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-β1 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-β1 and IFN-γ.

Results: In CRSwNP, we observed a significantly lower FOXP3 mRNA and TGF-β1 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-β1 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-β1 in CRSwNP versus controls and CRSsNP. (J Allergy Clin Immunol 2008;121:1435-41.)

Key words: *Chronic rhinosinusitis, FOXP3, nasal polyps, T-cell polarization, TGF-β1, 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-β1 concentrations,[1] whereas CRSwNP typically shows a T_H2 skewed eosinophilic inflammation with high levels of IL-5 and IgE and low TGF-β1.[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 cells[5]; 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-β1, 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] recent studies demonstrated the induction of FOXP3 in induced Treg cells,[16-18] positioning this transcription factor as a marker

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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

for both nTreg cells and iTreg cells. As indirect markers for Tr1 and T_H3 activity, we measured IL-10 and TGF-β1 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 Polyps[19] and American[20] guidelines. General exclusion criteria were based on the EP^{3}OS 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 μg 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-β1, 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 μL. 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-β1/IFN-γ ELISA

Tissue homogenates were assayed for total TGF-β1 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 ×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 controls. FOXP3-expressing cells were detectable in both