

Evidence for altered activity of the IL-6 pathway in chronic rhinosinusitis with nasal polyps

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Background: IL-6 activates T_H17 cells and regulates the response of B lymphocytes and regulatory T cells. The IL-6 receptor and the membrane protein, glycoprotein 130 (gp130), form an active signaling complex that signals through signal transducer and activator of transcription 3 (STAT3) and other signaling molecules. Both the IL-6 receptor (IL-6R) and gp130 can be found in soluble forms that regulate the pathway.

Objective: We measured IL-6 signaling components and IL-17 in chronic rhinosinusitis (CRS) with nasal polyps (CRSwNP), CRS without nasal polyps (CRSsNP), and controls to assess the IL-6 pathway in CRS.

Methods: IL-6, soluble IL-6R, soluble gp130 (sgp130), and IL-17 were measured in sinus tissue extracts and in nasal lavage fluid by either cytokine bead array or ELISA. phosphoSTAT3 (p-STAT3) was determined by Western blot and by immunohistochemistry.

Results: IL-6 protein was significantly ($P < .001$) increased in CRSwNP compared with CRSsNP and controls. Soluble IL-6R was also increased in nasal polyp compared with control tissue ($P < .01$). Despite elevated IL-6 and sIL-6R, IL-17A, E, and F were undetectable in the sinus tissue from most of the patients with CRS and controls. p-STAT3 levels were reduced in the polyp tissue, possibly indicating reduced activity of IL-6 in the tissue. sgp130 was elevated in CRSwNP compared with CRSsNP and controls.

Conclusion: p-STAT3 levels are decreased in CRSwNP despite increased levels of IL-6 and sIL-6R and are associated with the absence of an IL-17 response. This may be a response to elevated levels of sgp130, a known inhibitor of IL-6 signaling. These results indicate that IL-6 and its signaling pathway may be altered in CRSwNP. (*J Allergy Clin Immunol* 2010;125:397-403.)

Key words: Chronic rhinosinusitis, nasal polyps, IL-6, IL-6 receptor, soluble glycoprotein 130, IL-17, phospho-STAT3

Chronic rhinosinusitis (CRS) is a common clinical syndrome characterized by inflammation of the mucosa of the nose and the paranasal sinuses.¹ This disorder is typically classified into CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP). The etiology and pathogenesis of CRS is a matter of vigorous debate, but bacteria, viruses, and fungi have all been implicated in the establishment of the inflammatory process.²⁻⁵ Abnormalities in host response to these common agents, including defective cytokine and chemokine signaling of the nasal mucosa, have been suggested to underlie the persistence of the inflammatory state.⁶

In the current study, we investigated the role in CRS of IL-6, a cytokine implicated in the pathogenesis of various inflammatory diseases such as rheumatoid arthritis, Crohn disease, lupus, and asthma.⁷⁻¹¹ Early studies using RT-PCR and immunohistochemistry techniques indicated that IL-6 expression was increased in CRS.¹²⁻¹⁸ A recent report demonstrated elevated IL-6 protein in polyp tissue compared to middle turbinate in the same patients with CRSwNP.¹⁹ Although IL-6 has been proposed as a marker of inflammation in CRS, the role of IL-6 in CRS is not well defined. The published studies did not differentiate between CRSwNP and CRSsNP and none investigated the presence of IL-6 signaling components or the activation of upper airway tissue by IL-6.

IL-6 binds a specific 80-kd receptor (IL-6R, CD126), and then the complex associates with the 130-kd signal-transducing molecule, glycoprotein 130 (gp130, CD130).^{20,21} In general, the IL-6 receptor (IL-6R) is found primarily on hepatocytes and lymphocytes; however, gp130 is ubiquitously present on many cell types.^{22,23} IL-6 can affect gp130-positive cells that do not express the IL-6 receptor by binding to a 50-kd soluble IL-6 receptor (sIL-6R) that can complex with membrane bound gp130. This pathway has been termed “trans-signaling,” enabling IL-6 to regulate cells that would otherwise not respond to IL-6 because they lack the cognate IL-6R.²⁴ A soluble form of gp130 (sgp130) can inhibit trans-signaling by blocking the association of the IL-6/sIL-6R complex to the membrane-bound gp130 but does not inhibit signaling through the cell surface IL-6R.^{25,26}

Because of the potential importance of IL-6 in the pathogenesis of inflammatory diseases, we examined the distribution of IL-6 protein and the trans-signaling components in CRS tissue and cultured epithelial cells from patients with CRS. We also evaluated the level of activation of signal transducer and activator of

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Abbreviations used

CRS:	Chronic rhinosinusitis
CRSwNP:	Chronic rhinosinusitis with nasal polyps
CRSSNP:	Chronic rhinosinusitis without nasal polyps
Foxp3:	Forkhead box protein 3
gp130:	Glycoprotein 130
HIES:	Hyper-IgE syndrome
IL-6R:	IL-6 receptor
p-STAT3:	phospho-Signal transducer and activator of transcription 3
sgp130:	Soluble glycoprotein 130
sIL-6R:	Soluble IL-6 receptor
STAT3:	Signal transducer and activator of transcription 3
Treg:	Regulatory T

transcription 3 (STAT3), an IL-6-activated transcription factor, in sinonasal tissue. Because IL-6 is now known to be important in IL-17 production, we further extended our analysis and examined IL-17 production in CRS tissue.^{27,28} To the best of our knowledge, this is the first study to evaluate the IL-6 signaling pathway in CRS. Our results indicate increased levels of several components of the IL-6 pathway in sinus mucosa from patients with CRSwNP and CRSSNP. Interestingly, we detected evidence that IL-6 signaling may actually be blunted in nasal polyp tissue, on the basis of a reduced level of phosphorylation of STAT3 and increased sgp130.

METHODS**Subjects and specimens**

Sinonasal tissue and nasal lavage fluid were collected from subjects with CRSSNP and CRSwNP undergoing functional endoscopic sinus surgery. All subjects met the criteria for CRS as defined by the Sinus and Allergy Health Partnership.¹ All subjects had symptoms for 12 weeks or greater and had failed to respond to medical therapy. The presence of sinusitis or bilateral nasal polyps was confirmed by office endoscopy and sinus computed tomography scans. Most of the subjects were skin-tested before the procedure to pollens, dust mites, pets, molds, and cockroach by using Hollister Stier Canada (Toronto, Ontario, Canada) extracts. Further details are described in this article's Subject Selection section of the [Methods](#) in the Online Repository at www.jacionline.org. The Lund-Mackay scoring system (0-24) was used to grade the radiographic severity of sinus disease.²⁹ Clinical characteristics of subjects undergoing IL-6, sIL-6R, and sgp130 analyses in the sinus tissue are presented in [Table I](#). Detailed subject characteristics are also presented in [tables E1, E2, and E3](#) in the Online Repository at www.jacionline.org.

The control specimens included inferior turbinate tissue from individuals without history of CRS or asthma who were undergoing sinonasal surgery for unrelated reasons (eg, skull base tumor, cosmetic rhinoplasty, facial fracture). Nasal lavage was collected from control subjects without history of allergic rhinitis, CRS, or asthma. The Institutional Review Board of Northwestern University Feinberg School of Medicine approved the study protocol, and all subjects gave signed informed consent.

Measurement of IL-6, sIL-6R, sgp130, and IL-17A, E, and F in tissue and nasal lavage fluid

Extracts of sinonasal tissue or polyps were prepared by addition of 1 mL PBS-Tween 20 at 4°C to freshly obtained or fresh-frozen polyp/tissue samples. A cocktail of protease inhibitors (PIC) purchased from Sigma Chemical Co (St Louis, Mo) was added to the samples in a 1:100 dilution (10 μ L). The polyp/tissue samples were mechanically minced with a scalpel on ice and then homogenized for 30 to 60 seconds on ice with an IKA-WERKE Ultra-Turrax T8 Homogenizer (IKA Works Inc, Wilmington, NC). The suspensions were then centrifuged at 4000 rpm for 20 minutes at 4°C. The tissue extracts were collected and stored at -20°C until use. Nasal lavage was performed by instilling 5 mL warmed sterile

PBS into each nostril, holding for 10 seconds, and then expelling into a container. Of the 5 mL used for the nasal lavage, the recovery was about 3 to 4 mL. Samples were concentrated 2-fold by using Centriplus YM-10 filters (Millipore, Bedford, Mass) and stored at -80°C until analyzed. Levels of IL-6 protein were determined by cytometric bead array assay (BD Biosciences, San Diego, Calif). Levels of sIL-6R and sgp130 were determined by ELISA (R&D Systems, Minneapolis, Minn). According to the sIL-6R kit, the soluble form arises from proteolytic cleavage of membrane-bound IL-6R. The minimal detection limits for IL-6, sIL-6R, and sgp130 are 20 pg/mL, 31.2 pg/mL, and 0.125 ng/mL, respectively. IL-17A, E, and F concentrations were determined by ELISA (IL-17A and F from R&D Systems, Minneapolis, Minn, and IL-17E from PeproTech, Rocky Hill, NJ). The minimal detection limits for these kits are 31.2 pg/mL, 31.2 pg/mL, and 78 pg/mL, respectively. Zero value was taken when the samples were under the detection limit. All results were normalized to total protein content in the extracts as determined by the Bio-Rad protein assay kit (Bio-Rad, Hercules, Calif).

Cell culture and Immunohistochemistry

The methods for primary nasal epithelial cell culture and immunohistochemistry are described in the [Methods](#) in the Online Repository at www.jacionline.org.

Nasal tissue protein extraction and Western blot

The methods are described in the [Methods](#) in the Online Repository.

Statistical Analysis

Statistical comparisons were performed by nonparametric analysis using the Mann-Whitney *U* test and a value of *P* < .05 was accepted as being statistically significant.

RESULTS

Clinical and epidemiologic data are shown in [Table I](#) and this article's [Tables E1, E2, E3, and E4](#) in the Online Repository at www.jacionline.org. The mean Lund-Mackay scores were 14.7 ± 1 and 9.7 ± 1 in the CRSwNP and CRSSNP groups, respectively. Levels of IL-6 protein detected in nasal polyp extracts (21.5 ± 50.7 pg/mg protein; mean $\pm$ SD) were significantly increased compared to levels in sinonasal tissue extracts from individuals with CRSSNP (1.7 ± 2.1 pg/mg protein) and levels in controls (0.7 ± 1.2 pg/mg protein; *P* < .001; [Fig 1, A](#)). There was no statistically significant difference observed in the levels of IL-6 comparing the tissue from CRSSNP patients versus controls. There was no difference in the IL-6 levels from the nasal lavage fluid from individuals with CRSwNP (47 ± 114 pg/mg) compared with controls (12 ± 18 pg/mg; *P* = .27; see this article's [Fig E1, A](#), in the Online Repository at www.jacionline.org).

Levels of sIL-6R were higher in the polyp tissue compared with control tissue (*P* < .01) and marginally higher compared with sinonasal tissue from patients with CRSSNP (*P* = .06). CRSSNP tissue did not have elevated levels of sIL-6R levels compared with controls ([Fig 1, B](#)). sIL-6R was detectable in the nasal lavage, but the differences between the groups were not significant ([Fig E1, B](#)). To determine whether there is an intrinsic increase in either basal or stimulated IL-6 release in cells from patients with CRSwNP, cultured epithelial cells from the inferior turbinate and uncinate process of patients with CRSwNP, CRSSNP, and controls were challenged with either medium alone or the Toll-like receptor 3 ligand, dsRNA (double stranded RNA). Data in this article's [Fig E2](#) in the Online Repository at www.jacionline.org show that baseline levels of IL-6 secreted by epithelial cells from the inferior turbinate (*A*) or uncinate process (*B*) from

TABLE 1. Clinical characteristics of subjects undergoing IL-6, sIL-6R, and/or sgp130 analyses in the sinus tissue

Characteristic	CRSwNP	CRSSNP	Controls
Subject no.	38	30	18
Age, median (y) (range)	42 (22-70)	39 (26-61)	41 (26-71)
Sex	17 M/21 F	14 M/16 F	11 M/7 F
Race, no. (%)			
White	26 (68)	26 (87)	13 (72)
Black	6 (16)	2 (7)	1 (5)
Asian	0	1 (3)	1 (5)
Hispanic	3 (8)	0	0
Other	3 (8)	1 (3)	3 (17)
Atopic	23	13	2
Nonatopic	11	11	10
Atopy status unknown	4	6	6
Asthma	19	5	0
Aspirin intolerance	0	0	0

F, Female; M, male.

subjects with CRS and control subjects were not significantly different. Although there was a trend for greater activation of IL-6 production by stimulation with dsRNA in both CRS groups, the values were not different among the 3 groups of subjects.

Because tissue extracts from subjects with CRSwNP had significantly elevated levels of both IL-6 and sIL-6R, we hypothesized that both direct and trans-signaling may occur and that the sinonasal tissue from patients with CRSwNP would manifest evidence of activation by IL-6. One of the major pathways by which IL-6 activates the inflammatory response is receptor-mediated phosphorylation and activation of the transcription factor, STAT3.³⁰ We therefore analyzed the presence of total STAT3 and the phosphorylated form of STAT3 (p-STAT3) in nasal polyps and control tissue. Fig 2, A, demonstrates a representative Western blot showing that polyp tissue did not have increased STAT3 phosphorylation and may actually have reduced levels of p-STAT3 compared with control tissue. Fig 2, B, displays densitometric analysis of p-STAT3 in the subjects shown in Fig 2, A, and shows that levels of total STAT3 were not different, but p-STAT3 levels were lower in nasal polyps compared with control tissue. We pooled p-STAT3 data from 4 separate experiments. Normalized values for subjects with CRSwNP were converted to a percent of control subjects' values. Levels of p-STAT3 in polyp tissue from subjects with CRSwNP with high IL-6 levels were significantly lower than from normal control tissue (Fig 2, C). Because the tissue used from patients with CRSwNP was polyp tissue and the tissue in controls was inferior turbinate tissue, we compared polyp and inferior turbinate tissue from 3 subjects with CRSwNP. Data in Fig 2, D, show that levels of p-STAT3 were similar in tissue from both locations. Fig 2, E, I, shows representative staining for p-STAT3 in uncinate tissue from a control subject. We performed immunohistochemistry in tissue samples from 5 subjects with CRSwNP and 4 controls. We counted the number of p-STAT3-positive cells using a semiquantitative method. Although p-STAT3-positive cells were decreased in the epithelium (3200 ± 900 cells/mm²) from polyp tissue compared to the epithelium of uncinate from controls (4300 ± 2000 cells/mm²), this difference was not significant. This may be because of the small number of samples and also because p-STAT3 was highly expressed in the epithelium of both the polyp tissue and the uncinate tissue from normal controls. Epithelial p-STAT3-positive cells were not evenly distributed throughout the tissue; they were observed mostly in

clusters. In the figure shown, there is no such epithelial cluster present. The positive cells counts in the lamina propria were 1300 ± 500 cells/mm² in the normal control tissue and 1000 ± 100 cells/mm² in the polyp tissue.

These studies suggest that there is constitutive STAT3 activation in normal tissue and that there is no evidence that elevated levels of IL-6 and sIL-6R lead to additional activation of STAT3 in the sinonasal tissue of patients with CRSwNP. To the contrary, reduced STAT3 phosphorylation suggests reduced levels of signaling via this pathway. To investigate this further, we assayed levels of the inhibitory signaling factor sgp130 in tissue extracts. Levels of sgp130 were significantly elevated in the polyp tissue compared with the sinus tissue from controls ($P = .016$) but not compared with the sinus tissue from individuals with CRSSNP ($P = .19$), as shown in Fig 3. The levels of sgp130 were not different in the sinus tissue extracts from subjects with CRSSNP versus controls. sgp130 was detected in the nasal lavage fluid in all subject groups; however, we did not observe a significant difference among the 3 groups (see this article's Fig E3 in the Online Repository at www.jacionline.org).

Because IL-6 plays a pivotal role in the development of T_H17 cells from naive T cells, we investigated the presence of IL-17 in CRS tissue (see this article's Fig E4 in the Online Repository at www.jacionline.org). IL-17A, IL-17E, and IL-17 F were undetectable in all but 2 of the CRS and control tissues. To test stability in tissue extracts, we added IL-17A, E, or F to tissue extracts and incubated them overnight at 4°C. We failed to find evidence that the tissue extracts degraded the cytokines (data not shown). We also assessed IL-23, a member of the IL-12 family that is important in T_H17 cell differentiation. Similar to IL-6, STAT3 activation is required in IL-23-dependent T_H17 differentiation. IL-23 protein was undetectable in the sinus tissue in most patients with CRS and controls (data not shown).

There was significant variability in the results as shown on the graphs, and there was no correlation between IL-6 and its signaling components. We did subgroup analysis of subjects with and without allergy within the study groups. There were no differences in the levels of IL-6, sIL-6R, or sgp130 when comparing subjects with CRSwNP with and without allergy. Similarly, no significant differences were observed in the levels of IL-6, sIL-6R, or sgp130 in the sinus tissue from patients with CRSwNP with and without asthma (data not shown).

DISCUSSION

The current study demonstrates that the IL-6 pathway may be altered in CRS tissue. We found increased levels of IL-6, sIL-6R, and sgp130 proteins in nasal polyp tissue compared with extracts of sinus tissue from normal subjects or subjects with CRSSNP. Although there are reports of increased IL-6 mRNA and increased immunohistochemical staining for IL-6 in CRS, this is the first report of elevated levels of IL-6 and sIL-6R protein in CRSwNP compared with CRSSNP.¹⁶⁻¹⁹ Despite elevations of IL-6 and sIL-6R in CRSwNP, we observed reduced p-STAT3 levels in CRSwNP, suggesting that the signaling pathway may be blunted. Increased levels of sgp130, a signaling chain known to have inhibitory effects on IL-6 signaling, could help explain the reduced levels of p-STAT3 in nasal polyps. Although the cellular source of IL-6, sIL-6R, and sgp130 is unknown, we found that nasal epithelial cells from patients with CRSwNP did not produce elevated levels of IL-6 *in vitro*.

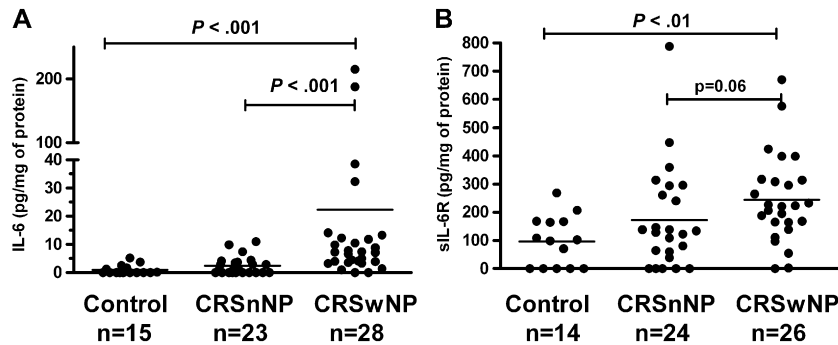


FIG 1. Assessment of IL-6 and sIL-6R levels in CRS by ELISA. **A**, IL-6 levels were increased in nasal polyps compared with sinus tissues from controls and CRSsNP. **B**, sIL-6R levels were increased in the polyp tissue compared to sinus tissue from controls. Levels were marginally increased ($P = .06$) in CRSwNP compared with CRSsNP.

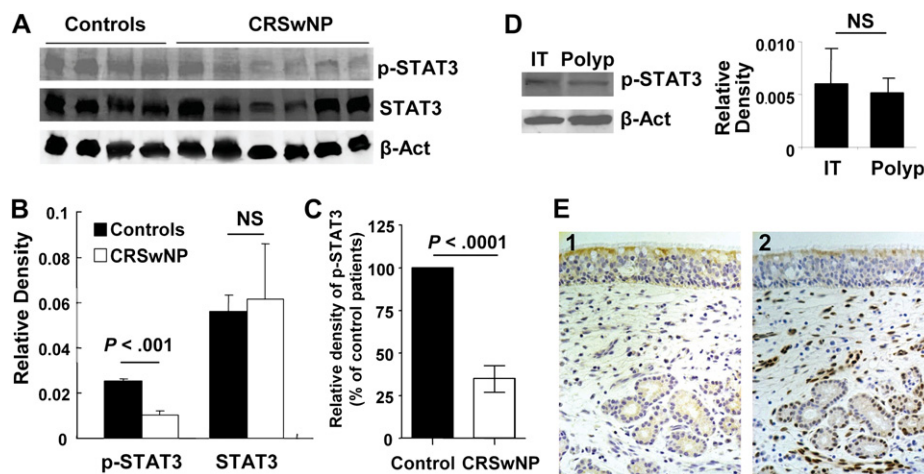


FIG 2. **A**, Representative Western blot of p-STAT3 and total STAT3 in nasal polyps and controls. **B**, Densitometric analysis of p-STAT3 from 4 controls and 6 subjects with CRSwNP shown in **A**. **C**, Densitometric analysis of p-STAT3 from 7 controls and 9 subjects with CRSwNP. **D**, Representative Western blot of p-STAT3 in nasal polyp ($n = 3$) and inferior turbinate tissue ($n = 3$) within the same patients with CRSwNP. **E**, (1) Isotype control antibody staining in control tissue; (2) representative immunostaining for p-STAT3 in control tissue. IT, inferior turbinate; β -actin, beta actin; NS, not significant.

Elevations of IL-6 and sIL-6R suggest that IL-6 plays a pathogenic role in CRS. Proinflammatory effects of IL-6/sIL-6R and IL-6 trans-signaling have been implicated in the transition of acute innate immune responses to adaptive chronic inflammatory reactions.³¹ IL-6 inhibits neutrophil recruitment during innate immune responses and enhances apoptosis of granulocytes.³²⁻³⁴ In adaptive immune responses, IL-6 trans-signaling is critical for T-cell recruitment and survival.³⁵ In rheumatoid arthritis, IL-6 and sIL-6R concentrations are increased in the synovial fluid and directly correlate with leukocyte influx into the joint.³⁶ Inhibition of T-cell apoptosis via IL-6 trans-signaling is believed to be responsible for the persistent T-cell-dependent inflammation in the colon that is characteristic of Crohn disease.³⁷ Similar resistance of T cells to apoptosis after IL-6 trans-signaling has been demonstrated in the inflammatory eye disease, uveitis.³⁸ It is possible that the increased levels of IL-6 and its soluble receptor in the polyp tissue that we report here may be partially responsible for the recruitment and retention of T cells that have been observed in CRS.^{2,39-41}

We also examined IL-17 in the sinus tissue because IL-6 is important in regulatory T (Treg) cell and T_H17 cell differentiation.^{42,43} IL-17-producing T_H17 cells are important in mucosal defense against extracellular organisms and are also involved in autoimmune diseases such as RA and Crohn disease.⁴⁴⁻⁴⁷ Treg cells, on the other hand, are important in preventing autoimmunity. The transcription factor forkhead box protein 3 (Foxp3) promotes Treg cell conversion from T_H cells and inhibits retinoic acid-related orphan receptor γ -induced T_H17 differentiation.^{48,49} IL-6 has been shown to inhibit Foxp3, which in turn leads to diminished inhibition of retinoic acid-related orphan receptor γ and consequently expansion of T_H17 cells.⁵⁰⁻⁵² Pasare and Medzhitov⁵³ have demonstrated Toll-like receptor-dependent suppression of Treg activity by IL-6 produced by dendritic cells. There is very limited literature regarding the role of Treg and T_H17 cells in CRS. Potentially, the increased levels of IL-6 and its soluble receptor in the polyps may be inhibiting Treg activity in CRSwNP. According to Van Bruaene et al, TGF β , a cytokine implicated in Treg formation and activity, and Foxp3

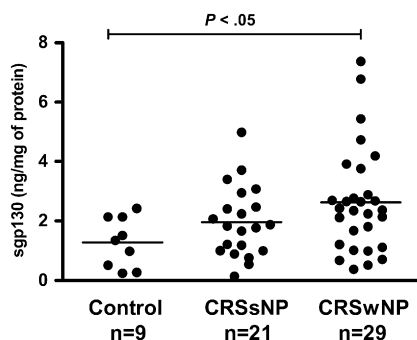


FIG 3. sgp130 levels are increased in polyp tissue compared with sinus tissue from controls by ELISA.

mRNA are decreased in CRSwNP compared with CRSsNP and controls.^{2,54} In terms of T_H17 cells, Molet et al⁵⁵ demonstrated increased expression of IL-17 in nasal polyps compared with normal control tissue by *in situ* hybridization, and Wang et al⁵⁶ detected IL-17 and IL-17 receptor expression in nasal polyps by immunohistochemical staining and Western blot analyses. Van Bruaene et al failed to find a difference in IL-17 mRNA expression among patients with chronic sinusitis with or without polyps and controls.⁵⁴ However, recently they reported an increase in IL-17A protein, which is responsible for neutrophil recruitment and activation, in polyp tissue from Chinese patients compared with Belgian patients with nasal polyps.⁵⁷ This is most likely because polyps from Chinese patients tend to be neutrophilic compared with polyps from Western patients, which tend to be eosinophilic. They did not investigate IL-17E, which is important in the T_H2 pathway and eosinophil recruitment. In our study of IL-17 protein, we did not detect high levels of IL-17, nor did we find a difference in levels of IL-17A, E, or F among CRSwNP, CRSsNP, or control tissues. Most of the samples yielded values below the level of detection, suggesting that if IL-17 is present in the polyp or sinus tissue, the quantity is small. Blunted IL-6 signaling, as indicated by reduced tissue p-STAT3 and increased sgp130, may be responsible for a local tissue environment unfavorable to the development of T_H17 cells in CRS. Although it would be appealing to correlate IL-6 and sIL-6R with IL-17 in our sample sets, it is unlikely that we would find correlations because so few samples had detectable levels of IL-17.

Any reduction of local IL-17 production could result in susceptibility to infections that are associated with CRS. Holland et al⁵⁸ have recently described mutations in the gene encoding STAT3 and defective IL-6 signaling in hyper-IgE syndrome (HIES). HIES is characterized by recurrent sinopulmonary infections, eosinophilia, eczema, and high IgE. Mutations in the DNA binding or SH (Src homology)2 regions of STAT3 are thought to lead to absent IL-17 production by T cells in HIES.⁵⁹ The low IL-17 in turn is suggested to elevate risk for the recurrent infections seen in these patients. Although HIES is a systemic immunodeficiency syndrome, CRSwNP shares certain features, because it is frequently characterized by elevated local IgE, eosinophilia, and colonization by *Staphylococcus aureus*.^{2,60-66} We speculate that the reduced local activation of STAT3 that we demonstrated here in polyps may promote this phenotype in the sinonasal mucosa. Future studies are required to determine whether the increased staphylococcal colonization, eosinophilia, and local IgE observed in CRSwNP occurs as a result of local STAT3 mutations in the sinus tissue.

In addition to effects on T cells, alterations in IL-6 signaling may influence the recruitment and activation of B cells in CRS. Gevaert et al have identified lymphoid follicle-like structures containing T cells, B cells, and plasma cells in CRSwNP.⁶⁶ In transgenic mice, overexpression of IL-6 leads to profound formation of lymphoid structures in the lungs.⁶⁷ Infiltration or recruitment of B cells and local production of IgE and possibly IgA are believed to be responsible for the local inflammation seen in both allergic and nonallergic airway diseases. IL-6 is a potent B-cell growth factor important for antibody synthesis and secretion. The local increase in IL-6 and its soluble receptor in the polyp tissue may contribute to the local formation of B-cell-rich follicles and production of IgE in the polyps.⁶⁶ We have recently observed increased levels of the B-cell activating factor of the TNF family in nasal polyp tissue.⁶⁸ Working together, B cell-activating factor of the TNF family and IL-6 may potentially expand and activate B cells in the sinuses of patients with CRS.

Limited tissue quantity made it unfeasible to assess all the analytes in all of the subjects. Other limitations in our study include lack of atopy information in some of the subjects and lack of smoking data in the subjects. In addition, glucocorticoids are known to affect IL-6 production and a weakness of our study is that we did not control for previous glucocorticoid use.

In conclusion, we have provided novel data suggesting that there may be important alterations in the IL-6 signaling pathway in CRSwNP. Because we have found elevations in both proinflammatory and downregulatory molecules, it is not possible yet to conclude whether the IL-6 pathway is inappropriately activated or blunted in CRSwNP; based on our findings that p-STAT3 levels are lower in samples from patients, we suspect the latter, although responses may vary among different cell types. Overactivity of the IL-6 pathway because of increased IL-6 and sIL-6R could increase T_H2 inflammation and disable T-regulatory cell responses, contributing to the persistence of chronic inflammation that is characteristic of CRSwNP. On the other hand, if the elevated levels of the inhibitor sgp130 are dominant and STAT3 phosphorylation is blunted in T cells, Treg cell responses may be excessive and T_H17 responses may be reduced, rendering adaptive immune responses inadequate and inappropriately skewed to T_H2 . Further investigations will be required to identify the cellular sources of IL-6, sIL-6R, and sgp130 as well as the impact of these molecules on disease pathogenesis in CRSwNP.

Clinical implications: The IL-6 signaling pathway may have a pathogenic role in CRSwNP.

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METHODS

Subject selection

All patients scheduled for surgery had previously failed to respond to adequate trials of conservative medical therapy (prolonged antibiotic regimens, nasal steroid sprays, oral steroids, saline irrigations, and decongestants). Nasal polyps were confirmed by sinus computed tomography (CT) scans and office nasal endoscopy by 1 of the 3 otolaryngologists who are coauthors of this article. Polyps were also noted in the operating room by the same surgeons. CRSwNP was characterized by abnormal sinus CT scans, and inflammation was documented on the nasal endoscopy by the same otolaryngologists. The CT scans were taken no more than 3 months before surgery. Four of the subjects in the CRSwNP group were undergoing repeat surgery. Polyp tissue was used for the specimens in the CRSwNP group, uncinate tissue was used in the CRSNP group, and inferior turbinate tissue was used in the control group unless noted otherwise. The median weight of the sinus tissue was 25 mg (4–121 mg). The median weight of the polyp tissue was 88 mg (4–484 mg). Subjects were skin-tested to pollens, dust mites, pets, molds, and cockroach using Hollister Stier Canada (Toronto, Ontario, Canada) extracts. A positive skin test response was defined as a wheal greater in size than that produced by the saline control by 3 mm or more. Histamine was used as a positive control. Subjects with allergic rhinitis had at least 1 positive skin reaction with the prick-puncture technique to the extracts. Atopic status was assessed in all subjects except if subjects declined or if the history did not suggest atopy. These subjects were not grouped into a nonatopic category because testing was not done at our institution. Other than topical corticosteroids, subjects were on a variety of medications. These included antihistamines, decongestants, and short-acting or long-acting β -agonists, among others.

Cell culture

Human primary nasal epithelial cells (PNECs) were collected from the inferior turbinate or uncinate tissue by curettage with a Rhinoprobe (Arlington Scientific, Inc, Springville, Utah) under a Northwestern University Feinberg School of Medicine Institutional Review Board–approved human subject research protocol. PNECs were maintained in unsupplemented serum-free bronchial epithelial cell growth medium (Cambrex, Walkersville, Md). PNECs were plated in 24-well culture plates coated with collagen (Vitrogen; Collagen Biomaterials, Palo Alto, Calif). When the cells reached 80% confluence, they were treated with medium or 25 μ g/mL dsRNA (Sigma, St Louis, Mo) and stimulated for 24 hours.

Nasal tissue protein extraction and Western blot

Nasal tissue protein extracts were prepared by using the T-PER Reagent (Pierce, Rockford, Ill) with protease and phosphatase inhibitor. Forty

micrograms of extract protein was subjected to 10% SDS-PAGE and immunodetected with specific antibodies to β -actin (Sigma) or β -tubulin (Santa Cruz Biotechnology, Santa Cruz, Calif), STAT3, and p-STAT3 (Cell Signaling, Beverly, Mass) by using a previously described protocol.^{E1} Immunoblots were digitalized and quantified with Odyssey imaging software (LI-COR, Lincoln, Neb). Data were pooled from 4 separate experiments. Within each experiment, densitometry values for p-STAT3 from CRSwNP and control subjects were normalized to the housekeeping genes. Normalized values for CRSwNP subjects were then converted to a percent of control subjects' values (which was set to 100%).

Immunohistochemistry

Nasal tissue was dehydrated, infiltrated, and embedded with paraffin, and tissue was sectioned at 3 μ m by using a Leica RM2245 Cryostat (Leica Microsystems, Bannockburn, Ill). Sections were rehydrated and endogenous peroxidase activity was blocked with 3% H₂O₂/methanol. After rinsing tissue sections, nonspecific binding was blocked with 1% goat serum/0.3% Tween-20/PBS. Tissue sections were then incubated with 0.25 μ g/mL rabbit anti-human p-STAT3 (Tyr 705) mAb (clone D3A7, IgG; Cell Signaling, Danvers, Mass) or 0.25 μ g/mL normal rabbit IgG negative control antibody (R&D Systems, Minneapolis, Minn) for 16 to 20 hours at 4°C. Sections were rinsed and then incubated in biotinylated secondary goat antirabbit antibody (Vector Laboratories, Burlingame, Calif) at a 1:500 dilution for 1 hour at room temperature. After another rinse, sections were incubated in ABC reagent (avidin–biotin–horseradish peroxidase complex; Vector Laboratories, Burlingame, Calif) for 1 hour at room temperature. Sections were rinsed again and incubated in DAB (diaminobenzidine) reagent (Invitrogen, Carlsbad, Calif) for 10 minutes at room temperature. They were then rinsed in deionized H₂O, counterstained with hematoxylin, dehydrated, cleared, mounted, and coverslipped by using Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, Mich) in preparation for microscopic analysis.

The number of p-STAT3–positive cells in epithelium, glands, and submucosae were counted using a magnification of $\times 400$. Each section was randomly selected and diagnosis was unknown to the observer. p-STAT3 photos were taken at a magnification of $\times 400$ by using an Olympus IX71 Inverted Microscope (Olympus America, Inc, Center Valley, Pa).

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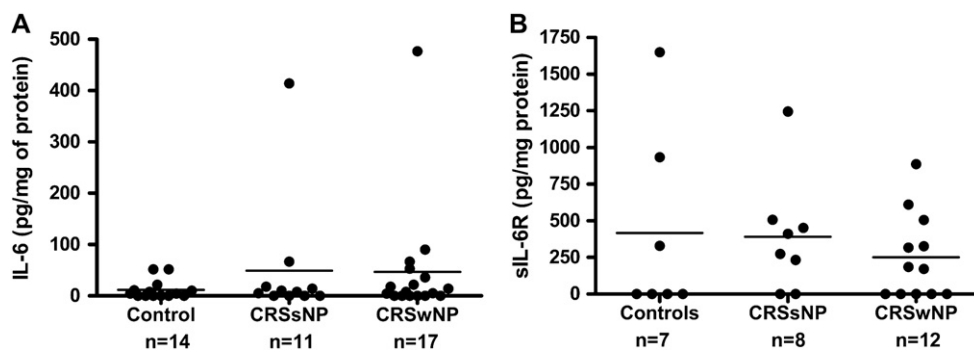


FIG E1. Assessment of IL-6 and sIL-6R levels in CRS by ELISA. **A**, Levels of IL-6 in nasal lavage samples were not significantly different among the groups. **B**, sIL-6R was present in the nasal lavage samples, but no differences were observed among the different groups.

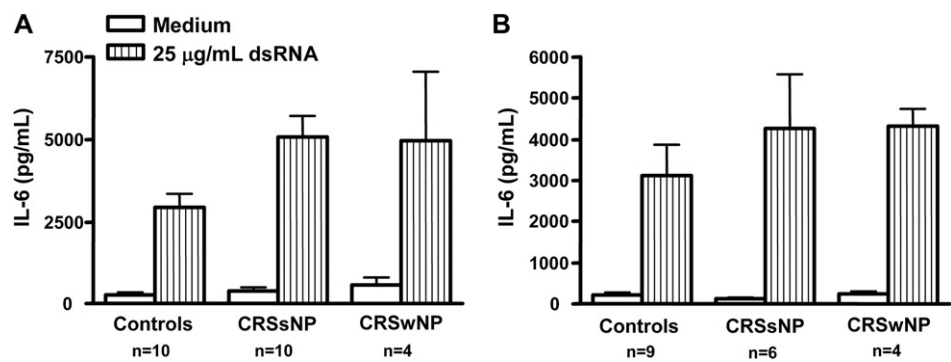


FIG E2. Secretion of IL-6 by primary nasal epithelial cells cultured from controls, patients with CRSsNP, and patients with CRSwNP at baseline and after stimulation with 25 µg/mL dsRNA. **A**, IL-6 secretion by epithelial cells from the inferior turbinate. **B**, IL-6 secretion by epithelial cells collected from the uncinate process.

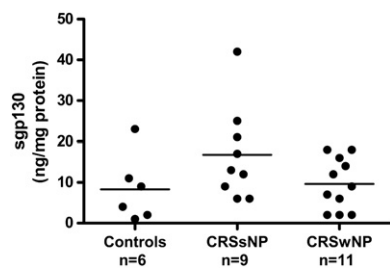


FIG E3. Levels of sgp130 by ELISA assessed in nasal lavage showed no significant differences among the groups.

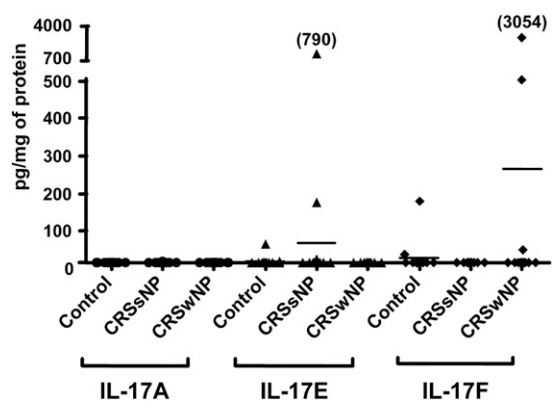


FIG E4. Assessment of IL-17 A, E, and F levels by ELISA in tissue extracts from controls (IL-17A, n = 17; IL-17E, n = 15; IL-17F, n = 16), CRSsNP (IL-17A, n = 20; IL-17E, n = 18; IL-17F, n = 17), and CRSwNP (IL-17A, n = 20; IL-17E, n = 12; IL-17F, n = 14).

TABLE E1. Clinical characteristics of subjects undergoing IL-6 measurement in sinus tissue

Characteristic	CRS _w NP	CRS _s NP	Controls
Subject no.	28	23	15
Age, median (y) (range)	46 (24-68)	38 (32-58)	32 (26-71)
Sex	13 M/15 F	9 M/14 F	9 M/6 F
Race, no. (%)			
White	18 (64)	18 (78)	10 (66)
Black	5 (18)	2 (9)	1 (7)
Asian	0	1 (4)	1 (7)
Hispanic	3 (11)	0	1 (7)
Other	2 (7)	2 (9)	2 (13)
Atopic	15	9	1
Nonatopic	10	10	8
Atopy status unknown	3	4	6
Asthma	15	3	0
Oral steroids	8	1	0
Topical steroids			
Nasal	9	8	1
Inhaled	15	2	0
Aspirin intolerance	0	0	0

F, Female; M, male.

TABLE E2. Clinical characteristics of subjects undergoing sIL-6R measurement in sinus tissue

Characteristic	CRSsNP	CRSsNP	Controls
Subject no.	26	24	14
Age, median (y) (range)	40 (22-70)	38 (26-61)	32 (26-71)
Sex	14 M/12 F	9 M/15 F	8 M/6 F
Race, no. (%)			
White	16 (62)	19 (79)	9 (64)
Black	5 (19)	3 (12)	1 (7)
Asian	0	1 (4)	1 (7)
Hispanic	2 (8)	0	1 (7)
Other	3 (11)	1 (4)	2 (14)
Atopic	16	7	1
Nonatopic	8	12	8
Atopy status unknown	2	5	5
Asthma	15	3	0
Oral steroids	8	1	0
Topical steroids			
Nasal	9	8	1
Inhaled	15	2	0
Aspirin intolerance	0	0	0

F, Female; M, male.

TABLE E3. Clinical characteristics of subjects undergoing sgp130 measurement in sinus tissue

Characteristic	CRSwNP	CRSSNP	Controls
Subject no.	29	21	9
Age, median (y) (range)	41 (22-70)	36 (32-58)	40 (27-50)
Sex	15 M/14 F	7 M/14 F	5 M/4 F
Race, no. (%)			
White	19 (66)	17 (81)	7 (78)
Black	4 (14)	2 (9)	0
Asian	0	1 (5)	0
Hispanic	3 (10)	0	1 (11)
Other	3 (10)	1 (5)	1 (11)
Atopic	16	6	1
Nonatopic	9	13	5
Atopy status unknown	4	2	3
Asthma	16	3	0
Oral steroids	8	0	0
Topical steroids			
Nasal	10	6	1
Inhaled	16	2	0
Aspirin intolerance	0	0	0

F, Female; M, male.

TABLE E4. Clinical characteristics of subjects undergoing nasal lavage

Characteristic	CRS _w NP	CRS _s NP	Controls
Subject no.	17	11	21
Age, median (y) (range)	53 (35-78)	30 (25-60)	41 (24-55)
Sex	8 M/9 F	5 M/6 F	8 M/13 F
Race, no. (%)			
White	10 (59)	7 (64)	9 (43)
Black	2 (12)	1 (9)	3 (14)
Asian	1 (6)	0	2 (10)
Hispanic	1 (6)	0	3 (14)
Other	3 (17)	3 (27)	4 (19)
Atopic	7	8	0
Nonatopic	6	3	21
Atopy status unknown	4	0	0
Asthma	7	3	0
Aspirin intolerance	0	0	0

F, Female; M, male.

TABLE E5. Clinical characteristics of subjects undergoing IL-17 measurement, immunohistochemistry, and Western blot analysis

Characteristic	CRSwNP	CRSsNP	Controls
Subject no.	35	25	34
Age, median (y) (range)	46 (28-66)	35 (32-60)	38 (26-68)
Sex	17 M/18 F	11 M/14 F	24 M/10 F
Race, no. (%)			
White	26 (74)	17 (68)	24 (71)
Black	4 (11)	4 (16)	2 (6)
Asian	2 (6)	0	0
Hispanic	1 (3)	0	1 (3)
Other	2 (6)	4 (16)	7 (20)
Atopic	12	9	2
Nonatopic	10	8	26
Atopy status unknown	13	8	6
Asthma	11	4	0
Oral steroids	8	1	0
Topical steroids			
Nasal	9	5	1
Inhaled	11	3	0
Aspirin intolerance	0	0	0

F, Female; M, male.