Peripheral blood and tissue T regulatory cells in chronic rhinosinusitis

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ABSTRACT

Background: *The pathogenesis of chronic rhinosinusitis (CRS) has not been fully elucidated. Increased inflammatory cell infiltration and decreased numbers and/or impaired function of T regulatory cells (Tregs) have been reported. This study aimed to determine the role of Tregs in CRS in peripheral blood (PB) and sinus tissue.*

Methods: *Sinus tissue was obtained from 16 CRS subjects and 5 controls. PB from additional 16 CRS subjects and total 20 controls was obtained. Immunohistochemical analysis ($CD3^+$, $CD4^+$, $CD8^+$, and Treg [$CD4^+$-$FoxP3^+$ and $CD25^+$-$FoxP3^+$] cells) of sinus tissue was performed. Percentage of PB Tregs ($CD4^+$-$CD25^+$-$FoxP3^+$ cells) was analyzed by flow cytometry. Spontaneous and phytohemagglutinin (PHA)-induced release of cytokines (IL-6, IL-4, IL-10, interferon gamma, transforming growth factor [TGF] beta1, and TNF-alpha) from PB mononuclear cells (PBMCs) was determined.*

Results: *PB flow cytometric analysis revealed a lower percentage of Tregs in subjects with CRS compared with healthy controls ($p = 0.0003$). Although no differences in the PB Treg counts were observed between the CRS subjects with nasal polyposis (CRSwNP) and without nasal polyposis (CRSsNP), immunohistochemical analysis performed on sinus tissue revealed a higher proportion of Tregs in CRSwNP subjects compared with CRSsNP ($p < 0.05$). Additionally, we failed to detect any Tregs from control sphenoid sinus tissue. Lower levels of regulatory cytokines (IL-10 and TGF-β1) and higher levels of proinflammatory cytokines (TNF-α and IL-6) were found from PBMCs from CRS subjects compared with controls ($p < 0.05$).*

Conclusion: *Our findings suggest that CRS subjects exhibit a decreased percentage of PB Tregs compared with normal controls. PBMCs from CRS subjects show a more proinflammatory and less regulatory phenotype.*

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Chronic rhinosinusitis (CRS) is a mucosal inflammatory disease of the upper airway that affects ~5–15% of the U.S. population and imposes a significant public health burden.[1,2] CRS has been divided into two broad categories—CRS with nasal polyposis (CRSwNPs) and without nasal polyposis (CRSsNPs).[3,4] Both forms of the disease display distinct features based on immunochemistry and the expression of inflammatory and remodeling mediators. Of these, CRSwNPs, the most commonly studied type of CRS, appears to be primarily a T_H2 response with an accumulation of eosinophils, T cells, neutrophils, and plasma cells associated with elevated levels of IL-5 and IgE and decreased levels of transforming growth factor (TGF) β1.[2,4–6] In contrast, CRSsNPs is characterized by a predominant T_H1 milieu with increased interferon (IFN) γ and TGF-β1 levels.[4,6] However, some data suggest a mix of both T_H1 and T_H2 inflammation with a predominance of one type in both forms of the disease—with and without polyposis.[7,8]

To date, the pathogenesis of CRS has not been fully elucidated, and few studies provide insights about dysregulated T_H1/T_H2 mucosal immunity that characterize this disorder. Given the crucial role of T regulatory cells (Tregs) in maintaining the balance between T_H1 and T_H2 polarization of immunity,[3,9,10] it is important to study the role of these cells in CRS.

Tregs are characterized by the presence of CD4 and CD25 surface markers and nuclear expression of the transcription factor forkhead box P3 (FoxP3). Two distinct subsets of Tregs are recognized: natural Tregs (nTregs), which mature in the thymus and represent a small proportion of the peripheral $CD4^+$ T-cell population, and peripherally induced Tregs (iTregs), which are $FoxP3^+$ or $FoxP3^-$ and generally secrete anti-inflammatory and immunosuppressive cytokines such as IL-10 and TGF-β1.[9–11] Tregs have been shown to control effector immune responses through a diverse array of mechanisms.[3,9,10] These cells play roles in the coordination and regulation of recruitment of effector T cells to sites of inflammation and also have the ability to impair the capacity of antigen-presenting cells (APCs) to prime adaptive immune responses.[9,10,12] Decreased numbers and/or impaired function Tregs have previously been implicated in the development of autoimmunity[13,14] and other immune diseases, including asthma and allergy.[9,12,15]

Previous studies of populations of Chinese and European origin have indicated a decreased expression of FoxP3 and TGF-β1 in tissue samples from subjects with CRSwNP compared with CRSsNP or control subjects assessed by a variety of methods.[3,6,16–18] Other studies investigating T-cell phenotypes in Chinese subjects with or without nasal polyps also confirm down-regulation of FoxP3 mRNA expression and a significantly decreased infiltration of $FoxP3^+$ Tregs into nasal polyp tissue, thereby implicating a role of Tregs in these disorders at the local tissue level.[19,20]

Classically, glucocorticoids are thought to mediate potent anti-inflammatory actions through the inhibition of transcription factors involved in cytokine regulation. Another mechanism of their immunomodulatory effects is the stimulation of Tregs. For example, studies have shown that inhaled or systemic glucocorticoid treatment restores low $CD4^+$-$CD25^+$ cell numbers, FoxP3 mRNA expression, and IL-10 production and suppressive function in asthma.[21] The expression of FoxP3 mRNA was down-regulated in the tissues of patients with allergic rhinitis and CRSwNP, whereas treatment with a topical

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Table 1 **Demographics and clinical characteristics of the subjects**

	Control Subjects	CRS Subjects (Group 1)	CRS Subjects (Group 2)
n	20	16	16
Sex (male/female)	8:12	11:5	7:9
Age (yr)	23–63	23–77	30–69
Positive *in vitro* allergy test	6/20	9/16	8/16
Asthma	0/20	7/16	6/16
Nasal polyposis	0/20	8/16	11/16
Recurrent surgery	0/20	5/16	7/16
Systemic and/or intranasal steroids use before surgery	0/20	8/16	8/16
Race			
White	12	9	7
Black American	3	5	6
Hispanic	1	—	1
Asian	3	—	1
Other	1	1	1

The CRS subjects in group 1 were used for immunohistochemical analysis, and group 2 CRS subjects and controls were used for the PB Treg study.
PB = peripheral blood; Treg = T regulatory cell.

steroid enhanced the expression of FoxP3 mRNA and IL-10 and increased Treg accumulation in nasal polyps.[22] These studies provide evidence that decreased Treg function might be responsible for dysregulation of the T_H1/T_H2 cytokine network in CRSwNP and that one reason behind the beneficial effect of intranasal steroids in this disease is to increase Tregs significantly.

In this study, we aimed to characterize (1) the histological features of CRSsNP and CRSwNP in a population recruited from Chicago and (2) the difference in the proportion of $CD4^+$ and $CD25^+$ cells costained with FoxP3 (the classic Treg cell marker) in affected tissue from these subjects. Also, because studies of immunologic function in CRS have been hampered by difficulties in accessing sinonasal tissue in human subjects and in conducting comparisons with appropriate normal controls, we aimed to investigate the proportion of $CD4^+$-$CD25^+$-$FoxP3^+$ cells in peripheral blood (PB) in subjects with CRS compared with healthy controls and the expression profiles of representative T_H1/T_H2/Treg cytokines from PB mononuclear cells (PBMCs).

METHODS

Subjects and Specimens

CRS subjects were recruited from the Otolaryngology–Head and Neck Surgery Clinic at The University of Chicago Medical Center in Chicago, IL, in two groups over two time intervals. Subjects in the first group ($n = 16$; 8 CRSsNP and 8 CRSwNP) were recruited over the period of 2006–2008 for study of the affected sinonasal tissues, whereas subjects in the second group ($n = 16$; 5 CRSsNP and 11 CRSwNP) were collected in 2008–2009 for analysis of the PB. All subjects met standard research criteria for the definition of CRS,[1] including history, the presence of symptoms for >12 weeks, and confirmatory nasal endoscopy and imaging. All subjects had previously failed to respond to adequate trials of conservative medical therapy including antibiotics, oral or intranasal steroids, and leukotriene modifiers, and were scheduled for endoscopic sinus surgery. All of our subjects had severe disease. For example, a large percentage of subjects with CRSwNP were undergoing revision surgery (Table 1). Additionally, withdrawal of medications (systemic and/or intranasal corticosteroids) before the study was not feasible clinically (8/16 subjects in both groups were on these medications) because of the disease severity and hence these subjects had been receiving steroids for 2 weeks to 1 month before surgery and blood draw. We excluded subjects with a diagnosis of cystic fibrosis, an established diagnosis of immunodeficiency, pregnancy, and classic allergic fungal rhinosinusitis from our study.

Control subjects ($n = 15$) for the study of PBMCs recruited for this study had no evident history of CRS or asthma and had normal sinonasal examinations by anterior rhinoscopy. The atopic status was confirmed by radioallergosorbent test to a standard screening panel of representative allergens in Chicago. Additional controls ($n = 5$) were recruited from subjects undergoing endoscopic transsphenoidal pituitary surgery for analysis of normal sphenoid sinus mucosa; these subjects were phenotyped in a similar manner. Thus, a total of 20 control subjects were recruited for the study.

Polyp tissue was used in the CRSwNP group, sinus mucosa from the ethmoid sinus was used in the CRSsNP group, and sphenoid sinus mucosa was used from transsphenoidal controls ($n = 5$). PB was collected from all subjects by venipuncture at surgery for CRS subjects and in the clinic for controls. The study protocol was approved by the Institutional Review Board of The University of Chicago. Written informed consent was obtained from all subjects.

Tissue Histology and Immunohistochemistry

Paraffin sections (5 μm) of sinus tissue were stained with hematoxylin and eosin, and the stained sections were analyzed at 400× magnification by two independent observers who were blinded to the clinical data. The numbers of eosinophils, mononuclear cells, plasma cells, and lymphocytes were assessed.

For immunohistochemistry, sinonasal tissue was dehydrated, infiltrated, and embedded with paraffin, and tissue was sectioned at 3 μm with a Leica RM2245 Cryostat (Leica Microsystems, Inc., Bannockburn, IL). Sections were rehydrated, incubated in antigen retrieval buffer (Tris-EDTA buffer, pH 9), and heated in a microwave oven at above 98°C for 20 minutes. After rinsing, tissue sections were blocked for nonspecific binding with 1% goat serum/0.3% Tween-20/PBS. For CD3 staining, tissue sections were then incubated with CD3 antibody (1:100 dilution; Neomarkers, Fremont, CA) for 1 hour at room temperature. The sections were rinsed and then incubated in biotinylated secondary goat anti-rabbit antibody (Dako, Glostrup, Denmark) at a 1:500 dilution for 1 hour at room temperature. After another rinse, antibody binding was visualized by use of the EnVision plus system (Dako). The sections were then rinsed in deionized water, counterstained with hematoxylin, dehydrated, cleared, mounted, and coverslipped.

For CD4-CD8 staining, tissue sections were incubated with CD4-CD8 antibody cocktail (Neomarkers) for 30 minutes at room temperature. A multiVision Polymer Detection System (Thermo Scientific, Fremont, CA) was used for visualization. The antigen–antibody binding for CD4 was detected by a polymer-horseradish peroxidase (HRP)-red system, whereas the antigen–antibody binding for CD8

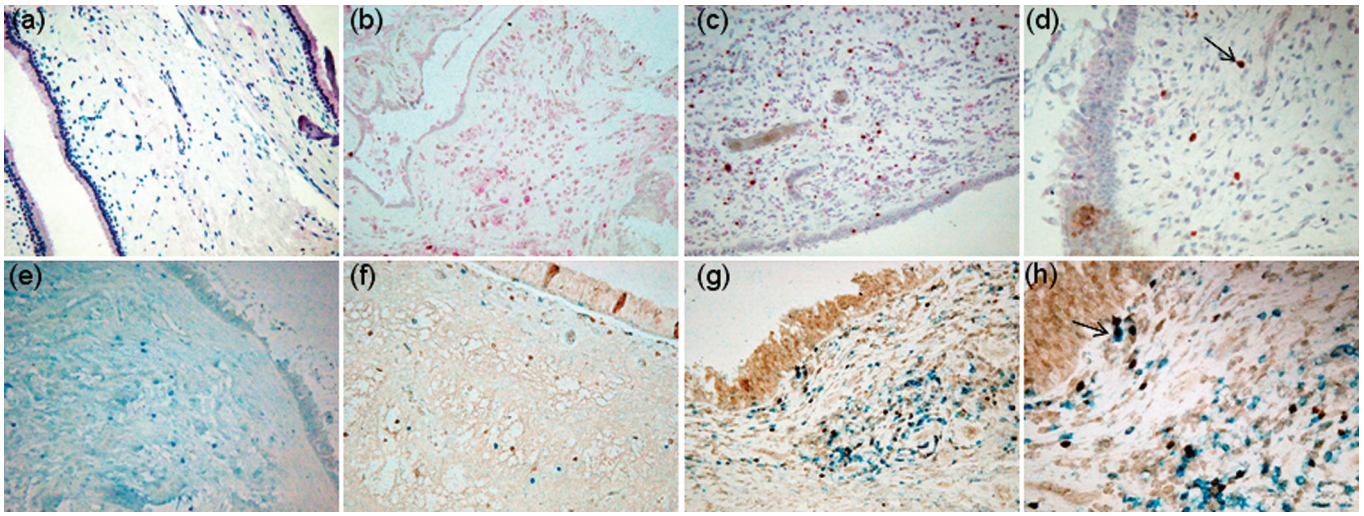

Figure 1. *Representative immunostaining for (a-d) $CD25^+$- forkhead box P3 ($FoxP3^+$) from (a) normal sphenoid sinus, (b) chronic rhinosinusitis without nasal polyposis (CRSsNP), and (c–d) chronic rhinosinusitis with nasal polyposis (CRSwNP) subjects, and (e-h) for $CD4^+$-$FoxP3^+$ cells from (e) normal sphenoid sinus, (f) CRSsNP, and (g and h) CRSwNP subjects (arrow: positive cells; magnification, ×200 for panels a–c and e–g and ×400 for panels d and h).*

was detected by a polymer-AP-blue system. The slides were then rinsed in deionized water, counterstained with hematoxylin, dehydrated, cleared, mounted, and coverslipped.

For CD4-FoxP3 staining, FoxP3 antibody (1:100 dilution; Abcam, Cambridge, U.K.) was applied on the tissue sections for 10 minutes of incubation at room temperature. Antigen–antibody binding was detected by Polymer/HRP (Envision G/2 Doublestain System; Dako) and DAB (Dako). After double stain, blocking was performed. CD4 (1:50 dilution; Vector Laboratories, Burlingame, CA) was used for 1 hour of incubation at room temperature. Antigen–antibody binding for CD4 was detected by a polymer-AP system and an alkaline phosphatase substrate kit III (Vector Laboratories). The sections were then rinsed in deionized water, counterstained with hematoxylin, dehydrated, cleared, mounted, and coverslipped.

For CD25-FoxP3 staining, FoxP3 antibody (1:100 dilution; Abcam) was applied on the tissue sections for 10 minutes at room temperature. The antigen–antibody binding was detected by a Polymer/HRP (Envision TM G/2 Doublestain System; Dako) and DAB. After a double-stain blocking was performed, CD25 (1:200 dilution; Novocastra, Newcastle upon Tyne, U.K.) was used for 1 hour of incubation at room temperature. Antigen–antibody binding for CD25 was detected by a polymer-AP system. The sections were then rinsed in deionized water, counterstained with hematoxylin, dehydrated, cleared, mounted, and coverslipped.

The number of $CD3^+$, $CD4^+$-$CD8^+$, $CD4^+$-$FoxP3^+$, and $CD25^+$-$FoxP3^+$ cells in the epithelium and submucosa were counted at a magnification of ×400. Ten high-power fields (HPFs) were randomly selected, and cells were counted in a blinded fashion by two observers, with median values (range) reported. We emphasize, here, that we failed to detect any $CD25^+$-$FoxP3^+$ (Fig. 1 *a*) and $CD4^+$-$FoxP3^+$ cells (Fig. 1 *e*) from sphenoid sinuses taken from the transsphenoidal controls recruited for our study; thus, all comparisons with controls were significantly different. We therefore opted to compare disease subgroups: CRSsNP and CRSwNP.

PBMC Separation and Treg Counts with Flow Cytometry

PBMCs were isolated from whole blood by use of Ficoll density gradient centrifugation. Briefly, whole blood was diluted 1:1 in Hanks'-buffered salt solution (Invitrogen, Carlsbad, CA) and layered over a Ficoll Paque gradient (Ficoll-Paque PLUS; GE Health care Bio-Sciences AB, Uppsala, Sweden). The PBMCs were centrifuged, washed, resuspended in freezing medium (RPMI-1640 [Invitrogen] supplemented with 20% FBS and 10% sterile dimethyl sulfoxide), and stored at −80°C until further use. After storage, the PBMCs were defrosted, and the cell viability was found to be >90%, as determined by Trypan blue staining. PBMCs (2×10^6 cells) were stained for $CD4^+$-$CD25^+$-$FoxP3^+$ cells with the use of a human Treg staining kit (eBioscience, Inc., San Diego, CA) as per the manufacturer's instructions. Briefly, the samples were stained for extracellular CD4 and CD25 markers by use of a cocktail of anti-human CD4-FITC and anti-human CD25-APC. After fixation and permeabilization (using the fix/perm buffers included in the kit), the cells were washed and blocked for nonspecific binding sites by use of normal rat serum and then stained with anti-human FoxP3-PE or rat IgG2a-PE isotype negative control. Samples were analyzed with the fluorescent activated cell sorter Canto flow cytometer (BD Biosciences, San Jose, CA), running DiVA acquisition software (BD Biosciences). Ten thousand gated events were acquired from each sample. Lymphoid cells were selected based on forward- and side-scatter properties. Appropriate isotype controls for FoxP3, CD4, and CD25 were used for setting quadrant markers. The percentage of Tregs was analyzed and compared with the use of FloJo software (Treestar, Ashland, OR).

Cell Culture

PBMCs were plated in a 12-well dish (2×10^6 cells/well) and were stimulated with phytohemagglutinin (PHA; 1 mg/mL; Sigma, St. Louis, MO) in RPMI-1640 supplemented with 10% FBS (Invitrogen). After 24 hours, the cell-free supernatants were collected and stored at −80°C. The levels of IL-6, IL-4, IL-10, IFN-γ, and TNF-α were determined by Milliplex bead array assay (Millipore, Billerica, MA). The minimal detection limits for IL-4, IL-6, IL-10, IFN-γ, and TNF-α were 0.13, 0.10, 0.15, 0.29, and 0.05 pg/mL, respectively. Levels of total TGF-β1 (both latent and active forms; R & D Systems, Minneapolis, MN) and IL-17A (eBioscience) were determined by ELISA according to the manufacturer's instructions. The minimal detection limits for the TGF-β1 and IL-17A ELISA kit were 4.61 and 4 pg/mL, respectively. The spontaneous and PHA-stimulated release of cytokines was compared between the groups.

Statistics

All data have been reported as medians with their interquartile range unless stated otherwise. Results of histological analysis have

Table 2 **Immunohistochemical analysis in CRSwNP (n =8) and CRSsNP (n =8) subjects from group 1**

	CRSwNP	CRSsNP	p Value
T cells ($CD3^+$)	132.1 (70.25, 161.85)	55.1 (22.05, 68.33)	<0.01
T_H cells ($CD4^+$)	52.41 (23.32, 57.68)	26.53 (6.33, 41.9)	NS
Cytotoxic T cells ($CD8^+$)	58.79 (39.12, 67.2)	33.9 (24.7, 54.85)	NS
Eosinophils	27.8 (11.65, 36.58)	14.83 (9.81, 21.29)	NS
Lymphocytes	65 (45.65, 92.88)	39.2 (23.31, 65.18)	<0.05
Plasma cells	18.9 (17.4, 28.15)	17 (11.06, 19.75)	NS
Mononuclear cells	28.5 (26.95, 47.45)	21.83 (16.14, 33.32)	NS

Values are expressed as median (interquartile range) of 10 HPFs. Mann-Whitney rank sum test was used for unpaired comparisons. The value of $p < 0.05$ was considered to indicate statistical significance.
CRSsNP = chronic rhinosinusitis without nasal polyposis; CRSwNP = chronic rhinosinusitis with nasal polyposis; HPFs = high-power fields; NS = not significant.

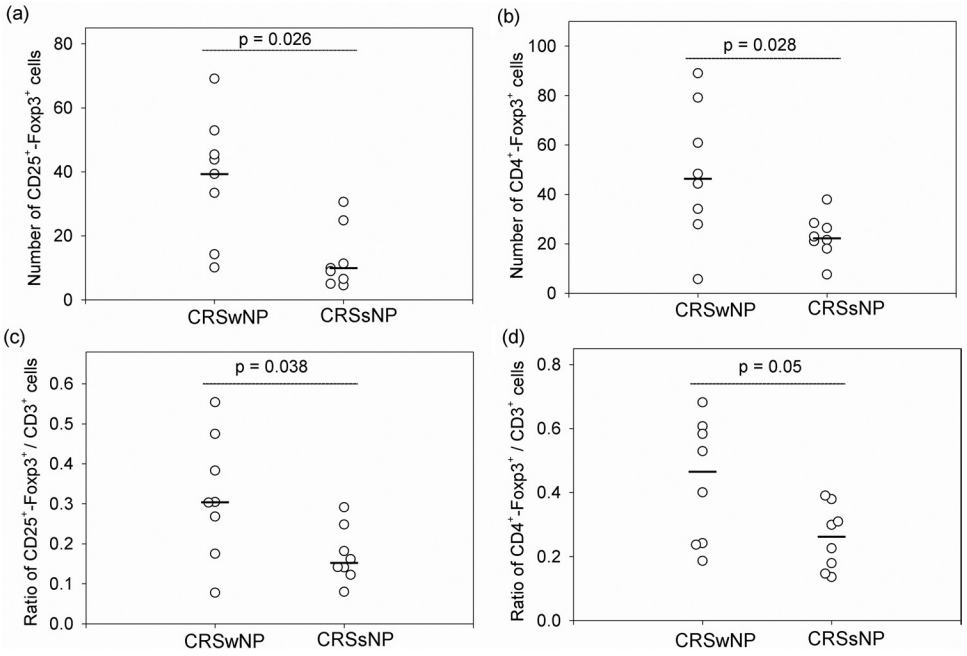

Figure 2. *The numbers of (a) $CD25^+$-forkhead box P3 ($FoxP3^+$) and (b) CD4-$FoxP3^+$ cells in chronic rhinosinusitis without nasal polyposis (CRSsNP) versus chronic rhinosinusitis with nasal polyposis (CRSwNP) subjects. The proportion of (c) $CD25^+$-$FoxP3^+$ and (d) $CD4^+$-$FoxP3^+$ cells to $CD3^+$ (total T) cells in CRSsNP versus CRSwNP subjects. The dark bars represent medians.*

been expressed as the median per 10 HPFs. Differences between groups were analyzed by use of the Mann-Whitney rank sum test. A value of $p \leq 0.05$ was considered significant.

RESULTS

Subject Characteristics

The subjects groups were similar in terms of their demographics including race, sex, and age (Table 1; $p > 0.05$, all). Subjects with CRS in both groups were more likely to have positive allergy *in vitro* allergy tests than were controls, and there were no asthmatic patients in the control subjects (Table 1).

Immunohistological Analysis of Inflammatory Cells In CRSwNP and CRSsNP

In the CRS subjects (eight CRSwNP and eight CRSsNP; Table 1), histological analysis showed a significantly increased number of lymphocytes ($p < 0.05$) and a trend toward increased eosinophils and mononuclear cells in the CRSwNP subjects when compared with CRSsNP (Table 2). The number of $CD3^+$, $CD4^+$ T helper (T_H), and $CD8^+$ T cytotoxic cells were also counted to estimate the presence of T cells in tissue samples. The number of $CD3^+$ T cells/10 HPF was higher in CRSwNP subjects when compared with CRSsNP subjects (median = 132.1 [70.25, 161.85] and 55.1 [22.05, 68.33]; $p < 0.01$; Table 2). Also, increased levels of $CD4^+$ T helper (T_H) and $CD8^+$ T cytotoxic cells were observed in subjects with CRSwNP (median = 52.41 [23.32, 57.68] and 58.79 [39.12, 67.2]); however, the differences did not attain statistical significance ($p > 0.05$, all).

Immunohistochemical Analysis of Tregs in CRS and Control Subjects

We double stained single tissue sections for both CD25 and FoxP3 (Fig. 1, *a–d*); and for both CD4 and FoxP3 (Fig. 1, *e–h*). We failed to detect any $CD25^+$-$FoxP3^+$ (Fig. 1 *a*) and $CD4^+$-$FoxP3^+$ cells (Fig. 1 *e*) from sphenoid sinuses taken from the transsphenoidal controls recruited for our study. On the other hand, a significant number of both $CD25^+$-$FoxP3^+$ and $CD4^+$-$FoxP3^+$ cells were observed in the CRS subjects (Fig. 1, *b–d*, and *f–h*, respectively).

Immunohistochemical Analysis of Tregs in CRSwNP and CRSsNP

A significant increase in the number per 10 HPFs of both $CD25^+$-$FoxP3^+$ and $CD4^+$-$FoxP3^+$ cells was observed in the CRSwNP group compared with CRSsNP (median = 39.3 range [13.17, 47.27] and 9.9 [5.97, 21.42]; p = .026; and median = 46.3 [31, 61.9]) and 22.2 [19.5, 27.4] per 10 HPFs; p = 0.028 for $CD25^+$-$FoxP3^+$ and $CD4^+$-$FoxP3^+$,

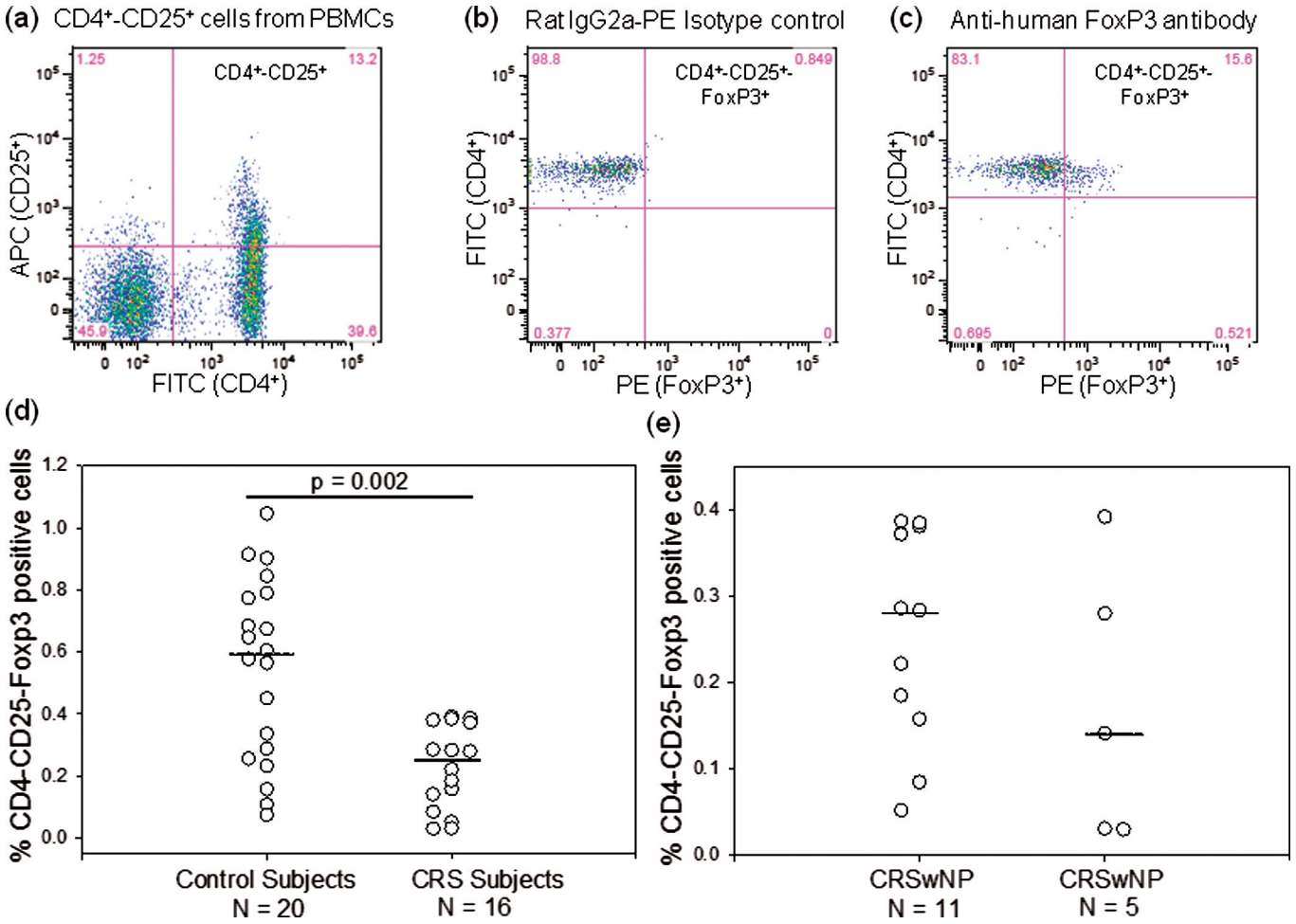

Figure 3. *Peripheral blood mononuclear cells (PBMCs) isolated from chronic rhinosinusitis (CRS) subjects and controls were stained. Representative scatter diagram of (a) $CD4^+$ (fluorescein isothiocyanate [FITC]) and $CD25^+$ (antigen-presenting cell [APC]) cells from the lymphocyte population in PBMCs. (b–c) The cells from the $CD4^+$-$CD25^+$ quadrant (marked) were then selected and analyzed; (b) forkhead box P3 (FoxP3$^+$; phycoerythrin [PE]) $CD4^+$-$CD25^+$ cells stained with use of a rat IGg2a-PE isotype control antibody; (c) FoxP3$^+$ (PE) $CD4^+$-$CD25^+$ cells ($CD4^+$-$CD25^+$-FoxP3$^+$ T regulatory cells [Tregs]) stained with use of anti-human FoxP3 antibody. (d) Percentage of $CD4^+$-$CD25^+$-FoxP3$^+$ Tregs in the PBMCs of CRS subjects and healthy controls. (e) Percentage of $CD4^+$-$CD25^+$-FoxP3$^+$ Tregs in the PBMCs of CRS without nasal polyposis (CRSsNP) and CRS with nasal polyposis (CRSwNP) subjects. The dark bars represent the medians in panels d and e.*

respectively; Fig. 2, *a* and *b*, respectively). Because the number of lymphocytes and $CD3^+$ cells was significantly higher in the CRSwNP group when compared with CRSsNP ($p < 0.05$; Table 2), the proportion of Tregs as a fraction of total T cells ($CD3^+$) was calculated. The proportion of $CD25^+$-FoxP3$^+$ (Fig. 2 *c*) to $CD3^+$ cells was significantly higher and the proportion of and $CD4^+$-FoxP3$^+$ Tregs (Fig. 2 *d*) to CD3+ cells was marginally higher in CRSwNP subjects when compared with CRSsNP (median = 0.30 range [0.22, 0.43] and 0.15 [0.13, 0.22]/10 HPFs; $p = 0.038$; and median = 0.465 [0.24, 0.60] and 0.262 [0.16, 0.34]/10 HPFs; $p = 0.05$ for $CD25^+$-FoxP3$^+$/$CD3^+$ cells and $CD4^+$-FoxP3$^+$/$CD3^+$ cells, respectively).

Treg Counts in PB by Flow Cytometry

Our results from CRS subjects pointed toward differences in the Treg numbers in the affected sinus tissue of CRSsNP and CRSwNP subjects. To determine whether there were differences in the systemic Treg counts in these subjects, we recruited another group of CRS subjects and normal controls. PBMCs were isolated from these subjects and the percentage of Tregs was determined by flow cytometry. $CD4^+$-$CD25^+$ cells were selected from the PBMCs (Fig. 3 *a*). Of these cells, the triple-positive ($CD4^+$-$CD25^+$-FoxP3$^+$) cells were selected (Figs. 3, *b* and *c*). There were no FoxP3$^+$ $CD4^+$-$CD25^+$ cells in the rat IgG2a-PE isotype control-stained cells (Fig. 3 *b*), whereas FoxP3$^+$ $CD4^+$-$CD25^+$ cells were observed when anti-human FoxP3 antibody was used (Fig. 3 *c*). The percentage of $CD4^+$-$CD25^+$-FoxP3$^+$ cells was lower in the PB of subjects with CRS (median = 0.25% [0.11–0.38]) than in healthy controls (median = 0.59% [0.27, 0.78]; $p = 0.002$; Fig. 3 *d*). However, there were no significant differences in PB Tregs between subjects with CRSwNP and those with CRSsNP (median = 0.28% [0.16, 0.38] and 0.14% [0.03, 0.31], respectively; $p = 0.31$; Fig. 3 *e*).

Cytokine Profile of the PBMCs from CRS Subjects versus Normal Controls

Because there were no differences in the Treg counts in the PB from CRSwNP and CRSsNP subjects, we combined them into one group CRS (affected) for the analysis of cytokine levels from PBMCs as an indicator of T-cell polarization and Treg function. Spontaneous release of cytokines from PBMCs (normal controls and CRS subjects) was determined for IL-10, TGF-β1, IL-6, TNF-α, IL-4, IFN-γ, and IL-17A. IL-17A was mostly undetectable in the unstimulated cells from our study subjects (data not shown). Lower levels of IL-10 and TGF-β1 and higher levels of IL-6 were observed in the CRS subjects

Table 3 **Spontaneous and PHA-induced cytokine release from PBMCs from CRS subjects (n =16) and controls (n =16)**

Cytokine	Spontaneous Release (pg/mL)			PHA-Induced Release (pg/mL)		
	Controls	CRS Subjects	p Value	Controls	CRS Subjects	p Value
IL-10	4.72 (1.76, 9.19)	1.98 (1.60, 4.06)	0.049	51 (2.13, 112.92)	2.52 (0.95, 20.56)	0.048
TGF-β1	300.04 (261.7, 359.09)	263.18 (240.18, 285.16)	0.020	8.76 (−13.66, 40.54)	−15.60 (−23.89, 7.47)	0.033
IL-6	17.17 (12.73, 25.74)	29.74 (19.24, 54.77)	0.011	1366.58 (815.50, 3055.40)	2629.12 (1496.12, 5271.89)	0.040
TNF-α	16.90 (7.21, 26.62)	12.85 (5.89, 16.99)	0.462	308.26 (190.35, 816.98)	774.36 (334.35, 1611.15)	0.048
IL-4	0.62 (0.27, 1.38)	0.85 (0.39, 1.27)	0.872	10.01 (3.23, 14.98)	3.32 (0.49, 37.18)	0.260
IFN-γ	0.39 (0.24, 0.71)	0.24 (0.18, 0.33)	0.118	33.83 (1.29, 197.30)	3.81 (0.47, 27.21)	0.126

The PBMCs were in culture for 24 hr with and without PHA before the measurement of the cytokines. Data are represented as median (interquartile range). All cytokine levels are in picograms per milliliter.
CRS = chronic rhinosinusitis; IFN = interferon; PHA = phytohemagglutinin; PBMCs = peripheral blood mononuclear cells; TGF = transforming growth factor.

when compared with healthy controls (Table 3). There was no significant difference in the spontaneous release of TNF-α, IL-4, and INF-γ in the two groups ($p > 0.05$; Table 3).

PHA-induced cytokine release was calculated by subtraction of the spontaneous release levels from levels obtained after stimulation with either PHA (Table 3). Again, we could detect IL-17A from the PHA-induced cells from four CRS subjects and seven healthy controls (data not shown). Consistent with our earlier observation of reduced Treg counts in the CRS subjects when compared with healthy controls, we found lower IL-10 and TGF-β1 and higher levels of IL-6 and TNF-α in the CRS subjects (Table 3). The disease status had no significant effect on the release of IL-4 and IFN-γ in the PHA-stimulated PBMCs from these subjects ($p > 0.05$; Table 3).

Tregs in CRS and Asthma

Reduced Treg numbers have previously been implicated in asthma.[12,15] Because, seven and six CRS subjects in both our groups had asthma as a confounding disease, we analyzed the Treg populations in these subjects stratified based on asthma. There were no significant differences in the number of $CD25^+$-$FoxP3^+$ (Fig. 4 *a*) and $CD4^+$-$FoxP3^+$ (Fig. 4 *b*) Tregs in the sinonasal tissues of CRS subjects stratified based on presence of asthma ($p > 0.05$). Similarly, we observed no influence of asthma as a disease on the PB Tregs in our CRS subjects (Fig. 4 *c*; $p > 0.05$).

Tregs, Cytokine Release, and Corticosteroid Usage

Intranasal and systemic corticosteroids have been considered a mainstay in the treatment of CRS.[23,24] Of our CRS subjects in both groups, 8/16 subjects had received systemic and/or intranasal corticosteroids within a month of surgery. When we stratified our CRS subjects (used for immunohistochemical analysis) based on corticosteroid administration before surgery, we observed a slight increase in the numbers of tissue $CD25^+$-$FoxP3^+$ (Fig. 5 *a*) and $CD4^+$-$FoxP3^+$-cells (Fig. 5 *b*) in CRS subjects administered corticosteroids before surgery, compared with those without corticosteroid treatment; however, the difference was not statistically significant ($p > 0.05$). Flow cytometric analysis of Tregs from PBMCs did show a marginal increase in the percentage of $CD4^+$-$CD25^+$-$FoxP3^+$ cells from CRS subjects who received steroids before surgery (median = 0.285% [0.23–0.38] compared with those who did not (median = 0.11% [0.04, 0.30]; $p = 0.05$; Fig. 5 *c*). However, no significant differences were observed in the spontaneous and PHA-simulated release of the cytokines IL10, TGF-β1, IL-6, TNF-α, IFN-γ, and IL-4 when CRS subjects were stratified based on corticosteroid use before surgery (Table 4).

DISCUSSION

In this study, we investigated the patterns of cellular inflammation in sinus tissue and PB from two independent groups of CRS subjects. Our histological analysis showed an increased accumulation of lymphocytes ($p < 0.05$) and eosinophils in CRSwNP subjects when compared with CRSsNP of the CRS subjects. This was further supported by immunohistochemical analysis, which showed an increased accumulation of $CD3^+$ ($p < 0.01$), $CD4^+$, and $CD8^+$ cells in the CRSwNP subjects. This is in accordance with earlier studies[6,18,20] and indicates a higher degree of inflammatory cell infiltration in CRSwNP.

Because Treg insufficiency and the dysregulation of the T_H1/T_H2 have been implicated in several autoimmune and allergic diseases, we analyzed the Treg population from these groups. Using immunohistochemistry to measure $CD4^+$-$FoxP3^+$ and $CD25^+$-$FoxP3^+$ cells (Tregs), we showed that subjects with CRSsNP ($n = 8$) had a significantly lower percentage of Tregs in the affected sinus tissue than did subjects with CRSwNP ($n = 8$). We failed to detect any $CD25^+$-$FoxP3^+$ or $CD4^+$-$FoxP3^+$ cells from sphenoid sinus tissues taken from the transsphenoidal controls recruited for our study. We next investigated PB as an accessible surrogate site for the study of inflammation in CRS. Our study indicates that there are fewer $CD4^+$-$CD25^+$-$FoxP3^+$ Tregs in the PB of CRS subjects when compared with controls. However, it is difficult to comment on the differences in the PB Treg counts between the CRSsNP subjects and CRSwNP subjects because of the small number of subjects used in our study ($n = 5$ for CRSsNP). Our results are in contrast to earlier studies performed on European and Chinese populations, which showed a decreased expression of FoxP3 in tissues of subjects with CRSwNP compared with subjects who had CRSsNP or controls.[3,16–20,22] These differences may be a consequence of several factors, including experimental differences (using CD4 and/or CD25 as additional markers to FoxP3 in contrast to FoxP3 alone; FoxP3 was initially considered a marker specific for the natural Tregs; however, recent studies show that FoxP3 can also be induced in iTregs peripherally)[25,26], inclusion or exclusion of subjects who underwent previous sinus surgery or were on sinonasal medications, and assessment method (mRNA versus protein by use of immunohistochemistry). We would also like to point out that 50% of the CRSwNP subjects in both of our groups were administered systemic and/or topical corticosteroids before surgery and 50% un-

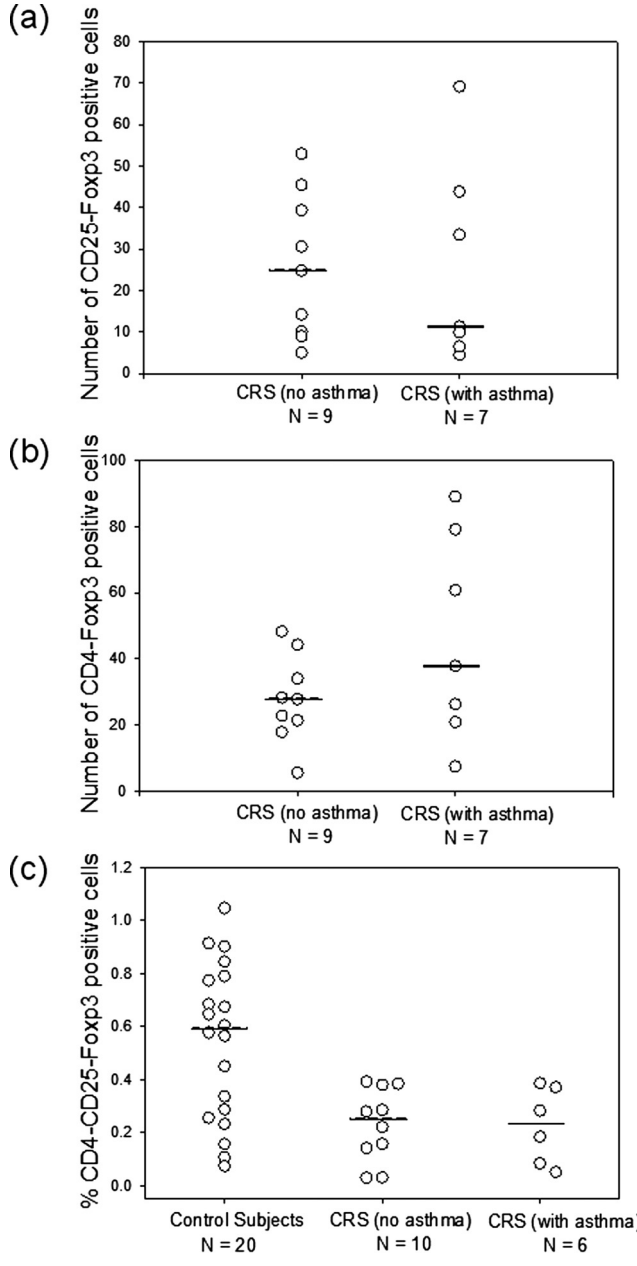

Figure 4. Distribution of T regulatory cells (Tregs) in chronic rhinosinusitis (CRS) subjects with respect to asthma. Immunohistochemical analysis of (a) $CD25^+$-forkhead box P3 ($FoxP3^+$) cells, (b) $CD4^+$-$FoxP3^+$ cells, and (c) flow cytometric analysis of $CD4^+$-$CD25^+$-$FoxP3^+$ cells (%). The dark bars represent the medians.

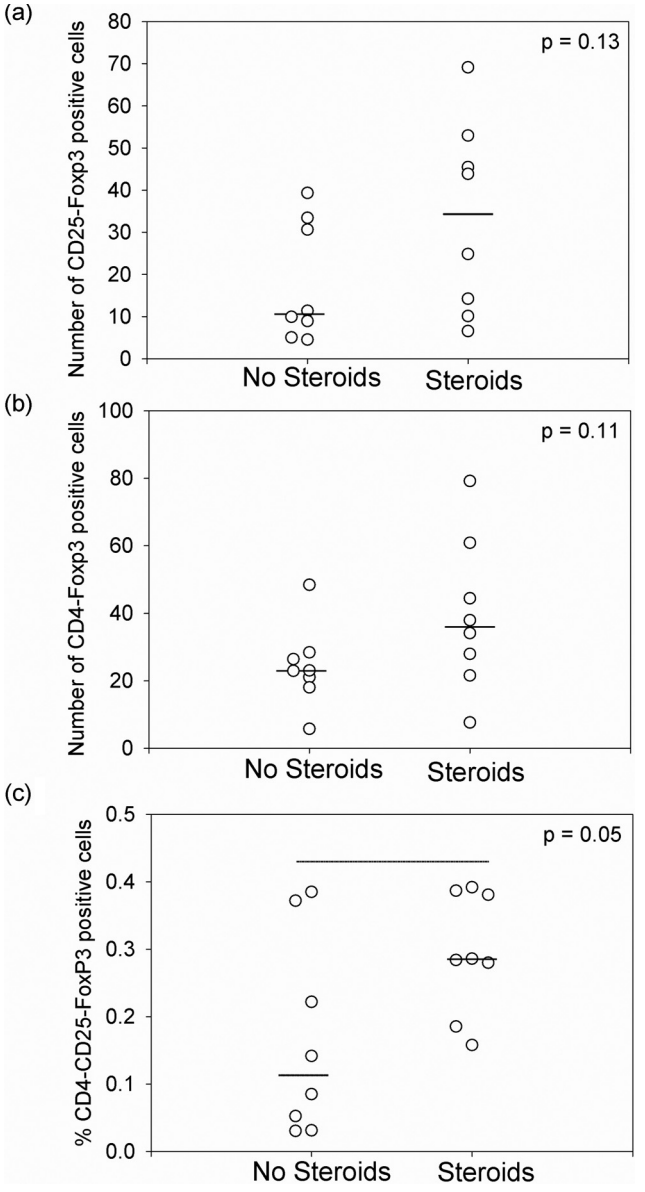

Figure 5. Distribution of T regulatory cells (Tregs) in chronic rhinosinusitis (CRS) subjects with respect to steroid usage (systemic and/or intranasal steroids) before surgery. Immunohistochemical analysis of (a) $CD25^+$- forkhead box P3 ($FoxP3^+$) cells, (b) $CD4^+$-$FoxP3^+$ cells, (c) flow cytometric analysis of $CD4^+$-$CD25^+$-$FoxP3^+$ cells (%). The dark bars represent the medians.

derwent revision surgery. Both of these clinical details indicate increased severity of disease, thereby possibly explaining the differences in findings. Another major difference between our study and prior publications is that prior studies had all subjects off medications for a month before surgery.

Tregs play important roles in maintaining immune homeostasis,[9,26] and impairment in the number and/or function of these cells could result in defective suppressor function, accounting for the persistent inflammation in CRS subjects. Our data do not distinguish between subtypes of Tregs (nTregs versus iTregs), and they do not incorporate newer markers for these cells,[11] something that will remain for future work. Similar studies investigating T-cell phenotypes in Chinese subjects with or without nasal polyps confirm a down-regulation of FoxP3 mRNA expression and significantly decreased numbers of FoxP3-positive Tregs in nasal polyp tissue.[6,19,20] Another study of Chinese subjects showed a similar decrease in the $FoxP3^+$-$CD3^+$ cells from the tissue samples from CRSwNP subjects compared with control subjects.[18] However, this study failed to detect a difference in PB $CD4^+CD25^{high}CD127^{low}$ cells (defined as Tregs) in CRSwNP subjects compared with control subjects. The investigators also found an increased number of PB $CD4^+CD25^{high}CD127^{low}$ cells in the CRSsNP subjects when compared with controls, which indicated that the chronic and persistent inflammatory state associated with CRSsNP may affect the number of peripheral Tregs. The discrepancies in the results of our study compared with some, but not all, prior work may be explained based on the clinical characteristics of the subjects. Our

Table 4 **Spontaneous and PHA-induced release of cytokines from PBMCs from CRS subjects stratified by steroid use before surgery**

Cytokine	Spontaneous Release (pg/mL)			PHA-Induced Release (pg/mL)		
	No Steroids ($n = 8$)	Steroids ($n = 8$)	p Value	No Steroids ($n = 8$)	Steroids ($n = 8$)	p Value
IL-10	2.51 (1.6, 5.64)	1.98 (1.6, 3.15)	0.878	0.95 (0.04, 15.85)	3.39 (1.75, 34.43)	0.195
TGF-β1	247.68 (237, 272.41)	277.37 (253.33, 301.92)	0.161	−11.7 (−26.71, 1.86)	−15.60 (−22.27, 15.8)	0.798
IL-6	32.32 (25.55, 48.76)	25.28 (16.19, 81.15)	0.798	2223.55 (1496.12, 5717.66)	3171.195 (1570.68, 5271.89)	0.798
TNF-α	14.72 (8.86, 23.16)	12.14 (4.74, 14.21)	0.328	1222.12 (526.52, 1836.4)	391.11 (159.74, 1176.55)	0.234
IL-4	0.95 (0.64, 1.86)	0.44 (0.38, 1.02)	0.383	12.78 (1.25, 44.97)	2.08 (0.34, 3.46)	0.383
IFN-γ	0.32 (0.19, 0.33)	0.21 (0.17, 0.25)	0.24	0.47 (0.21, 42.61)	5.79 (3.39, 11.8)	0.24

The PBMCs were in culture for 24 hr with and without PHA before the measurement of the cytokines. Data are represented as median (interquartile range). All cytokine levels are in picograms per milliliter.

CRS = chronic rhinosinusitis; IFN = interferon; PHA = phytohemagglutinin; PBMCs = peripheral blood mononuclear cells; TGF = transforming growth factor.

study included subjects who had severe CRS (mostly white and black Americans from an urban, academic center) who failed to respond to adequate trials of medical therapy. The study by Kim *et al.* excluded all subjects who had undergone previous sinus surgery, and all subjects stopped oral and topical application of corticosteroids for at least 1 month before surgery, and they did not take any other sinonasal medications.[18] Our study included subjects with severe disease and withdrawal of medications before the study for this lengthy period was not feasible clinically in a U.S. study population. In addition, ethnic differences could also account for the observed differences because previous studies have indicated differences in immunopathological characteristics of NPs between Asian and white patients (*e.g.*, eosinophil versus neutrophil predominance).[6]

To further our observations about the decreased percentages of the Tregs in the PB of CRS subjects, we measured levels of T-cell cytokines from the PHA-stimulated PBMCs as an indicator of T-cell polarization and of Treg function. The fact that FoxP3 and cytokines including TGF-β1 and IL-10 can originate in cells other than Tregs leads to difficulty in interpreting our results, although this marker has been considered previously as specific for Tregs.[27] We found decreased IL-10 and TGF-β1 production and increased levels of IL-6 and TNF-α from the CRS subjects. These results are consistent with our findings of decreased Treg numbers. However, we failed to find any differences in the levels of IFN-γ (a characteristic T_H1 cytokine) and IL-4 (a characteristic T_H2 cytokine) in our subjects. In our study, we used PBMCs for the stimulation studies, and we concede that further functional studies and quantitative real-time polymerase chain reaction experiments with these purified $CD4^+$-$CD25^+$ cell populations from PBMCs in the future would provide greater insights into the function of these cells in CRS.

Earlier studies showed a similar decreased expression of TGF-β1 and IL-10 from the sinonasal tissue of CRSwNP subjects when compared with controls.[16,18,20] Most previous studies conducted on affected tissue samples showed increased TGF-β1 and IL-10 levels from CRSsNP when compared with CRSwNP.[3,16] TGF-β1 is a pleiotropic and multifunctional growth factor that is a key regulator of extracellular matrix production and remodeling.[3,16] The low levels of TGF-β1 from the PBMCs of CRS subjects may reflect a lack of tissue repair, indicated by loose connective tissue and edema formation in severely inflamed tissue. Additional studies at the tissue level will be required that will clarify this discrepancy.

Elevation in IL-6 levels has also been observed in the sinonasal tissue from CRSwNP subjects compared with controls and CRSsNP.[28] IL-6 plays important roles in regulating Treg and T_H17 differentiation by inhibiting FoxP3, which, in turn, leads to diminished inhibition of the retinoic acid-related orphan receptor RORγT and consequently expansion of T_H17 cells.[28] We failed to detect significant amounts of IL-17A, a key cytokine produced by the T_H17 cells in all of our samples, and we did not count T_H17 cells. Nevertheless, the elevation of IL-6 may indicate another mechanism whereby Treg function is inhibited in CRS *via* a T_H17 mechanism.

Corticosteroids (oral and/or topical) are the recommended treatment for both CRSwNP and CRSsNP. Our study included CRS subjects who failed to respond to these and other conservative medical therapies and therefore required surgery. Several required revision surgery as well, indicating severe disease. Using flow cytometry, we found a marginally reduced percentage of the $CD4^+$-$CD25^+$-$FoxP3^+$ Tregs in the PB from CRS subjects who were not administered corticosteroids before surgery, compared with those who did take these medications. However, using immunohistochemistry, we found no significant difference in the number of Tregs in the tissues subjects administered corticosteroids before surgery compared with the rest. An earlier study by Li *et al.*[22] showed that intranasal steroids increased the number and activity of Tregs (increased FoxP3 and IL-10) in subjects with CRSwNP. Similar results have been obtained in other diseases, including asthma and lupus.[21,29,30] Therefore, the increased number and activity of the Tregs after corticosteroid treatment may contribute to the anti-inflammatory effects. A controlled trial would be required in the future to address this issue.

In conclusion, our results indicate that subjects with CRS exhibit a decreased percentage of Tregs in their PB when compared with healthy normal controls, a novel finding. Additionally, a higher proportion of Tregs was observed in the nasal polyps when compared with sinonasal mucosa of CRSsNP and no Tregs were detected in the sphenoid sinuses taken from controls. Our study thus provides support for the possibility that Tregs play a role in the dysregulation of immune responses that characterize CRS.

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