

Imbalance of Follicular Helper and Follicular Regulatory T Cells in Chronic Rhinosinusitis with Nasal Polyps

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ABSTRACT

Objective: Data regarding the imbalance in follicular helper T (T_{fh}) and follicular regulatory T (T_{fr}) cell responses in patients having chronic rhinosinusitis with nasal polyps (CRSwNP) is so far limited. Thus, we aimed to assess the changes in circulating T_{fh} and T_{fr} in CRSwNP patients.

Methods: This case-control study included 21 patients having CRSwNP and 20 age and sex-matched healthy blood donors as a control group. Lund-Mackay staging system was used for radiologic scoring of chronic rhinosinusitis. Two milliliters of peripheral blood samples were collected from all participants into EDTA-containing vacutainer tubes to assess the levels of T_{fh} and T_{fr} cells using flow cytometry.

Results: Patients having CRSwNP did not show significant differences in the percentages of CD4⁺ T cells and total CD4⁺CXCR5⁺ T cells from healthy controls. Meanwhile, levels of both activated circulating T_{fh} and T_{fr} showed a marked rise in patients than controls. In addition, a positive correlation was observed between the levels of both activated T_{fh} and T_{fr} cells.

Conclusion: An imbalance in circulating T_{fh}/T_{fr} levels was detected in patients having CRSwNP. A significant rise in the levels of T_{fh} and T_{fr} was detected in patients proposing a possible role of this imbalance in disease pathogenesis.

Keywords: Follicular helper T cells, Follicular regulatory T cells, Chronic rhinosinusitis, Nasal polyps.

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INTRODUCTION

Chronic Rhinosinusitis (CRS) is a complex heterogeneous disease distinguished by sinonasal chronic inflammation. Endoscopic examination and imaging procedures of the nasal cavity classify CRS phenotypically into Chronic Rhinosinusitis with Nasal Polyps (CRSwNP) and Chronic Rhinosinusitis without Nasal Polyps (CRSsNP). Nasal polyps appear as visible sinonasal inflammatory masses that gradually obstruct nasal airflow and sinus drainage¹.

The diversity of T cell subtypes has been described in chronic sinusitis and nasal polyposis, and their biological function has been identified. CD4⁺ T cells may differentiate into one of several lineages, including T helper cells (Th) 1, Th2, Th17, Th9, and follicular helper T (Tfh) cells^{2,3}. The equilibrium between these T-cell subsets is essential for the mucosal physiological immune response, which is dysregulated by persistent inflammation⁴.

A distinctive feature of CRSwNP is the prevalence of eosinophils and Th2 cytokines in the sinus mucosa⁵. The factors implicated in inducing this mucosal eosinophilia remain largely unknown. Nevertheless, elevated IgE production in polyp tissues mediates mucosal mast cell activation and subsequently causes eosinophilic inflammation⁶. Even though Th2 cells have long been regarded as the primary cell type inducing B cell class-switching to IgE, more recent studies showed that this process mainly depends on Tfh cells⁷.

Tfh cells are a specialized subpopulation of CD4⁺ T cells that help B cells, promoting Germinal Central (GC) responses in B cell follicles of secondary lymphoid organs^{8,9}. Besides, Tfh cells have also been recognized in peripheral blood. They share phenotypic and functional similarities with their bona fide Tfh cells in secondary lymphoid organs and predominantly have a memory phenotype¹⁰. Three subsets of circulating Tfh have been identified; Tfh1, Tfh2, and Tfh17 cells, which secrete cytokines produced by the conventional helper-T cell subsets¹¹. Between Tfh subsets, the Tfh2 cells are considered the primary producer of interleukin (IL)-4, promoting the B cell class-switching to IgE. Increased levels of circulating or tissue Tfh2 cells have been described in some inflammatory airway disorders, such as allergic rhinitis, nasal polyposis, and asthma^{12,13}.

Follicular regulatory T cells (Tfr) are a unique population of regulatory T cells (Tregs) that suppress GC responses by inhibiting Tfh cell activation^{14,15}. Phenotypically, Tfr cells co-express the Tfh and Treg cell's characteristic markers¹⁶. Data regarding the imbalance in Tfh/Tfr cell responses in patients with CRSwNP is limited. Thus, we aimed to assess the changes in the levels of circulating Tfh and Tfr in CRSwNP patients.

METHODS

The current case-control study included 21 patients having CRSwNP enrolled from the Department of Otorhinolaryngology & Head and Neck Surgery, Assiut

University. Twenty age and sex-matched healthy blood donors were also recruited as a control group. The Ethics Committee of the Faculty of Medicine, Assiut University, appraised and accepted the study protocol (IRB. No.17300110) following the Helsinki Declaration. Written informed consent was collected from all patients before participation.

For radiologic evaluation of chronic rhinosinusitis, a non-contrast computed tomography was performed using standard bone and soft-tissue window settings, coronal, axial, and sagittal cross-sections of 3 mm thickness. Lund-Mackay staging system was used for chronic rhinosinusitis scoring¹⁷. Each sinus was assigned a value of 0 to 2; 0 for totally patent, 1 for partially opacified, or 2 for wholly opacified. The osteomeatal complex (OMC) was given a score of either 0 or 2 (not occluded or occluded, respectively). The maximum score for each side is hence 12, with a total score up to 24 (all sinuses are completely opacified). Lund Mackay scores of 4 and above broadly support the clinical diagnosis of CRS¹⁸.

Flow Cytometric Assessment of Follicular Helper and Follicular Regulatory T Cells: Preoperatively, two milliliters of peripheral blood samples were obtained from all participants into EDTA containing vacutainer tubes to assess the levels of Tfh and Tfr cells using flow cytometry. First, 100 μ l of whole blood samples were incubated for 20 minutes with 10 μ l of Fluoroisothiocyanate (FITC)-conjugated anti-CXCR5 (R&D systems, USA), Phycoerythrin (PE) conjugated anti-ICOS (R&D systems, USA), and Peridinium-Chlorophyll-Protein (PerCP)-Cy5.5-conjugated anti-CD4 (R&D systems, USA), monoclonal antibodies. Next, red blood cells were lysed, then samples were washed with Phosphate-Buffered Saline (PBS). Fixation solution was added with 10-minute incubation. After PBS rewash, 10 μ l of Allophycocyanin (APC)-conjugated anti-Foxp3 (eBioscience, USA) were added together with the permeabilizing solution and incubated for 20 minutes. Anti-human IgG isotype-matched negative control was used for each sample to evaluate the non-specific staining. Nearly 50,000 events were collected and analyzed by the FACSCanto flow cytometer using FACSDiva software (Becton Dickinson Biosciences, USA), South Egypt Cancer Institute, Assiut University. Figure 1 illustrates the gating strategy used for the study. The total Tfh cells were defined as CD4⁺CXCR5⁺ T lymphocytes, activated Tfh cells as CD4⁺CXCR5⁺ICOS⁺Foxp3⁻, and Tfr cells as CD4⁺CXCR5⁺ICOS⁺Foxp3⁺.

Statistical Analysis: IBM Statistical Package for the Social Sciences (IBM SPSS statistics, version 20) was used for the statistical analysis. Categorical data were presented as numbers and percentages, while quantitative data were expressed as the mean and standard error. The Mann-Whitney U test was applied to compare groups, and Spearman rank-order correlation was employed to evaluate the association between different variables. P-value is significant if <0.05.

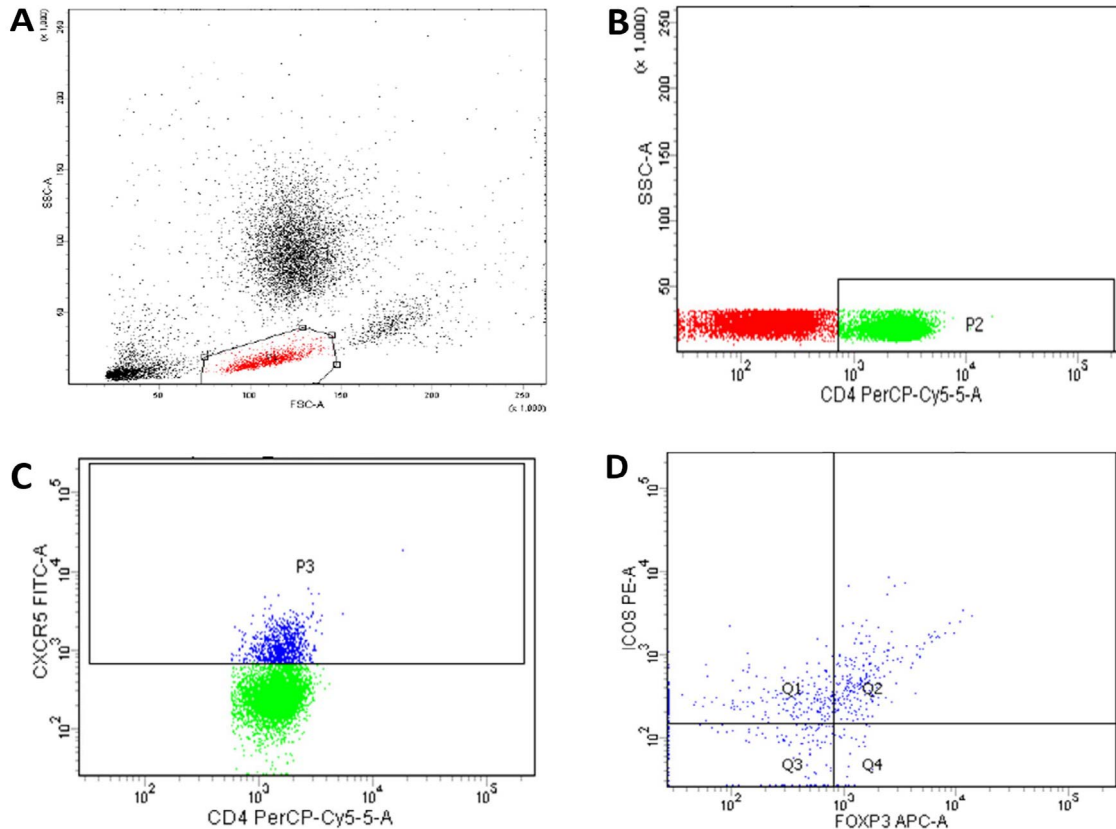


Figure 1: Flow cytometry gating strategy of follicular helper T cells (Tfh) and follicular regulatory T cells (Tfr):
 A: P1 was drawn to select lymphocytes based on their forward and side scatter properties.
 B, C: CD4⁺ T cells were gated by drawing (P2) to analyze CD4⁺CXCR5⁺ Tfh cells (P3) further. D: The percentages of activated Tfh cells (CD4⁺CXCR5⁺ICOS⁺Foxp3⁻) and Tfr cells (CD4⁺CXCR5⁺ICOS⁺Foxp3⁺) were assessed within the CD4⁺CXCR5⁺ Tfh cell population.

Table 1: Patients' Laboratory and radiologic features.

Variables	Patients (n=21)	Control (n=20)
Age (years)	32.6 ± 4	36 ± 2
Sex Males	14 (66.7%)	13 (65%)
Females	7 (33.3%)	7 (35%)
Hematological findings		
TLC (X10 ⁶ /L)	7 ± 0.5	7.5 ± 0.4
Neutrophil percent/ count (X10 ⁶ /L)	53.3 ± 3/3.9 ± 0.5	50.6 ± 2/3.4 ± 0.2
Eosinophil percent/count (X10 ⁶ /L)	4 ± 0.8/0.3 ± 0.05	2.7 ± 0.4/0.2 ± 0.04
Total radiologic score		
8	2 (9.5%)	
13	1 (4.8%)	
14	2 (9.5%)	
15	1 (4.8%)	
16	1 (4.8%)	
18	4 (19%)	NA
19	1 (4.8%)	
21	1 (4.8%)	
22	2 (9.5%)	
24	6 (28.6%)	

TLC Total Leukocyte Count, NA not applicable

RESULTS

Patients' Laboratory and Radiologic Features: The mean age of the enrolled CRSwNP patients was 32.6±4. The average total leukocyte count was 7±0.5X10⁶/L, Neutrophil count was 3.9±0.5X10⁶/L (53.3±3%), and eosinophil count was 40.8X10⁶/L (0.3±0.05%). Total radiologic scores

varied from 8 to 24, and about half of the patients had a score greater than 18. Laboratory and radiologic features of patients are demonstrated in the Table 1.

Comparison of the Frequencies of Circulating Follicular Helper and Follicular Regulatory T Cells in Patients and Healthy Controls: Patients having CRSwNP

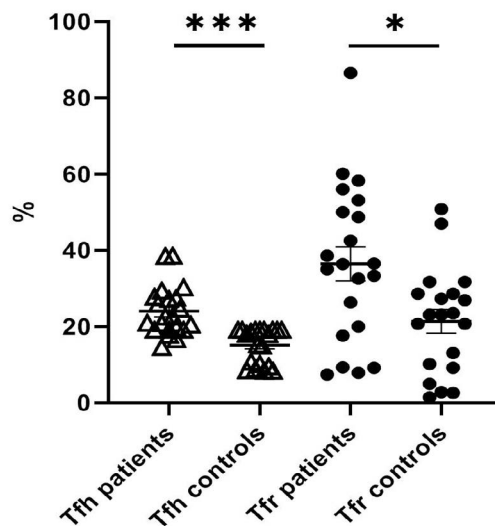


Figure 2: Levels of circulating activated follicular helper T cells (Tfh) and follicular regulatory T cells (Tfr) in patients having CRSwNP and healthy controls (p-value: * significant <0.05 & *** highly significant <0.0001).

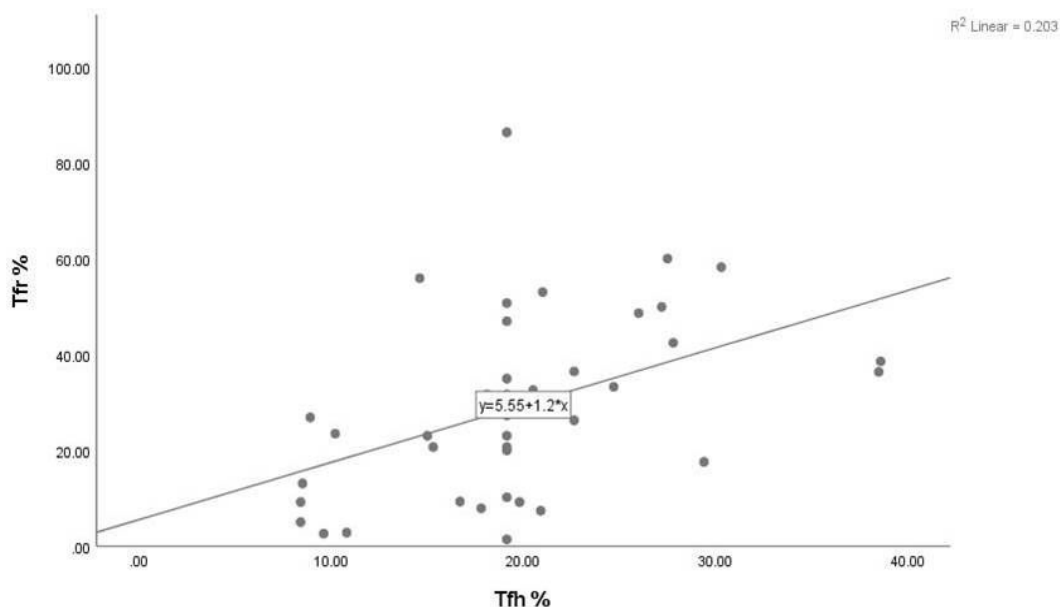


Figure 3: Correlation between the levels of circulating activated follicular helper T cells (Tfh) and follicular regulatory T cells (Tfr).

didn't show significant differences in the percentages of CD4⁺ T cells and total CD4⁺CXCR5⁺ Tfh cells from healthy controls (34.6±2 vs. 38.7±1, p=0.2 and 8.9±1 vs. 9.3±1, p=0.5, respectively). Meanwhile, levels of both activated Tfh and Tfr showed a marked rise in patients than controls (24±1 vs. 15±1, p<0.0001 and 36.4±4 vs. 21.4±3, p=0.01), as illustrated in Figure 2. In addition, a positive correlation was observed between the levels of both activated Tfh and Tfr cells (r=0.4, p<0.0001), Figure 3. Tfh and Tfr cells didn't show any significant correlations with either eosinophil count or CT score.

DISCUSSION

Data regarding the imbalance in Tfh and Tfr cell responses in patients having CRSwNP is so far limited. Thus, we

aimed to assess the changes in circulating Tfh and Tfr in CRSwNP patients. The CRSwNP group didn't show significant differences in the percentages of CD4⁺ T cells and total CD4⁺CXCR5⁺ Tfh cells from healthy controls. Meanwhile, levels of both activated Tfh and Tfr showed a marked rise in patients than controls. In addition, a positive correlation was observed between the levels of both activated Tfh and Tfr cells.

CRSwNP has been considered a Th2-mediated inflammation, characterized by high IL-4, IL-5, and eosinophilic inflammation. In comparison, CRSsNP is predominantly Th1 inflammation, with increased expression of interferon-gamma (IFN-γ) and Transforming Growth Factor-beta (TGF-β). A previous study reported that Th2 cells within the sinonasal mucosa of CRS patients were only detected in patients with nasal

polyposis and were higher in patients having asthma and/or *Staphylococcus aureus* enterotoxin specific IgE¹⁹.

The central role of type-2 cytokines in the pathogenesis of CRSwNP is emphasized by the excellent clinical efficacy of dupilumab, a fully humanized IgG4 monoclonal antibody against the IL-4 receptor α (IL-4R α) subunit²⁰. Yet, the relevant concepts have gradually become more complex, since authors described the probable involvement of other T cell subsets, rather than Th2, as Th17 and Th22 in the pathogenesis of CRSwNP²¹.

Emerging evidence suggests that IgE class switch recombination largely relies on Tfh cells^{7, 22}. In addition, patients with allergic diseases have shown an upregulation of Tfh cell activity, including a Tfh phenotype with skewed differentiation toward Tfh2 cells and IL-13-producing Tfh13 cells²³.

An earlier study²⁴, showed that a higher frequency of B-cell lymphoma-6 (Bcl-6)⁺CD4⁺ Tfh cells in polyp tissues was accompanied by B cell accumulation, activation and IgA and IgE production in polyp tissue. They also found that levels Bcl-6⁺CD4⁺ cells and immunoglobulins were significantly higher in CRSwNP patients with GC-like structure than those without GC-like structure. Additionally, the frequencies of Bcl-6⁺CD4⁺ cells correlated with the tissue eosinophilia, asthma comorbidity and recurrence after surgery, suggesting that Tfh cells may present a potential biomarker of disease severity.

In another study²⁵, a significant increase was observed in the levels of total Tfh cells and Tfh cell subsets secreting IL-21, IFN- γ , and IL-17 in both eosinophilic and non-eosinophilic nasal polyp tissues than in normal nasal tissues. However, Tfh cells secreting IL-4 have only increased in the eosinophilic nasal polyp tissues and strongly correlated with the local IgE levels. Authors deduced that IL-4 and IL-21 were implicated in polyp Tfh cell-induced IgE production. Moreover, Bcl-6⁺ Tfh cells secreting IL-4 were found in ectopic lymphoid tissue in eosinophilic nasal polyps. Tfh cells are also directly correlated with the GC B cells and plasma cells in the nasal tissues. On the contrary, they didn't detect meaningful differences in the frequencies of total Tfh cells, most of the Tfh cell subsets, and B cells in peripheral blood among the studied groups. On the other hand, another study showed no differences in CD4⁺ T cell, activated CD4⁺ T cell, CD8⁺ T cell, Tfh cells, Tregs, B cells, and IgA⁺ B cells between nasal polyp tissue and sinus mucosa²⁶.

IL-21 mRNA expression was upregulated in the CRSwNP group compared to the control group, and Bcl-6 and B-lymphocyte-induced maturation protein-1 were elevated in CRSwNP versus CRSsNP. Furthermore, IL-21 mRNA expression and Tfh cells secreting IL-21 increased significantly in nasal polyp tissue and increased more after stimulation with *S. aureus* enterotoxin B. They concluded that Tfh cells and IL-21 are central in the pathophysiology of CRSwNP²⁷.

Lately, Bergantini and coauthors reported higher levels of total Tfh and Tfh2 and lower Tfr in peripheral blood of patients with nasal polyps than the controls. They also noticed a significant reduction of Tfh and Tfh2 frequencies after six months of Omalizumab therapy, a humanized IgG1k monoclonal antibody against IgE²⁸.

The Tfr cells, in B cell follicles, restrain Tfh-mediated help in B-cell activation, IgM, IgA, IgG, and IgE production, and GC responses that give rise to long-lived plasma cell and affinity-matured memory B cell differentiation²². The increased levels of both circulating Tfh and Tfr observed in our patients, in addition to the positive correlation between the two cells, might be a counter-regulatory mechanism in a trial to control the increased Tfh numbers in those patients. This explanation was in line with that in a previous SLE study²⁹ but needed further investigations.

CONCLUSION

An imbalance in circulating Tfh/Tfr levels was detected in patients having CRSwNP. Levels of both Tfh and Tfr were elevated in patients proposing a possible role of this imbalance in disease pathogenesis.

CONFLICT OF INTEREST

The authors declare having no conflict of interests.

ROLE OF FUNDING

This work was not supported by grants from any funding agency.

DATA AVAILABILITY STATEMENTS

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request after taking permission from our ethical committee.

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