

T-cell regulation in chronic paranasal sinus disease

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Background: Chronic rhinosinusitis is an inflammatory disease with distinct cytokine and remodeling patterns. Chronic rhinosinusitis with nasal polyps (CRSwNP) is characterized by a T_H2-skewed eosinophilic inflammation, whereas chronic rhinosinusitis without nasal polyps (CRSsNP) represents a predominant T_H1 milieu.

Objective: We aimed to study the direct tissue expression of transcription factors for T-cell subpopulations, including T regulatory cells, in relation to the cytokine expression patterns in the different disease subgroups.

Methods: The expression of forkhead box P3 (FOXP3), T-box transcription factor (T-bet), GATA-3, retinoid acid-related orphan receptor C (RORc), the suppressive cytokines TGF- β 1 and IL-10, and T_H1/ T_H2/ T_H17 cytokines (IFN- γ , IL-4, IL-5, IL-13, IL-17) were analyzed by means of RT-PCR in 13 CRSsNP, 16 CRSwNP, and 10 control samples. Additional protein measurements were performed for TGF- β 1 and IFN- γ .

Results: In CRSwNP, we observed a significantly lower FOXP3 mRNA and TGF- β 1 protein expression, but a significantly higher T-bet, GATA-3, IL-5, and IL-13 mRNA expression compared with controls, whereas RORc was not significantly different compared with controls. In CRSsNP, FOXP3, T-bet, GATA-3, and RORc expression was not significantly different from controls, whereas TGF- β 1 mRNA, IFN- γ mRNA, and protein were significantly higher in CRSsNP compared with controls. For IL-17, no significant differences were noted among all groups.

Conclusion: We demonstrate for the first time a decreased FOXP3 expression accompanied by an upregulation of T-bet and GATA-3 and a downregulation of TGF- β 1 in CRSwNP versus controls and CRSsNP. (J Allergy Clin Immunol 2008;121:1016-1024.)

Key words: Chronic rhinosinusitis, FOXP3, nasal polyps, T-cell polarization, TGF- β 1, transcription factors, T regulatory cell

Chronic rhinosinusitis without nasal polyps (CRSsNP) and with nasal polyps (CRSwNP) are chronic sinus diseases, both characterized by persistent inflammation of the nasal and paranasal mucosa. Recent research has demonstrated that these pathologies can be differentiated into distinct subgroups on the basis of the expression of inflammatory and remodeling mediators.^{1,2} CRSsNP is characterized by a predominant T_H1 milieu with high IFN- γ and TGF- β 1 concentrations,¹ whereas CRSwNP typically shows a T_H2 skewed eosinophilic inflammation with high levels of IL-5 and IgE and low TGF- β 1.^{3,4} However, little is known regarding the intracellular mechanisms behind this initial T-cell polarization. Naive T cells differentiate toward different T-cell subtypes on the basis of the expression of certain transcription factors. T-box transcription factor (T-bet) involves commitment toward T_H1 cells⁵; GATA-3 is critical for commitment toward T_H2 cells and controls the expression of IL-4 and IL-5.^{6,7} Moreover, the balance between T_H1 and T_H2 is controlled by an intriguing subset of T cells called T regulatory (Treg) cells.⁸ A number of recent studies indicate that Treg cells play an important role in diseases characterized by T_H2-biased immune responses such as asthma and atopic dermatitis.⁹⁻¹¹ Until now, no data were available regarding expression and regulation in CRSsNP, in particular CRSwNP. It was tempting to speculate that in CRSwNP, characterized by a massive T_H2-driven eosinophilic inflammation, a deregulated Treg function might be involved. Knowledge of Treg cells in human disease is scarce so far, and studies mostly are based on *in vitro* experiments using peripheral blood-derived Treg cells.

In this study, we analyzed T-cell transcription factors and downstream events at the level of the sinonasal mucosa in controls and chronic sinus disease.

Two main populations of Treg cells have been defined. One is made up of the naturally occurring Treg (nTreg) cells, characterized by the CD4⁺CD25⁺ forkhead box P3 (FOXP3)⁺ phenotype. They functionally mature in the thymus, and their development is controlled by the transcription factor FOXP3. It is hypothesized that nTreg cells can migrate to sites of inflammation at mucosal surfaces and inhibit T_H2 and T_H1 cells via cell-cell contact.¹¹ Another group of Treg cells is made up of the induced Treg (iTreg) cells, generated from naive T cells in the periphery. The most important subsets of iTreg cells are Tr1 and T_H3 cells. They suppress immune function by secretion of predominantly IL-10 and TGF- β 1, respectively.¹² It is probable that both natural and inducible populations have complementary and overlapping functions. Although FOXP3 was initially thought to be a specific marker for nTreg cells and could not be activated in peripheral T cells,¹³⁻¹⁵

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Supported by grants to C.B. from the Flemish Scientific Research Board, Fonds Wetenschappelijk Onderzoek, no. A12/5-HB-KH3 and G.0436.04, the Global Allergy and Asthma European Network, and the Interuniversity Attraction Poles Program-Belgian State-Belgian Science Policy, Nr. IAP P6/35, to P.G. (postdoctoral grant of the Research Foundation – Flanders [FWO]), and to Cezmi Akdis from the Swiss National Science Foundation, grant no. 32-105865.

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication October 25, 2007; revised February 14, 2008; accepted for publication February 18, 2008.

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0091-6749/\$34.00

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doi:10.1016/j.jaci.2008.02.018

Abbreviations used

CRSsNP: Chronic rhinosinusitis without nasal polyps
 CRSwNP: Chronic rhinosinusitis with polyps
 FOXP3: Forkhead box P3
 IQR: Interquartile range
 iTreg: Induced T regulatory
 nTreg: Naturally occurring T regulatory
 RORc: Retinoid acid-related orphan receptor C
 T-bet: T-box transcription factor
 Treg: T regulatory

recent studies demonstrated the induction of FOXP3 in induced Treg cells,¹⁶⁻¹⁸ positioning this transcription factor as a marker for both nTreg cells and iTreg cells. As indirect markers for Tr1 and T_H3 activity, we measured IL-10 and TGF- β 1 mRNA expression.

In this study, we aimed to investigate the expression of key transcription factors for Treg and T_H1/T_H2/T_H17 cells, in relation to the mRNA and protein expression of representative cytokines, in a T_H2-biased and T_H1-biased sinus disease.

METHODS**Patients**

Sinonasal mucosa from 13 patients with CRSsNP, 16 patients with CRSwNP, and 10 control patients was obtained at the Department of Otorhinolaryngology of the Ghent University Hospital, Belgium. Inferior turbinates from patients without sinus disease undergoing septoplasty or rhinoseptoplasty were collected as controls. For CRSsNP, tissue samples originated from ethmoidal mucosa. For CRSwNP, samples of ethmoidal polyp tissue were used.

None of the control and CRSsNP patients had a history of asthma or a positive skin prick test result to common inhalant allergens. In the CRSwNP group, 5 of the 16 patients had asthma in the history. Two of these patients had a positive skin prick test result, with 1 patient reporting aspirin intolerance. The diagnosis of sinus disease was based on history, clinical examination, nasal endoscopy, and computed tomography of the paranasal cavities according to the current European EAACI Position Paper on Rhinosinusitis and Nasal Polyps¹⁹ and American²⁰ guidelines. General exclusion criteria were based on the EP³OS definition for research (cystic fibrosis, gross immunodeficiency, congenital mucociliary problems, noninvasive fungal balls and invasive fungal disease, systemic vasculitis and granulomatous diseases). Patients with nonallergic rhinitis with and without eosinophilia and vasomotor rhinitis were also excluded. All patients stopped oral and topical application of corticosteroids for at least 1 month before surgery. Patients did not take any other relevant medication. Patients who underwent previous sinus surgery were excluded. The study was approved by the local ethical committee of Ghent University Hospital, Belgium. An informed consent was obtained from each patient and control subject before collecting material.

Gene expression analysis: quantitative real-time PCR

cDNA was synthesized from 2 μ g of RNA with the iScript cDNA synthesis kit (BioRad Laboratories, Hercules, Calif) following the manufacturer's instructions. Levels of the transcription factors FOXP3, GATA-3, T-bet, and retinoid acid-related orphan receptor C (RORc) and cytokines IL-4, IL-5, IL-10, IL-13, IL-17, TGF- β 1, and IFN- γ were determined by real-time PCR. Amplification reactions were performed on an iCycler iQ Real-Time PCR Detection System (BioRad Laboratories) by using specific primer sequences (see this article's Table E1 in the Online Repository at www.jacionline.org). PCR reactions contained 30 ng cDNA (total RNA equivalent), 250 nmol/L primer pairs, 1X SYBR Green I Master mix (BioRad Laboratories), or 1X TaqMan mix with 100 nmol/L TaqMan probe in a final volume of 20 μ L. PCR protocol consisted

of 1 cycle at 95°C for 10 minutes followed by 40 cycles at 95°C for 30 seconds and at 60°C for 1 minute, and for reactions using TaqMan probes, of 1.5 minutes at 95°C followed by 50 cycles: 15 seconds at 95°C and 1 minute at 60°C.

The expression of 3 housekeeping genes—actin beta (ACTB), hydroxymethyl-bilane synthase (HMBS), and elongation factor 1—was used to normalize for transcription and amplification variations among samples after a validation using the geNorm software (Ghent University Hospital Center for Medical Genetics, Ghent, Belgium).^{21,22} The relative expression units of each gene per 30 ng of cDNA sample was determined by using the qBase program (version 1.3.5, Ghent University, Belgium), and results are expressed as the logarithm of normalized relative expression units/30 ng cDNA.

TGF- β 1/IFN- γ ELISA

Tissue homogenates were assayed for total TGF- β 1 and IFN- γ by using commercially available ELISA kits from R&D Systems (Minneapolis, Minn). All data were expressed as nanograms per milliliter. For TGF- β , acid was added during the ELISA procedure, resulting in physicochemical activation of latent TGF- β . Total TGF- β concentrations are reported including both active and latent forms.

Immunohistochemistry

CD3 staining. Sections were immunohistochemically stained with the mouse mAb CD3 (clone UCHT1; Dako Cytomation, Glostrup, Denmark). For immunohistochemical staining, specimens were fixed in acetone and incubated with primary antibody or isotype control for 1 hour and detected by using the LSAB+ kit (Dako Cytomation).

The number of positive cells was analyzed by using a magnification $\times 400$ and scored by 2 independent observers who did not know the diagnosis and clinical data. A grading scale from 0 to 3 was applied, ranging from absent to numerous stained cells. Score 0 represents no positive cells; score 1, <10 positive cells/field; score 2, 10 to 100 positive cells/field; and score 3, >100 positive cells/field. All areas of the section were analyzed, and for each sample, 10 high-power fields were scored.

FOXP3 staining. Tissue frozen sections were permeabilized with FOXP3 Fix/Perm solution (320501; BioLegend, San Diego, Calif) and blocked with 10% normal goat serum (X0907; Dako Cytomation) and incubated with primary polyclonal rabbit antihuman FOXP3 Ab (ab10563; Abcam, Cambridge, United Kingdom) overnight at 4°C. Slides were incubated with peroxidase-labeled polymer followed by 3-amino-9-ethyl carbazole. Sections were counterstained with hematoxylin (Sigma, St Louis, Mo) and permanently mounted with Ultramount (S1964; Dako Cytomation). FOXP3 blocking peptide (ab14151; Abcam) was used as a control to block anti-FOXP3 binding. Human tonsil sections were used as a positive controls on each staining run. Counting of 10 random high-power fields was performed by 2 independent observers. For more information on material and methods, see this article's Online Repository at www.jacionline.org.

Statistical analysis

Statistical analysis was performed with MEDCALC software v 9.2.0.1 (F. Schoonjans, Mariakerke, Belgium). Data are expressed in box-and-whisker plots. When comparisons were made between groups, the Kruskal-Wallis test was used to assess significant intergroup variability. The Mann-Whitney *U* 2-tailed test was used for between-group comparison. The significance level was set at $\alpha = 0.05$.

RESULTS**Immunohistochemistry for FOXP3 and CD3**

Immunohistochemical staining was used to determine the presence of FOXP3-expressing cells in healthy and diseased sinonasal mucosal tissue. Representative sections of control, CRSsNP and CRSwNP samples stained for FOXP3 are shown in Fig 1 ($n = 6$ per group). Tonsil sections were used as positive

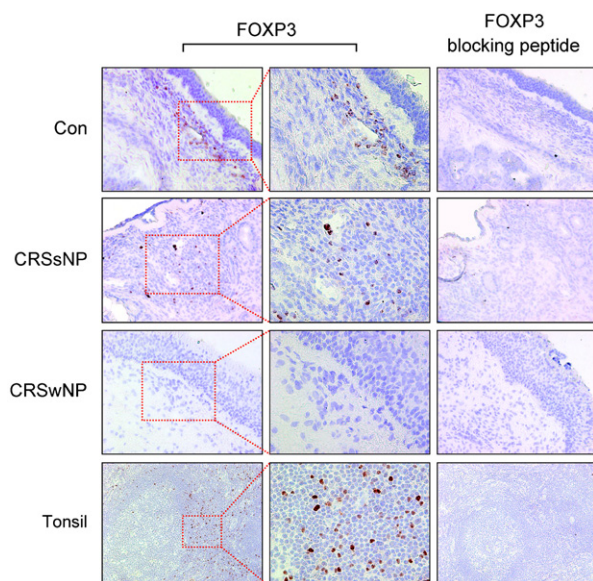


FIG 1. Expression of FOXP3⁺ cells in nasal mucosa from healthy individuals (controls, CON) and subjects with CRSsNP or CRSwNP. Lack of staining is observed by blocking with FOXP3-immunizing peptide. Human tonsil sections served as a positive control with FOXP3⁺ cells located in the parafollicular cortex (T-cell zone). Original magnification was $\times 200$ or $\times 400$. For human tonsil magnification was $\times 100$ or $\times 400$. Representative slides are shown ($n = 6$ per group).

controls. FOXP3-expressing cells were detectable in both healthy nasal mucosa and CRSsNP, but not in CRSwNP tissue (Fig 1). The median (interquartile range [IQR]) counts for FOXP3 positive cells were significantly lower in CRSwNP (0; 0-4) compared with controls (39; 21-41; $P < .0001$) and CRSsNP (52; 37-85; $P < .0001$). In addition, to estimate the total number of T cells present in tissues, we quantified the number of CD3-positive cells present in the tissues. The median (IQR) of CD3-positive cell number (sum of 10 high-power fields) was similar in CRSsNP (22; 20-26) and CRSwNP (23; 19-30), but significantly higher compared with controls (20; 18-21; $P = .01$ and $.001$, respectively).

Tissue expression of T reg and T_H1/T_H2/T_H17 transcription factors

The expression levels of transcription factors for Treg (FOXP3) and T_H1 (T-bet)/T_H2 (GATA-3)/T_H17 (RORc) populations were directly analyzed on tissue biopsies by means of quantitative real-time PCR. This revealed a significantly lower FOXP3 mRNA expression level in nasal polyps (CRSwNP) compared with controls ($P = .026$), whereas the expression of T-bet ($P = .005$) and GATA-3 ($P = .035$) was significantly higher in CRSwNP compared with controls (Fig 2). RORc was not significantly different between the latter 2 groups, but was significantly lower in CRSwNP compared with CRSsNP ($P < .001$). In contrast, in chronic rhinosinusitis without polyps (CRSsNP), FOXP3 mRNA expression was higher compared with controls, and significantly higher compared with CRSwNP ($P = .005$). No significant differences were noted for T-bet, GATA-3, and RORc compared with controls (median values and IQRs for all transcription factors are specified in this article's Table E2 in the Online Repository at www.jacionline.org).

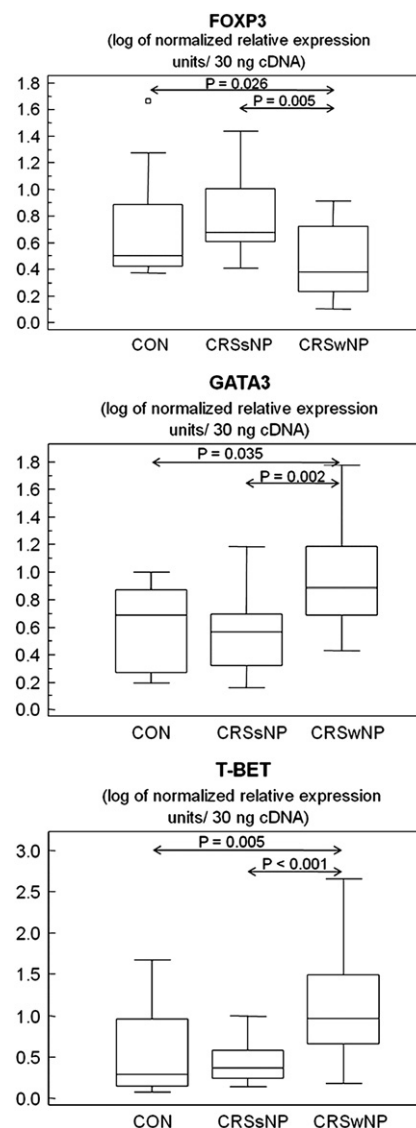


FIG 2. mRNA expression of transcription factors (FOXP3, T-bet, GATA-3) involved in naive T-cell differentiation in controls (CON), CRSwNP, and CRSsNP expressed as logarithm of normalized relative expression units/30 ng cDNA.

T_H1/T_H2/T_H17 and iTreg-related cytokine expression

Differences in T_H1/ T_H2/ T_H17 cytokine pattern and iTreg-related TGF- β 1/IL-10 were assessed at the mRNA level between CRSsNP, CRSwNP, and controls (Fig 3, A and B). Because of the relevance of posttranslational regulation mechanisms, additional protein measurements were performed for TGF- β 1 and IFN- γ (Fig 3, B).

In CRSwNP, we found a significantly higher mRNA expression of the T_H2 cytokine IL-5 compared with controls ($P < .001$) and with CRSsNP ($P < .001$) and a significantly higher mRNA expression of IL-13 in CRSwNP compared with controls ($P = .029$). For IL-4, no significant differences were noted among controls, CRSsNP, and CRSwNP (Fig 3, A). In contrast, both mRNA and protein levels of the T_H1 cytokine IFN- γ were significantly higher in CRSsNP compared with controls ($P = .009$ for mRNA; $P = .001$ for protein) and with CRSwNP ($P = .001$ for mRNA; $P = .01$ for protein).

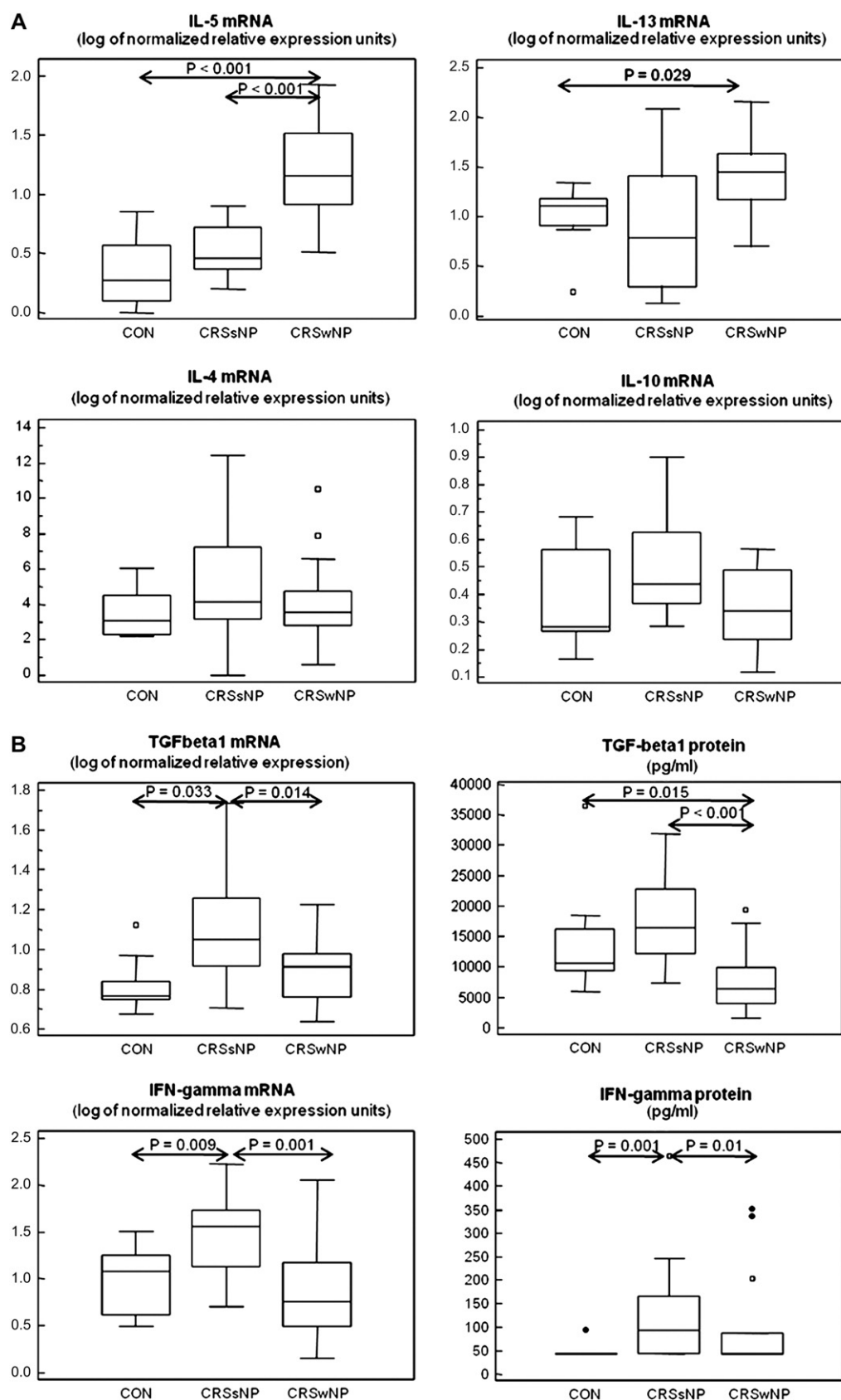


FIG 3. mRNA expression of key cytokines IFN- γ (T_H1), IL-4, IL-5, IL-13 (T_H2), and iTreg-related TGF- β 1 and IL-10 in controls (CON), CRSsNP, and CRSwNP, expressed as logarithm of normalized relative expression units/30 ng cDNA. For TGF- β 1 and IFN- γ , additional protein measurements were performed, expressed as picograms per milliliter.

protein). mRNA expression of TGF- β 1 was significantly higher in CRSsNP compared with controls ($P = .033$). At the protein level, TGF- β 1 showed higher concentrations in CRSsNP compared with controls and with CRSwNP, reaching statistical significance for the latter ($P < .001$). Furthermore, TGF- β 1 protein levels were significantly lower in CRSwNP compared with controls ($P = .015$), although this difference was not observed at the mRNA level (Fig 3, B). For IL-10 mRNA expression, no significant differences were noted between groups (Fig 3, A).

The mRNA expression of IL-17 was not significantly different among all groups (see Table E2).

Within the CRSwNP group, patients with and without comorbid asthma were compared for the expression of FOXP3, T-bet, GATA-3, RORc, IL-4, IL-5, IL-13, IL-10, IL-17, IFN- γ , and TGF- β 1. This revealed no significant differences between both nasal polyp subgroups.

DISCUSSION

In this study, we analyzed at the sinonasal mucosal tissue level the expression of the transcription factors FOXP3, T-bet, GATA-3, and RORc in relation to T_H1/T_H2/T_H17 cytokines and suppressive iTreg-related cytokines.

We here demonstrate for the first time a significantly lower expression of FOXP3 in CRSwNP compared with controls and with CRSsNP. The reduced expression of FOXP3 in CRSwNP at the mRNA level was consistent with the immunohistochemical findings. FOXP3-positive cells were almost undetectable in CRSwNP tissue sections and appear to be focally present.

We used FOXP3 as the most widely accepted marker for Treg cells, which, although still controversial, also has been associated with suppressive function. It was demonstrated in a mouse model of colitis that retroviral transfer of FOXP3 converts naive T cells into functional CD25⁺ Treg cells.²³ Importantly, mutations in FOXP3 result in the absence or dysfunction of Treg cells and lead to the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome and its murine homolog, the scurfy mouse.^{24,25} Moreover, the role of Treg cells in controlling allergic diseases became apparent. In allergic rhinitis, a reduced number of FOXP3-positive cells was reported compared with controls.²⁶ Treg cells play an important role in controlling T_H2 immune responses, and an impaired expansion of natural and/or inducible Treg cells has been suspected to result in the development of allergy and asthma.¹¹ Similar to asthma, CRSwNPs are typically associated with a T_H2-driven eosinophilic inflammation, characterized by high concentrations of eosinophilic cationic protein, eotaxin, and IL-5, but not correlated to atopy.⁴ We suggest that a defective suppressive function of Treg cells in CRSwNP, indicated here by the low FOXP3 expression, might account for the often severe persistent eosinophilic inflammation. Because Treg cells are assumed to control the pathogenic T_H1 and T_H2 cells, the reduced expression of FOXP3 in CRSwNP together with the upregulation of T-bet and GATA-3 suggests either a deficiency or a dysfunction of T regulation in nasal polyp disease. However, this statement can only be of general nature at this moment. The exact functional implications of these findings need further investigation.

In contrast, in CRSsNP, adequate expression of FOXP3 and unchanged expression of the transcription factors for T_H1 and T_H2 lymphocyte populations compared with control mucosa suggest active Treg function. Because corticosteroids are known to

upregulate FOXP3 and Treg cells in asthma, only samples of patients who ceased oral and topical steroids for at least 1 month before surgery were selected.

GATA-3 is both necessary and sufficient for commitment toward T_H2 cells and controls the expression of IL-5.^{6,7} Moreover, GATA-3 has the capacity to inhibit IFN- γ promoter activity directly, resulting in repression of T_H1 and development of a T_H2 phenotype.²⁷⁻²⁹ The upregulation of GATA-3 in CRSwNP was reflected by the subsequent increase of the IL-5 mRNA signal. These findings at the mRNA level confirm previous protein data.³⁰ In CRSsNP, IL-5 mRNA was not upregulated compared with controls, in line with the unchanged expression of GATA-3. In addition, the T_H2 cytokines IL-4 and IL-13 were analyzed. IL-4 mRNA was not found to be significantly different among CRSsNP, CRSwNP, and controls, confirming previous data.³¹ However, IL-13 was significantly increased in CRSwNP compared with controls, which, under certain circumstances, has been described to replace IL-4.³² It should be noted that other cell types such as eosinophils and mast cells may contribute to GATA-3 expression.³³ IL-4, IL-5, and IL-13 levels can also variably be produced by eosinophils and mast cells. Although the coincidence of low FOXP3 levels and the upregulation of GATA-3 together with the IL-5 and IL-13 signal suggest a functional relation, the contribution of individual cell types to GATA-3 and cytokine expression could not be quantified at the whole tissue level.

T-bet is a T_H1-specific T-box transcription factor that controls the expression of the hallmark T_H1-cytokine IFN- γ , and T-bet expression correlates with IFN- γ expression in T_H1 cells.⁵ The upregulation of T-bet in CRSwNP was not reflected by a significant upregulation of IFN- γ at the mRNA expression and protein level in CRSwNP, confirming previous protein data.¹ Different from T-bet, the expression of which is mainly restricted to the T_H1 cell type, IFN- γ can be produced by several cell types including CD4⁺T_H1 cells, CD8, and natural killer cells, but also B cells. In addition, IFN- γ expression can also be influenced by exogenous and endogenous factors (IL-12/IL-12 receptor, and IL-18) that can act together or independently of the T-bet signaling pathway. Accordingly, the expression of T-bet was not expected to correlate directly with IFN- γ expression.

As a general remark, we wish to mention that inferior turbinates from healthy persons were used as control tissue, because it is unethical to resect ethmoidal tissue from healthy persons. However, it should be noted that the differences observed between controls and chronic rhinosinusitis with or without nasal polyps might be influenced by comparing these different tissue localizations. However, clear differences are observed between ethmoidal tissue from CRSsNP and CRSwNP.

As a marker for T_H17 cells, we analyzed RORc, a transcription factor that controls the differentiation toward proinflammatory T_H17 cells and regulates IL-17 production.^{34,35} The expression of RORc was not significantly different in diseased tissue compared with controls, although there was a significantly lower expression of RORc in CRSwNP compared with CRSsNP. For IL-17, no significant differences were found at the mRNA level among CRSwNP, CRSsNP, and controls.

In CRSsNP, IFN- γ mRNA expression was significantly upregulated compared with controls. At the protein level, this was also true when compared with CRSwNP, confirming the previously described T_H1-polarized inflammation. However, the transcription signal of T-bet, critical for commitment toward the T_H1 phenotype, was not found upregulated in CRSsNP.

As markers for induced T regulatory activity of T_H3 cells, we analyzed the expression of the suppressive cytokine TGF- β 1. T_H3 cells are known to exert their immune-suppressive effect via the production of this growth factor. However, numerous other cell types can express TGF- β , such as macrophages, mast cells, neutrophils, eosinophils, endothelial and epithelial cells, and fibroblasts. Furthermore, TGF- β 1 induces FOXP3 expression in CD25⁻ naive T cells to enforce transition to Treg cells and is a critical factor in the development of peripheral Treg cells.³⁶ There was no significant difference in TGF- β 1 mRNA expression in CRSwNP tissue versus controls. However, regulation of TGF- β 1 mainly occurs at the posttranscriptional level. TGF- β 1 is secreted from cells as small latent complexes, preventing binding of TGF- β 1 to ubiquitously expressed receptors, assuring an extracellular reservoir of TGF- β that can be activated on demand.³⁷ Therefore, we performed additional protein quantification by means of ELISA, measuring both active and latent forms of TGF- β 1. Latent forms were released from extracellular matrix by adding acid. This confirmed previously published findings of our group⁴ and others^{38,39} of a downregulation of TGF- β 1 protein in polyp tissue. Although the coincidence of low levels of TGF- β 1 protein and the downregulation of FOXP3 may imply a functional link, the effect of local TGF- β 1 on Treg function has to be confirmed in CRSwNP.

In contrast, we found a significant upregulation of TGF- β 1 at the mRNA level in CRSsNP, just not reaching significance at the protein level, compared with controls. Furthermore, normal TGF- β 1 protein levels coincided with adequate FOXP3 expression in CRSsNP tissue.

IL-10, a cytokine that is deeply involved in the regulation of inflammatory and immune responses, was measured at the mRNA level. The induction of peripheral regulatory T cells by IL-10 points toward a crucial role in the establishment of peripheral tolerance.⁴⁰ Specifically, IL-10 is instrumental in Tr1-mediated suppression of proliferation and cytokine production of naive CD4⁺CD25⁻ T, T_H1 , and T_H2 cells. Activation of T cells in the presence of IL-10 induces a long-lasting state of nonresponsiveness or anergy.⁴¹ Here, no significant differences in IL-10 mRNA expression were found among controls, CRSsNP, and CRSwNP.

TGF- β and IL-10 are both suppressor cytokines that frequently occur together at sites of inflammation, and both cytokines cooperate in the resolution of inflammation. TGF- β can induce IL-10, and IL-10 facilitates TGF- β regulatory activity.⁴² The striking coincidence of low TGF- β 1 protein levels, together with basal levels of IL-10 mRNA (not higher compared with controls) in CRSwNP, points to a lack of regulatory effect in the resolution of inflammation, hence contributing to the chronicity of this disease.

Conclusion

In this study, we demonstrate a decreased expression of FOXP3 in nasal polyp tissue, reflecting a deficiency or a dysfunction of Treg cells in an often persistent, severely inflamed sinus disease, CRSwNP. In line with the low FOXP3 expression in CRSwNP, we describe low levels of TGF- β 1 protein expression and an upregulation of the transcription signals for T_H1 (T-bet) and T_H2 (GATA-3) subpopulations, pointing toward a defective suppression of their upregulation by Treg cells. We suggest that this lack in Treg cell function may contribute to the severe

persistent T_H2 -skewed airway inflammation often observed in patients with CRSwNP. In contrast, upregulated TGF- β 1 protein levels compared with CRSwNP coincide with an adequate expression of FOXP3 and maintained control over T-bet and GATA-3 expression in CRSsNP, suggesting adequate Treg cell function in this sinus disease subgroup.

Clinical implications: This study reveals a new understanding in chronic sinus disease, pointing toward a deficient Treg cell function in CRSwNP, but not in CRSsNP.

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METHODS

Gene expression analysis

RNA preparation and cDNA synthesis. Clinical tissue samples (30 mg) were homogenized in Lysis Buffer (BioRad Laboratories, Hercules, Calif). Total RNA isolation was performed by using the Aurum Total RNA kit (BioRad Laboratories) following the manufacturer's instructions. RNA quality and percent of degradation were assessed with the Agilent 2100 Bioanalyzer system using the RNA 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, Calif) for representative results. cDNA was synthesized from 2 μ g RNA with the iScript cDNA synthesis kit (BioRad Laboratories) following the manufacturer's instructions.

Quantitative real-time PCR. mRNA levels of the transcription factors FOXP3, GATA-3, T-bet, and RORc and cytokines IL-4, IL-5, IL-10, IL-13, IL-17, TGF- β 1, and IFN- γ were determined by real-time PCR. Amplification reactions were performed on an iCycler iQ Real-Time PCR Detection System (BioRad Laboratories) using specific primer sequences (Table E1). All primers were purchased from Invitrogen (Carlsbad, Calif) with the exception of IL-5 and RORc, which were obtained from SuperArray BioScience Corp (Frederick, Md), and the sequence of which was not revealed. FOXP3, GATA-3, and T-bet were obtained from Swiss Institute of Allergy and Asthma Research. PCR reactions contained 30 ng cDNA (total RNA equivalent) of unknown samples, 1X SYBR Green I Master mix (BioRad Laboratories), and 250 nmol/L primer pairs in a final volume of 20 μ L. The PCR protocol consisted of 1 cycle at 95°C for 10 minutes followed by 40 cycles at 95°C for 30 seconds and at 60°C for 1 minute.

PCR tubes contained 30 ng cDNA with 1X TaqMan mix (BioRad Laboratories), 250 nm each pair primer and 100 nmol/L TaqMan probe. Amplification reactions consisted in a cycle of 1.5 minutes at 95°C followed by 50 cycles: 15 seconds at 95°C and 1 minute at 60°C.

The expression of 3 housekeeping genes—actin beta (ACTB), hydroxymethyl-bilane synthase (HMBS), and EF1—was used to normalize for transcription and amplification variations among samples after a validation using the geNorm software (Ghent University Hospital Center for Medical Genetics, Ghent, Belgium).^{E1,E2} The relative expression units of each gene per 30 ng of cDNA sample was determined by using the qBase program (version 1.3.5, Ghent University, Belgium), which consists of a collection of Microsoft Excel sheets that automatically analyses real-time quantitative PCR data, combining the Δ cycle threshold (Δ CT) relative quantification model with PCR efficiency correction and multiple reference gene normalization. Results are expressed as the logarithm of normalized relative expression units/30 ng cDNA.

TGF- β 1/IFN- γ ELISA

Samples were snap-frozen in liquid nitrogen and stored at -80°C until homogenization.

For homogenization, the tissue was thawed and weighed, and 1 mL 0.9% NaCl and a protease inhibitor Complete Roche (Mannheim, Germany) was added per every 0.1 g tissue. The tissue was then homogenized by using a B. Braun (Melsungen, Germany) homogenizer for 5 minutes. The homogenates were centrifuged at 3000g, 4°C for 10 minutes. After centrifugation, 250- μ L aliquots were made and stored at -80°C until needed for ELISA. Total TGF- β 1 and IFN- γ level in the supernatant of tissue were determined by using a commercially available ELISA kit from R&D Systems (Minneapolis, Minn). All data were expressed as nanograms per milliliter. For TGF- β , acid was added during the ELISA procedure, resulting in physicochemical activation of latent TGF- β . Total TGF- β concentrations are reported including both active and latent forms.

Immunohistochemistry

CD3 staining. Cryostat sections were prepared (6 μ m) and mounted on SuperFrost Plus glass slides (Menzel Glaeser, Braunschweig, Germany), packed in aluminum paper, and stored at -30°C until staining. Sections were immunohistochemically stained with the following mouse mAb: CD3 (clone UCHT1; Dako Cytomation, Glostrup, Denmark). For immunohistochemical staining, specimens were fixed in acetone. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in TRIS-buffered saline containing 0.1% sodium azide for 20 minutes. Primary antibody or negative control, consisting of the corresponding isotype control, were incubated for 1 hour and detected using the LSAB+ technique (labeled streptavidin-biotin; Dako Cytomation) conjugated with peroxidase according to the manufacturer's instructions. The peroxidase activity was detected by using AEC Substrate chromogen (Dako Cytomation), which results in a red-stained precipitate. Finally, sections were counterstained with hematoxylin and mounted.

The number of positive cells was analyzed by using a magnification of $\times$ 400 and scored by 2 independent observers who did not know the diagnosis and clinical data. A grading scale from 0 to 3 was applied, ranging from absent to numerous stained cells. Score 0 represents no positive cells; score 1, <10 positive cells/field; score 2, 10 to 100 positive cells/field, and score 3, >100 positive cells/field. All areas of the section were analyzed, and for each sample, 10 high-power fields were scored.

FOXP3 staining. The frozen sections were air-dried, fixed with 4% paraformaldehyde, and stained by using combined FOXP3 Flow Kit (BioLegend, San Diego, Calif) and ready-to-use EnVision+ System-HRP Kit (K4010; Dako Cytomation). Briefly, samples were permeabilized with FOXP3 Fix/Perm solution (320501; BioLegend) and blocked with 10% normal goat serum (X0907; Dako Cytomation). Primary polyclonal rabbit antihuman FOXP3 Ab (ab10563; Abcam, Cambridge, United Kingdom) was applied overnight at 4°C. Activity of endogenous peroxidase was quenched with 0.3% H₂O₂ in PBS. Then, slides were incubated with peroxidase-labeled polymer followed by 3-amino-9-ethyl carbazole. Sections were counterstained with hematoxylin (Sigma, St Louis, Mo) and permanently mounted with Ultramount (S1964; Dako Cytomation). FOXP3 blocking peptide (ab14151; Abcam) was used as a control to block anti-FOXP3 binding. Human tonsil sections were used as a positive control on each staining run. Slides were viewed with a light microscope (Axiovert 405 M; Carl Zeiss, Feldbach, Switzerland).

Counting of 10 random high-power fields was performed by 2 independent observers. The immunohistochemical stainings were performed at Swiss Institute of Allergy and Asthma Research, Switzerland.

Statistical analysis

Statistical analysis was performed with MEDCALC software v 9.2.0.1 (F. Schoonjans, Mariakerke, Belgium). Data are expressed in box-and-whisker plots. When comparisons were made between groups, the Kruskal-Wallis test was used to assess significant intergroup variability. The Mann-Whitney *U* 2-tailed test was used for between-group comparison. The significance level was set at $\alpha = 0.05$.

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TABLE E1. Primer sequences used for real-time PCR amplifications

	Forward (5' → 3')	Reverse (5' → 3')	Probe (6-FAM-5' → 3-TAMRA-3')	Amplicon size (bp)	Accession number
TGF-β1	CAGCAACAATTC-CT GGCGATA	AAGGCGAAAGCCCTCAATTT		135	NM_000660.3
FOXP3†	GAAACAGCACATTC CCAGAGTTC	ATGGCCCAGCGGATGAG		100	NM_014009
T-bet†	GATGCGCCAGGA AGTTTCAT	GCACAATCATCTGGGTCACATT		83	NM_013351
GATA-3†	GCGGGCTCTATCACA AAATGA	GCTCTCCTGGCTGCAGACAGC		79	NM_002051
EF-1	CTGAACCATCCAGG CCAAAT	GCCGTGTGGCAATCCAAT		59	NM_001402
IFN-γ	ACTGACTTGAATGTC CAACGCA	ATCTGACTCCTTTTTCGCTTCC		101	NM_000619
Actin beta*	CTGGAACGGTGAAG GTGACA	AAGGGACTTCCTGTAACAATGCA		139	NM_001101
HMBS*	GGCAATGCGGCTGCAA	GGGTACCCACGCGAATCAC		154	NM_00319
IL-4	ACTTTGAACAGCCTCA CAGAG	TTGGAGGCAGCAAAGATGTC-	CTGTGCACCGAGTTGACCGTA	73	NM_000589
IL-13	TGAGGAGCTGGTCAA CATCA	CAGGTTGATGCTCCATACCAT	AGGCTCCGCTCTGCAATGGC	75	NM_002188
IL-17	AATCTCCACCGCAAT GAGCA	ACGTTCCCATCAGCGTTGA	CGGCACTTTGCCTCCCAGATCACA	94	NM_002190

HMBS, Hydroxymethyl-bilane synthase.

*Sequences were obtained from the real-time PCR primer and probe database (<http://medgen.ugent.be/rtprimerdb/>).

†Sequences were provided by the Swiss Institute of Allergy and Asthma Research.

TABLE E2. Medians and IQRs for Treg/T_H1/T_H2/T_H17 transcription factors and cytokines

	Controls	Versus	CRSsNP	Versus	CRSwNP	Versus controls
mRNA transcription factors (relative expression units/30 ng DNA)						
FOXP3	0.499 (0.426-0.884)	NS	0.677 (0.529-1.016)	<i>P</i> = .005	0.3775 (0.230-0.720)	<i>P</i> = .026
GATA-3	0.687 (0.272-0.873)	NS	0.562 (0.322-0.695)	<i>P</i> = .002	0.888 (0.689-1.185)	<i>P</i> = .035
T-bet	0.289 (0.154-0.959)	NS	0.376 (0.245-0.583)	<i>P</i> < .001	0.969 (0.661-1.499)	<i>P</i> = .005
RORc	4.300 (3.610-5.160)	NS	5.060 (4.835-5.418)	<i>P</i> < .001	3.580 (2.538-4.488)	NS
mRNA cytokine (relative expression units/30 ng DNA)						
IL-5	0.273 (0.100-0.567)	NS	0.459 (0.368-0.722)	<i>P</i> < .001	1.154 (0.912-1.517)	<i>P</i> < .001
IL-13	1.112 (0.913-1.183)	NS	0.796 (0.315-1.410)	NS	1.450 (1.055-1.626)	<i>P</i> = .029
IL-4	3.090 (2.300-4.510)	NS	4.120 (3.177-7.248)	NS	3.565 (2.795-4.740)	NS
IL-10	0.283 (0.267-0.562)	NS	0.4392 (0.367-0.626)	NS	0.341 (0.236-0.490)	NS
TGF-β1	0.765 (0.746-0.841)	<i>P</i> = .033	1.053 (0.915-1.258)	<i>P</i> = .014	0.911 (0.760-0.977)	NS
IFN-γ	1.078 (0.614-1.251)	<i>P</i> = .009	1.563 (1.126-1.734)	<i>P</i> = .001	0.763 (0.491-1.178)	NS
IL-17	7.530 (5.733-8.983)	NS	8.358 (6.239-9.069)	NS	5.993 (3.689-8.177)	NS
Protein cytokine (pg/mL)						
TGF-β1 protein	10,690 (9319-16,267)	NS	16,508 (12,229-22,856)	<i>P</i> < .001	6478 (4025-9893)	<i>P</i> = .015
IFN-γ protein	42.5 (42.475-43.255)	<i>P</i> = .001	101.2 (43.55-165.75)	<i>P</i> = .01	43.1 (42.7-54.1)	NS

Mann-Whitney *U* test was used for unpaired comparisons. *P* < .05 was considered statistically significant NS, Not significant).