 cycles of denaturation at 95°C for 15 seconds and annealing and extension cycles at 60°C for 45 seconds each. The target product was detected with the SYBR Premix Ex Taq II kit (Takara, Dalian, China). Three housekeeping genes were used to normalize for transcription and amplification variations among the samples: β -actin, hydroxymethylbilane synthase (HMBS), and elongation factor 1 (EF1). The relative quantities expressed were assessed by using Gene Expression Macro Version 1.1 software (Bio-Rad Laboratories).

Western blotting

Protein was extracted with the Protein Extraction Reagent (Kaiji, Shanghai, China) from the nasal tissue of 35 patients with CRSwNP and 12 control subjects. Protein concentrations were measured by using the Bicinchoninic Acid Assay kit (Kaiji). The following antibodies were used for Western blotting: anti-SOCS3 (1:700), anti-STAT3 (1:800), anti-p-Tyr-STAT3 (Tyr705; 1:700), anti-STAT5 (1:600), and anti-p-Tyr-STAT5 (Tyr694; 1:600). All the above-mentioned antibodies were purchased from Cell Signaling Technology (Danvers, Mass). Anti-Foxp3 antibody (1 μ g/mL) was purchased from Abcam (Cambridge, United Kingdom). The horseradish peroxidase-conjugated secondary antibody (1:10,000) was from Santa Cruz Biotechnology (Santa Cruz, Calif). The relative band densities of the target protein to β -actin (Boaosen, Beijing, China) were estimated by using Bio-Rad Quantity One Analysis Software (Bio-Rad Laboratories).

Cell isolation

Primary mononuclear cells were isolated from the blood of 3 healthy donors by using Ficoll-Hypaque density gradient centrifugation, according to the standard protocol. Cells were plated at 2×10^6 cells per well in a 24-well plate and cultured at 37°C in 5% CO₂ for 5 days in 1 mL of RPMI 1640 culture medium alone or with recombinant human IL-2 (10 ng/mL) and recombinant human IL-15 (10 ng/mL; Miltenyi Biotec, Bergisch Gladbach, Germany).

Single-cell suspension and flow cytometry

Single-cell suspensions of human nasal mucosa were prepared, as previously described.¹⁷ The cultured cells and single cells of tissue from patients with CRSwNP ($n = 3$) were collected and incubated with anti-CD3-phycoerythrin-Cy, anti-CD4-PerCP-Cy5.5, and anti-CD25-fluorescein isothiocyanate antibodies for 30 minutes to analyze the coexpression of Foxp3 and SOCS3 proteins in Treg cells. After fixation and permeabilization, the cells were stained intracellularly with anti-Foxp3-phycoerythrin and a 1:100 rabbit anti-human SOCS3 antibody (Abcam) for 30 minutes, followed

by goat anti-rabbit allophycocyanin secondary antibody (Invitrogen, Merelbeke, Belgium) for 20 minutes. PBMCs from healthy donors were stained with anti-CD4–fluorescein isothiocyanate, anti-CD25–PerCP-Cy5-5 (BD Biosciences, Erembodegem, Belgium), and anti-Foxp3–phycoerythrin antibody (BD Biosciences), and Treg cells were sorted by using standard protocols and a FACSria instrument (BD Biosciences).

RNA interference experiments

Three SOCS3-specific small interfering RNAs (siRNAs; siG0933161433, siG0933161455, and siG0933161508) and a negative control siRNA (siN058151221487) were synthesized (RiBo Bio, Shanghai, China), and the target sequence was validated (5'-GAC CCA GTC TGG GAC CAA G-3'). The following sequences were used: SOCS3 sense, GAC CCA GUC UGG GAC CAA G dTdT; SOCS3 antisense, CUU GGU CCC AGA CUG GGU C TdT. The transfection efficacy of siRNA in nasal polyp tissue was estimated by using siR-Ribo Transfection Control (siN05815122148, RiBo Bio). The result is shown in Fig E1 in this article's Online Repository at www.jacionline.org. More detailed information about transfection efficiency has been provided in the Methods section in this article's Online Repository at www.jacionline.org. Nasal polyp tissue ($n = 5$) was washed with PBS containing 100 IU/mL penicillin and 100 μ g/mL streptomycin 3 times and sliced into cubes. The 80 mg of tissue cubes were placed into each well of a 5-well plate containing 1.3 mL of RPMI 1640 medium and transfected with SOCS3-specific siRNA (40 nmol/L) or negative control siRNA (40 nmol/L) using 5 μ L of the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, Calif), according to the standard protocol. After 4 hours of incubation, fetal serum (10% final concentration; Hyclone, Logan, Utah) was added to each well to terminate transfection, and tissues were incubated at 37°C in humidified air with 5% CO₂ for 48 hours. The tissue cubes were collected for RT-PCR and Western blotting.

Transient expression of SOCS3 in pancreatic carcinoma epithelial-like cells

Human pancreatic carcinoma epithelial-like cell line (PANC-1) cells, a cancer cell line with strong gene expression of Foxp3,¹⁸ were used for transient expression of SOCS3. pEF-FLAG-I/m vector and pEF-FLAG-I/m SOCS3 plasmid (2 μ g) were separately transfected into 5×10^6 PANC-1 cells by using Lipofectamine 2000 transfection reagent. Total RNA and proteins were extracted from transfected PANC-1 cells after 48 hours of incubation. These experiments were repeated 3 times.

Statistics

A 1-way ANOVA test, Mann-Whitney test, and *t* test were performed with SPSS 12.0 software (SPSS, Inc, Chicago, Ill). A *P* value of less than .05 was considered statistically significant.

RESULTS

SOCS3 expression is increased in patients with CRSwNP

Immunohistochemical analysis showed that patients with CRSwNP had significantly stronger expression of SOCS3 than control subjects (Fig 1). In tissue from patients with CRSwNP, SOCS3 protein was preferentially expressed in inflammatory cells, epithelium, and submucosal glandular epithelium. Quantitative analysis performed by 2 independent observers of the histologic sections showed a significant increase in SOCS3⁺ cell numbers in nasal polyp tissue; $33.3\% \pm 9.06\%$ of these cells were positively stained compared with only $6.67\% \pm 3.58\%$ in tissue from control subjects ($P < .0001$). Levels of SOCS3 gene expression were higher in patients with CRSwNP, and this was confirmed by means of Western blotting (Fig 2, A-C).

Importantly, the expression of Foxp3 protein in patients with CRSwNP was significantly increased (Fig 2, B and D).

Foxp3⁺ Treg cells express SOCS3 protein

In nonstimulated conditions 86% of CD4⁺CD25⁺Foxp3⁺ Treg cells in human peripheral blood expressed SOCS3 (Fig 3, A-B). In PBMCs treated with 10 ng/mL IL-2 and IL-15 for 5 days (Fig 3, A-C), 96% of CD4⁺CD25⁺Foxp3⁺ Treg cells coexpressed SOCS3. Therefore most CD4⁺CD25⁺Foxp3⁺ cells coexpressed SOCS3 in both stimulated and nonstimulated conditions. In addition, CD4⁺CD25⁺Foxp3⁺ cells were sorted from PBMCs of 3 healthy donors and stained with anti-SOCS3 antibody. The result confirmed the coexpression of these proteins in 96% of Treg cells (Fig 3, B and C). In human nasal mucosal tissue of patients with CRSwNP, 45.1% of CD3⁺CD4⁺CD25⁺ cells were SOCS3⁺Foxp3⁺ (Fig 3, D).

Modulation of SOCS3 levels and Foxp3 expression

Because immunohistochemical analysis showed high levels of SOCS3 in the epithelium, glandular epithelium, and inflammatory cells in tissue from patients with CRSwNP, human nasal mucosal tissues were used in silencing experiments. Three different SOCS3-specific siRNAs were designed to block the function of SOCS3 protein. The siRNA construct that knocked down SOCS3 protein most efficiently was selected. Blocking SOCS3 gene expression by siRNA decreased SOCS3 gene expression by 60% (Fig 4, A). Analysis of Foxp3 gene expression after blocking SOCS3 expression demonstrated a 3-fold increase in the same samples of tissue from patients with CRSwNP (Fig 4, A). The RNA expression results were confirmed by using Western blot analysis: Foxp3 protein expression increased significantly on silencing SOCS3 in tissue from patients with CRSwNP (Fig 4, B and C).

SOCS3 mRNA and protein levels were increased in PANC-1 cells on transfection with the SOCS3-encoding plasmid (Fig 4, D-F), whereas Foxp3 protein expression was attenuated by up to 80%, as evident from measuring the relative expression levels by using Western blotting (Fig 4, E). Taken together, our data demonstrate that SOCS3 negatively modulates Foxp3 expression.

SOCS3 regulation of Foxp3 might depend on the presence of pSTAT3

Expression of the STAT3 and STAT5A/B genes was investigated in nasal tissue to further study the regulation of Foxp3 expression by SOCS3. STAT3, STAT5B, and STAT5A gene expression was not different between tissue from patients with CRSwNP and control tissue (data not shown). However, SOCS3 might attenuate the phosphorylation of STATs, which are the activated forms of STATs in the JAK-STAT pathway. We demonstrate that pSTAT3 was significantly downregulated in the nasal mucosa of patients with CRSwNP in comparison with control subjects (Fig 5, A and B). STAT5 and pSTAT5 protein levels were undetectable in Western blots. As shown in Fig 5, C and D, the relative expression of pSTAT3 was upregulated by 65% after silencing SOCS3 protein expression in nasal mucosal tissue. In contrast, the relative expression of pSTAT3 was decreased by 75% when SOCS3 was overexpressed from a plasmid (Fig 5, E and F).

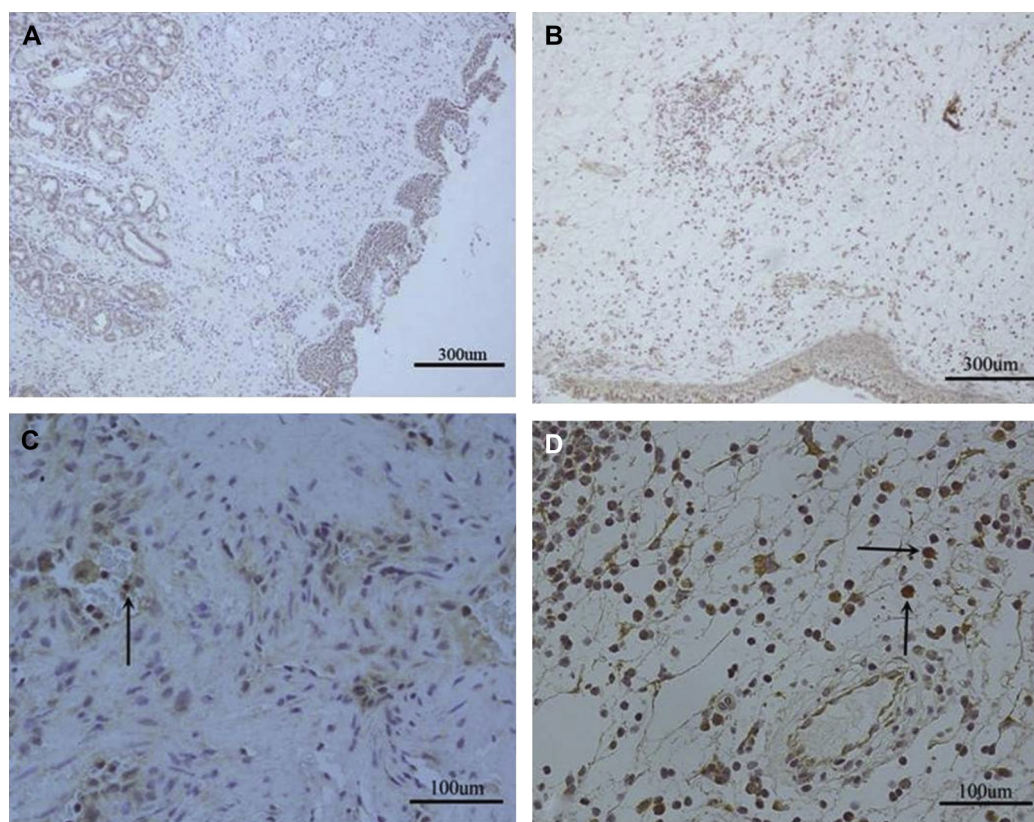


FIG 1. SOCS3 expression in tissue from patients with CRSwNP. Representative images of SOCS3 immunohistochemistry in tissue from patients with CRSwNP and control subjects are shown. **A** and **C**, SOCS3⁺ cells (*arrows*) in control subjects. **B** and **D**, SOCS3⁺ stained cells (*arrows*) in patients with CRSwNP. Magnification $\times 100$ (Fig 1, **A** and **B**) and $\times 400$ (Fig 1, **C** and **D**).

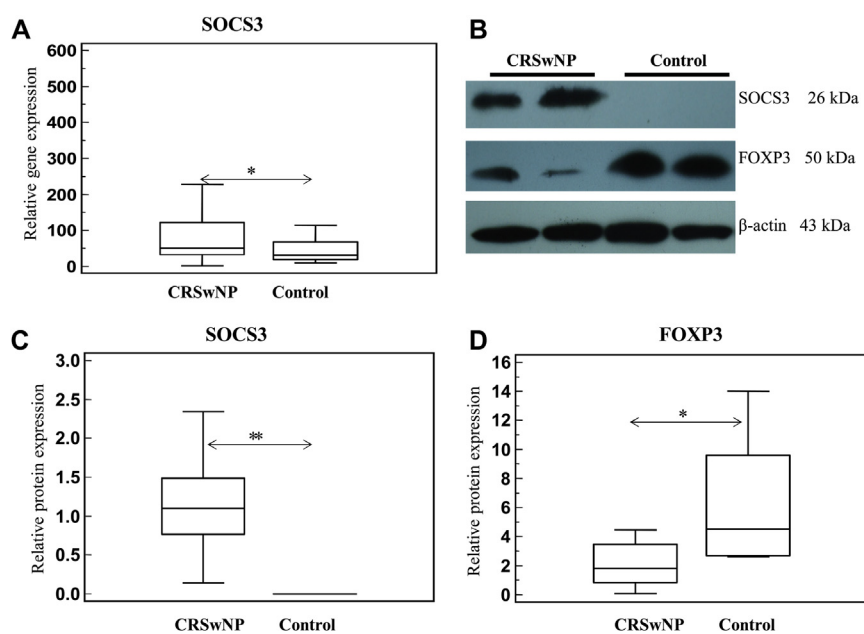


FIG 2. Expression of SOCS3 and Foxp3 in patients with CRSwNP. **A**, RT-PCR showing SOCS3 expression in patients with CRSwNP and control subjects. **B**, Western blots for SOCS3 and Foxp3 proteins in patients with CRSwNP and control subjects. **C**, Quantification for SOCS3 protein in tissues from patients with CRSwNP. **D**, Quantification for Foxp3 protein in tissues from patients with CRSwNP. * $P < .05$ and ** $P < .01$.

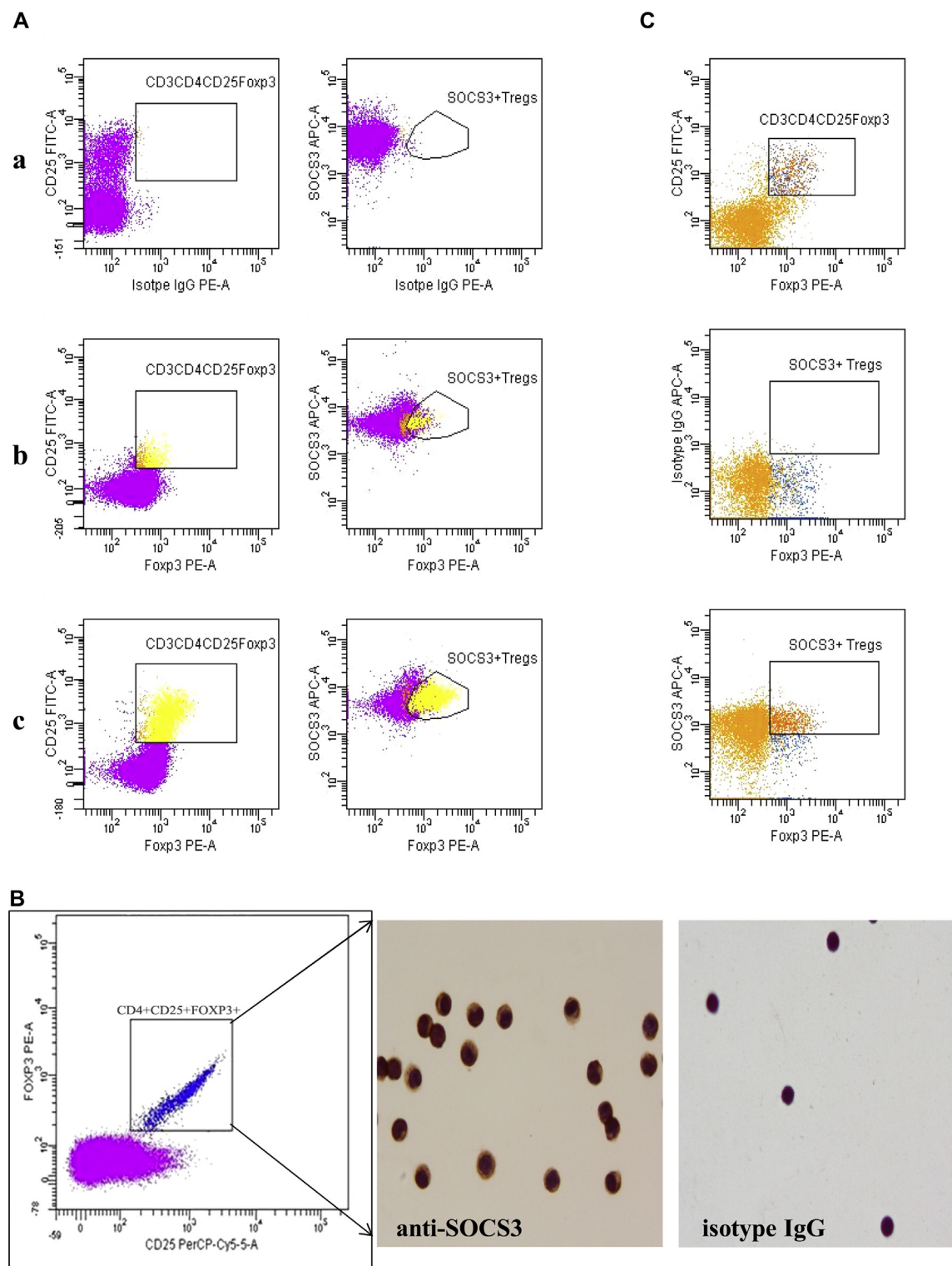


FIG 3. Expression of SOCS3 and Foxp3 proteins in Treg cells. **A**, PBMCs of healthy donors gated on living $CD3^+CD4^+CD25^+Foxp3^+$ cells cultured in RPMI: **A-a**, negative control; **A-b**, nonstimulated; **A-c**, stimulated with IL-2 and IL-15. **B**, $CD4^+CD25^+Foxp3^+$ cells sorted from PBMCs of healthy donors and stained with anti-SOCS3 antibodies. **C**, The single cells of tissue from patients with CRSwNP gated on living $CD3^+CD4^+CD25^+Foxp3^+SOCS3^+$ cells.

DISCUSSION

CRSwNP is characterized by T_H2 -skewed eosinophilic inflammation in European patients and by a T_H17 -biased inflammatory

profile in Asian patients. However, regardless of the inflammatory profile, significantly weaker Foxp3 mRNA expression and significantly stronger T-bet and GATA-3 mRNA expression

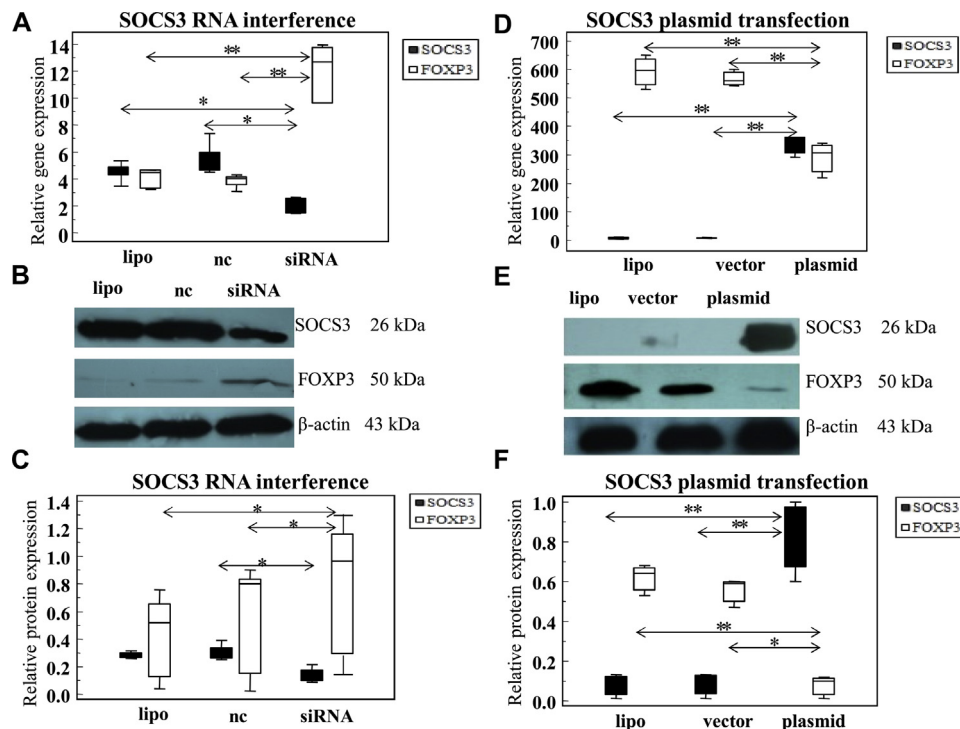


FIG 4. SOCS3 regulates Foxp3 expression in tissue from patients with CRSwNP. **A-C**, SOCS3 and Foxp3 expression in patients with CRSwNP on addition of Lipofectamine 2000 (*lipo*), transfection with negative control of siRNA (*nc*), or transfection with SOCS3-specific siRNA (*siRNA*). **D-F**, SOCS3 and Foxp3 expression on PANC-1 cells on addition of Lipofectamine 2000 (*lipo*), transfection with pEF-FLAG-I/m vector (*vector*), or transfection with pEF-FLAG-I/m SOCS3 plasmid (*plasmid*). * $P < .05$ and ** $P < .01$.

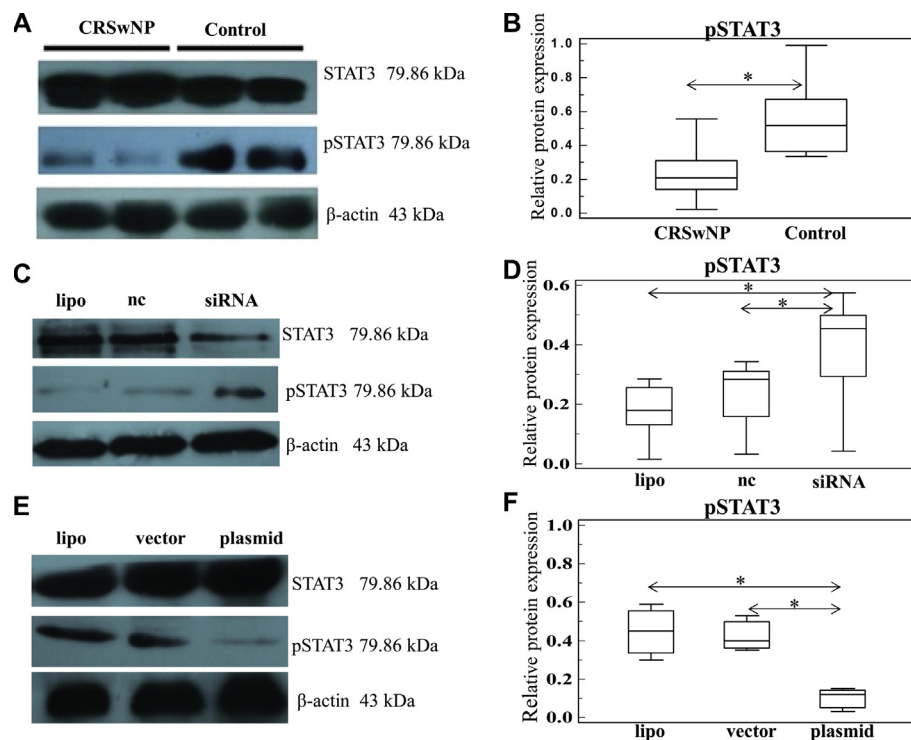


FIG 5. Role of pSTAT3 in regulation of Foxp3. **A and B**, pSTAT3 expression in nasal tissue. **C and D**, pSTAT3 expression in patients with CRSwNP on addition of Lipofectamine 2000 (*lipo*), transfection with negative control of siRNA (*nc*), or transfection with SOCS3-specific siRNA (*siRNA*). **E and F**, pSTAT3 expression on PANC-1 cells on addition of Lipofectamine 2000 (*lipo*), transfection with pEF-FLAG-I/m vector (*vector*), or transfection with pEF-FLAG-I/m SOCS3 plasmid (*plasmid*). * $P < .05$.

were observed in both groups of patients.³ The mechanisms of altered T-cell plasticity in patients with CRSwNP are not yet clear. We propose that SOCS3 protein is an important regulator of Foxp3 in patients with CRSwNP. We have demonstrated increased levels of SOCS3 protein in patients with CRSwNP compared with those seen in control subjects. The expression of SOCS3 protein was mostly observed in inflammatory cells, epithelium, and glandular epithelium in the submucosa.

Importantly, SOCS3 expression is not limited to effector T cells.^{14,15} Also, CD4⁺CD25⁺Foxp3⁺ Treg cells stained intensely for SOCS3. To our knowledge, this is the first study to demonstrate the coexpression of SOCS3 and Foxp3 proteins in Treg cells from human nasal mucosal tissue. In contrast, Pillemer et al⁹ did not find SOCS3 protein expression in mouse Treg cells. However, in mice lacking SOCS3 expression in T cells, a preferential T_H3 and Treg cell differentiation was observed.^{19,20} This T-cell polarization bias could be explained by increased production of IL-10 and TGF- β in conditions of SOCS3 deficiency. In addition, the impaired expression of SOCS3 in APCs indirectly induces the promotion of CD4⁺CD25⁺Foxp3⁺ cells.⁷ In recent experiments Nguyen et al²¹ showed that induction of SOCS3 expression in Treg cells by pharmacologic means abrogated a Treg cell proliferative response induced by serum amyloid A and monocytes. However, the mechanism of SOCS3-Foxp3 interaction and its role in Treg cell development are not completely understood.

The role of SOCS3 protein in T-cell differentiation and function is well established,²² but its role in Treg cell function is just being elucidated. The differentiation and survival of Treg cells are regulated by Foxp3. The nuclear factor κ B family members cAMP response element modulation protein (CREB)/activating transcription factor 1 (ATF1), v-ets avian erythroblastosis virus E26 oncogene homolog 1 (ETS-1), and forkhead box 1/3 (FOXO1/3) were shown to facilitate Foxp3 transcription and to bind to its promoter and other regulatory elements, as previously reviewed.²³ Additionally, STAT3 and STAT5 phosphorylation is critical for Foxp3 induction in precursor cells.²⁴ Phosphorylated STAT3 and STAT5 were also shown to interact with the Foxp3 promoter and to modulate Foxp3 gene expression.²⁵ SOCS3 protein acts mainly through phosphorylated STAT3 and STAT5.²⁶

In our RNA interference experiments, abolishing SOCS3 expression in the nasal polyp tissue increased the levels of Foxp3. In contrast, when overexpression of SOCS3 was achieved by means of transient transfection, Foxp3 levels were downregulated. These data confirm that in human nasal polyp tissue SOCS3 serves as a negative regulator of Foxp3 expression. Intranasal administration of Foxp3 strongly prevents ovalbumin-induced allergic airway inflammation in mice.²⁷ Moreover, upregulation of Foxp3 by corticosteroids in asthmatic patients restores Treg cell function to suppress the T_H2 response.²⁸ Therefore we suggest that upregulation of Foxp3 expression by SOCS3 downregulation might present a therapeutic approach in nasal polyps. In addition, we found that pSTAT3 protein levels are reduced in patients with CRSwNP. We also confirm that phosphorylation of STAT3 increased on silencing SOCS3 expression in inflamed mucosa and decreased on SOCS3 overexpression from a plasmid transfected in PANC-1 cells. The regulatory role of pSTAT3 on Foxp3, unlike pSTAT5, is still controversial. pSTAT3 is reported to play a negative role on Foxp3 in allergic or graft-versus-host mouse models^{29,30};

however, pSTAT3 signaling also can promote Foxp3 expression in the same graft-versus-host mouse model.²⁵ Furthermore, induced Foxp3 in human Treg cells is pSTAT3 dependent,³¹ which is in accordance with our results in human nasal polyp tissue. Consequently, we suggest that phosphorylation of STAT3 might be involved in the SOCS3-induced regulation of Foxp3.

The following limitations have to be taken into account. In this study we used patients with CRSwNP from China, and there is evidence that nasal polyps from Chinese subjects are different from polyps from white subjects, so that our findings might not be readily transferable to all patients; however, there is evidence that the same downregulation of Foxp3 applies to polyps from both populations, and we also demonstrated that upregulation of SOCS3 can be demonstrated in polyp tissue from white subjects (data not shown). Therefore we believe that our data are valid for all patients with nasal polyps. Our patients were asked to stop taking oral corticosteroids for at least 2 months and topical medication for at least 4 weeks before surgery; however, we could not control compliance. Also, we speculate on a possible therapeutic effect of silencing SOCS3 to increase the activity of Foxp3 Treg cells, which might lead to a suppression of the inflammatory process, but we have not yet proved this here; further studies are ongoing. We also noticed that there is a difference in the percentage of Foxp3 expression in SOCS3⁺ cells in nasal polyp tissue versus PBMCs from healthy donors. It is currently unclear whether this difference is due to the fact that we compare peripheral blood with tissue or healthy with diseased patients; further studies are needed to investigate this question.

In summary, we demonstrate that SOCS3 negatively regulates Foxp3 expression in patients with CRSwNP and that this regulation might involve pSTAT3. Because downregulation of SOCS3 leads to an increase in Foxp3 expression, this might improve the suppressive capacities of Treg cells.³² Therefore SOCS3 could be a potential target for restoring Foxp3 expression in Treg cells to consecutively suppress persistent mucosal inflammation. Thus SOCS3 siRNA or other promising therapeutic technology, such as SOCS3-specific DNzyme, a new class of antisense molecules that combines the specificity of DNA base pairing with an inherent RNA-cleaving enzymatic activity,³³ could possibly be used to inhibit SOCS3 expression for therapeutic intervention in patients with nasal polyp disease.

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Clinical implications: SOCS3 and Foxp3 are coexpressed in Treg cells. SOCS3 negatively regulates Foxp3 expression in patients with CRSwNP by interfering with pSTAT3. SOCS3 downregulation restores Foxp3 expression and might represent a novel therapeutic approach.

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METHODS

Nasal polyp tissue was cut thoroughly in tissue culture medium consisting of RPMI 1640 and antibiotics (50 IU/mL penicillin and 50 μ g/mL streptomycin). The tissue was passed through a mesh to achieve a comparable fragment. Tissue fragments (± 0.9 mm³) were resuspended and preincubated in tissue culture medium for 1 hour. Eighty milligrams of tissue fragment was placed into each well of a 6-well plate containing 1.3 mL of RPMI 1640 medium without antibiotics. FAM-labeled siRNA, siR-Ribo Transfection Control (40 nmol/L), was transfected into the tissue by using 5 μ L of the Lipofectamine 2000 transfection reagent. After 4 hours of incubation, fetal serum (10% final concentration)

was added to each well to terminate transfection, and tissues were incubated at 37°C in humidified air with 5% CO₂ for 48 hours. Tissue fragments with or without FAM-labeled siRNA were washed 3 times with PBS before collection, frozen, and prepared for cryosections. Five-micrometer-thick cryosections were fixed in 70% ethanol and dried at room temperature before being mounted with DAPI (1:50 dilution). The transfection efficiency was evaluated by using the following formula: Transfection efficiency = Cells expressing green fluorescence/Total accounted cells $\times$ 100%. Images were obtained at 2 magnifications ($\times 100$ and $\times 400$) by using a fluorescence microscope (Axioplan 2; Carl Zeiss, Gottingen, Germany).

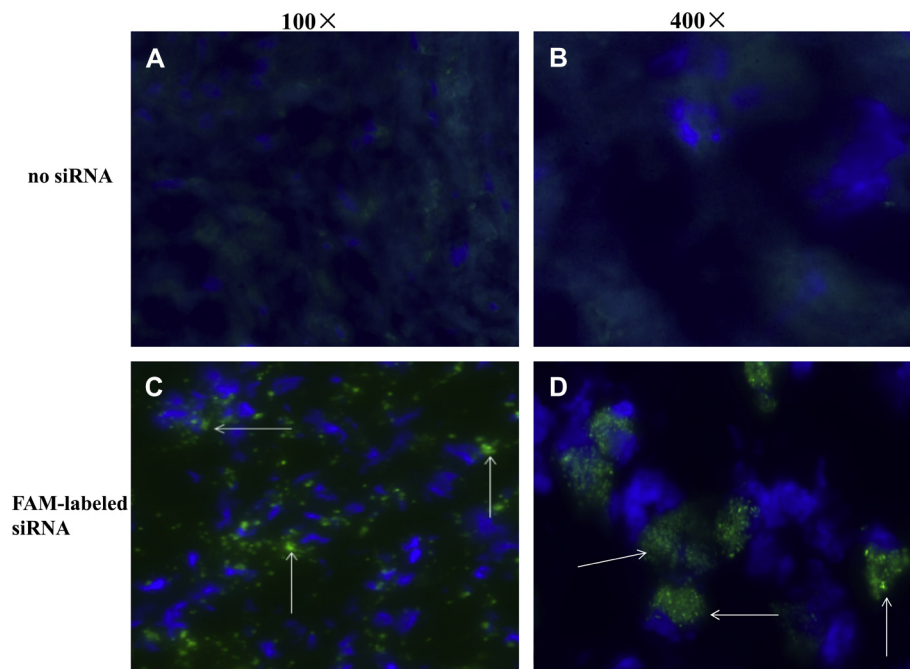


FIG E1. Transfection efficiency of siRNA in nasal polyp tissue fragments. The blue fluorescence represents the nuclei of cells, and the green dots represent the FAM-labeled siRNA transfection control. **A** and **B**, Fluorescence images of nasal polyp tissue fragments without FAM-labeled siRNA. **C** and **D**, Fluorescence images of tissue fragments from patients with CRSwNP with FAM-labeled siRNA. The merged version of green and blue fluorescence indicates the siRNA intracellular location (*white arrows*). Magnification: $\times 100$ (Fig E1, **A** and **C**) and $\times 400$ (Fig E1, **B** and **D**).

TABLE E1. Patients' characteristics and symptom scores

	Control subjects	Patients with CRSwNP
No. of patients	12	71
Age (y), mean (SD)	29.75 (17-60)	38.2 (17-66)
Female/male sex, no.	3/9	35/36
Asthma in history, n/N	0/12	7/71
Aspirin intolerance, n/N	0/12	0/71
Duration of symptoms (y), mean (SD)	NA	8.0 (0.5-28)
Bilateral CT score (Lund-Mackay), mean (SD)	NA	16.08 (11-24)
Bilateral polyp score (Davos), mean (SD)	0	4.16 (2-6)
Total symptom score, mean (SD)	0.9 (0-4)	4.7 (2-10)
Nasal congestion, mean (SD)	0.8 (0-3)	2.1 (0-3)
Sneezing, mean (SD)	0	0.6 (0-3)
Rhinorrhea, mean (SD)	0.2 (0-1)	1.2 (0-3)
Loss of smell, mean (SD)	0	0.8 (0-3)

CT, Computed tomographic.