

Increased expression of transcription factor Bcl-6 in chronic rhinosinusitis with nasal polyps

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Abstract B cell activation and excessive immunoglobulin (Ig) production were suggested as the key molecular events of chronic rhinosinusitis with nasal polyp (CRSwNP). However, whether T follicular cells (Tfh cells) were involved in this process has not been documented. In this study, 22 CRSwNP patients and 12 normal controls were enrolled, Bcl-6 (the key transcription factor for Tfh cell differentiation) immunoreactivity was examined by immunohistochemical staining, and the mRNA and protein expression of Bcl-6 and IL-21 was examined using qPCR, ELISA and Western blot, respectively. Moreover, the frequencies of Bcl-6⁺CD4⁺ cells (Tfh cells) in polyp tissues and normal controls were measured by flow cytometry. We found that Bcl-6 mRNA and protein levels, as well as the frequencies of Bcl-6⁺CD4⁺ cells were significantly increased in polyp tissues compared with normal controls. The frequencies of Bcl-6⁺CD4⁺ cells were found to be significantly associated with B cell cluster formation, tissue eosinophilia, asthma comorbidity and polyp recurrence.

These findings thus added a new insight into the molecular mechanisms underlying CRSwNP and raise the possibility that Tfh cells could be a novel therapeutic target for difficult-to-treat CRSwNP.

Keywords Chronic rhinosinusitis · Nasal polyps · T follicular cells · Bcl-6 · B cell · Immunoglobulin

Introduction

Chronic rhinosinusitis with nasal polyp (CRSwNP) is a common clinical syndrome characterized by Th2-skewed mucosal inflammation with marked tissue eosinophilia [1]. Accordingly, IL-5 and eosinophil cationic protein have been implicated in the induction of mucosal eosinophilia in CRSwNP because these cytokines are significantly elevated in polyp tissues compared with sinonasal tissues from patients with chronic rhinosinusitis without nasal polyp (CRSsNP) or healthy subjects [2]. Aside from mucosal eosinophilia, increased accumulation of B cells and excessive immunoglobulin (Ig) production (IgA, IgG and IgE) are also observed in polyp tissues of CRSwNP patients when compared with CRSsNP patients or healthy controls [3, 4], suggesting that local B cell accumulation, maturation and IgA and IgE production may be of great importance to promote mucosal inflammation of CRSwNP patients. However, few data were available to understand the local B cell immunity in polyp tissues.

T follicular helper (Tfh) cells represent the subpopulation of CD4⁺ T cells that provides help for antigen-specific B cells in the germinal center (GC) response [5]. Tfh cells express CXCR5, ICOS, PD-1, IL-21 and other molecules [6–8], and B cell lymphoma 6 (Bcl-6) was recently identified as a master regulator of Tfh differentiation [9]. We

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thus hypothesized that Tfh cells may be involved in the pathophysiology of CRSwNP by promoting B cell maturation and Ig production. To address this issue, we examined the expression of Bcl-6, IL-21 and the frequency of Bcl6⁺CD4⁺ T cells (Tfh cells) in polyp tissues of CRSwNP patients in this study.

Materials and methods

Subjects

This study was approved by the local ethical committee, and written informed consent was obtained from all the subjects. Adult CRSwNP patients ($n = 22$) were recruited from the Department of Otorhinolaryngology, Shandong Provincial Hospital Affiliated to Shandong University. The diagnosis of CRSwNP was made according to the international EPOS consensus [1]. Twelve non-atopic patients undergoing transnasal skull surgery because of anterior skull base tumors were considered as controls. The atopic status of the patients and control subjects was evaluated by allergen skin prick tests, and asthma was diagnosed by a pneumologist based on disease history and airway responsiveness testing. Polyp size was scored on a scale of 1–3: (1) polyps in the middle meatus but not reaching below the inferior border of the middle turbinate; (2) polyps reaching below the inferior border of the middle turbinate but not to the inferior border of the inferior turbinate; and (3) extensive large polyps congesting the inferior meatus. Recurrence of NP was defined as the newly presence of nasal polyps detected by nasal endoscopy after sinus surgery for more than 12 weeks. Tissue eosinophilia (eosinophilic and non-eosinophilic phenotypes) was identified by immunostaining of major basic protein (MBP) as described elsewhere [10]. None of the subjects used oral or nasal corticosteroids 4 weeks before sampling. All subjects were nonsmokers and free from upper respiratory tract infection for 4 weeks preceding the study. The demographic data from the CRSwNP patients and normal

controls enrolled in this study are listed in Table 1. The polyp tissues in CRSwNP patients and sinus tissues in normal controls were sampled during endoscopic inspection. Tissue samples were divided into several portions for RNA and protein isolation, immunohistochemical (IHC) staining and flow cytometric analysis.

IHC staining

Paraffin sections (4 μ m) were prepared and IHC staining was performed using the SABC method. The sections were incubated overnight at 4 °C in the presence of anti-Bcl-6 (1:100; Abcam, Cambridge, MA, USA) and anti-CD20 (1:200; Dako, Glostrup, Denmark). Then, the antibodies were detected via streptavidin–biotin–horseradish peroxidase complex formation. The immunostaining was considered to be positive when brown cells appeared following reaction with the reagent 3', 3'-diaminobenzidine. Replacement of the primary antibodies with isotype-matched IgG was used as a negative control. The sections were examined via light microscopy by two blinded investigators who were aware of the clinical data. The number of positive cells was counted in 10 high-power fields (HPFs, 400 $\times$) and averaged.

qPCR

The mRNA levels were detected through real-time qPCR analysis. In brief, total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Reverse transcription was performed, and the complementary DNA (cDNA) was synthesized from 2 mg of total RNA using an oligo (dT)18 primer and M-MLV reverse transcriptase (Takara, Syuzou, Shiga, Japan) for quantitative PCR. The RNA integrity and the success of the reverse transcription reaction were monitored by PCR amplification of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts. The mRNA expression was determined using ABI PRISM 7500 Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR Premix TaqTM (Takara). The sequences of the primer were as follows: Bcl-6 forward, 5'- GTG AGC CGT GAG CAG TTT-3'; Bcl-6 reverse, 5'- CTC AGG GTT GAT CTC AGG AT -3'; IL-21 forward, 5'-AAG GTC AAG ATC GCC ACA T-3'; IL-21 reverse, 5'-AGG GAC CAA GTC ATT CAC ATA A-3'; GAPDH forward: 5'-GAA GGT GAA GGT CGG AGT-3'; GAPDH reverse 5'- GAA GAT GGT GAT GGG ATT TC-3'. All PCR reactions were performed in duplicate. Melting curve analysis was used to control for amplification specificity. Expression of target gene was expressed as fold increase or decrease relative to the expression of GAPDH. The mean value of the replicates for each sample was calculated and

Table 1 Clinical characteristics of CRSwNP patients and Normal subjects

Characteristic	CRSwNP	Control
Subject no.	22	12
Age (years/mean; range)	35 (22–57)	29 (21–46)
Gender	14 M/8F	8 M/4F
Duration (year; range)	4 (2–7)	0
Asthma in history	7	0
Aspirin intolerance	0	0

M male, F female

expressed as cycle threshold (Ct). The amount of gene expression was then calculated as the difference (ΔCt) between the Ct value of target gene and the Ct value of GAPDH. Fold changes in target gene mRNA were determined as $2^{-\Delta\Delta\text{Ct}}$.

ELISA

For tissue homogenization, 1 mL of PBS containing a protease inhibitor cocktail (Keygentec Inc., Nanjing, China) was added for every 100 mg of tissue and the sample was homogenized for 1 min on ice. After that, the suspension was centrifuged at 4,000 rpm for 20 min at 4 °C, and the supernatants were stored at −80 °C for further use. A commercially available ELISA kit for IL-21 (eBioscience) and IgA (Bethyl, Montgomery, TX, USA) was used according to the manufacturer's protocols. The IgE level was measured using the Unicap System (Phadia, Uppsala, Sweden). The detection limits for the assays were as follows: IL-21, 39.0 pg/mL; IgA, 7.8 ng/mL and IgE, 0.35 KIU/L. For convenient analysis, all values below the detectable limit were considered 0.

Western blot analysis

Total protein was extracted in 100 μL of radioimmuno-precipitation assay buffer lysis buffer per 100 mg nasal tissue at 4 °C for 30 min. The protein concentration was determined by the Bradford method. Samples containing 10 μg of protein were boiled, subjected to SDS-PAGE in 10 % Tris–glycine gels, and transferred electrophoretically to a polyvinylidene fluoride membrane. The membrane was incubated with 5 % fat-free skim milk in Tris-buffered solution containing 0.05 % Tween-20 (1 h, room temperature) and then incubated with mouse anti-human primary antibodies (Bcl-6, 1:500; Abcam) (overnight, 4 °C). The membrane was then incubated with horseradish peroxidase-linked secondary antibody (goat antimouse immunoglobulin G, 1:1,000) and finally processed using the ECL chemiluminescence reaction kit (Cell Signaling, Danvers, MA), followed by exposure on medical film. The relative band densities of Bcl-6 compared with β -actin (Santa Cruz Biotech, Santa Cruz, CA, USA) were quantified with the Bio-Rad Quantity One 1-D Analysis Software (Bio-Rad, Hercules, CA, USA).

Flow cytometric analysis

Nasal tissue samples were cut into pieces of approximately 1–2 mm, digested in incomplete RPMI-1640 of total RNA using an oligo I (2 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) and DNase I (5U/mL, Takara) for 30 min at 37 °C. The digested fragments were filtered through a 100-mm

cell nylon mesh to disperse the cells. Dispersed nasal cells were incubated with anti-CD4 (eBioscience, San Diego, CA, USA) at 4 °C in the dark for 30 min. Following fixation and permeabilization with Permeabilization/Fixation buffer (eBioscience), these cells were stained with conjugated mAbs for Bcl-6 (eBioscience) according to the protocol of the Permeabilization/Fixation Kit. The stained cells were washed twice before analysis using BD FACSCalibur or FACS Aria II cytometer.

Statistical analysis

Data were expressed as the median and interquartile range (IQR), and were analyzed via nonparametric Mann–Whitney *U* test except for additional note. A *P* value of less than 0.05 was considered statistically significant.

Results

Increased Bcl-6 expression in polyp tissues of CRSwNP patients

To investigate whether Bcl-6 expression was involved in the formation of CRSwNP, we firstly evaluated the immunoreactivity of Bcl-6 in CRSwNP patients and normal controls. We found Bcl-6⁺ cells were extensively distributed in the polyp lamina propria of CRSwNP patients (Fig. 1a). The number of Bcl-6⁺ cells in polyp tissues was significantly increased compared to the normal controls ($P < 0.01$, Fig. 1b). Moreover, when correlated the immunoreactivity of Bcl-6 with the NP score, we found the number of Bcl-6⁺ cells in polyp tissues of score 3 was significantly greater than those in polyp tissues of score 2 ($P < 0.01$, Fig. 1c). Next, we examined the mRNA levels of Bcl-6 and IL-21 in polyp tissues of CRSwNP patients. We found both the Bcl-6 and IL-21 mRNA levels were significant upregulated in polyp tissues compared with normal controls ($P < 0.01$, Fig. 2a, b). The level of Bcl-6 mRNA correlated significant with the level of IL-21mRNA in polyp tissues ($P < 0.01$, Fig. 2c). Accordingly, we found the protein levels of Bcl-6 and IL-21 in polyp tissues were significant higher than those in normal controls ($P < 0.01$, Fig. 2d, e).

Increased frequencies of Tfh cells (Bcl-6⁺CD4⁺ cells) in polyp tissues of CRSwNP patients

Since Bcl-6 was considered the key transcription factor of Tfh cells, we examined the frequencies of Tfh cell (Bcl-6⁺CD4⁺ cells) in polyp tissues of CRSwNP patients by flow cytometric analysis. As shown in Fig. 3a, b, we observed the existence of Bcl-6⁺CD4⁺ cells in polyp tissues,

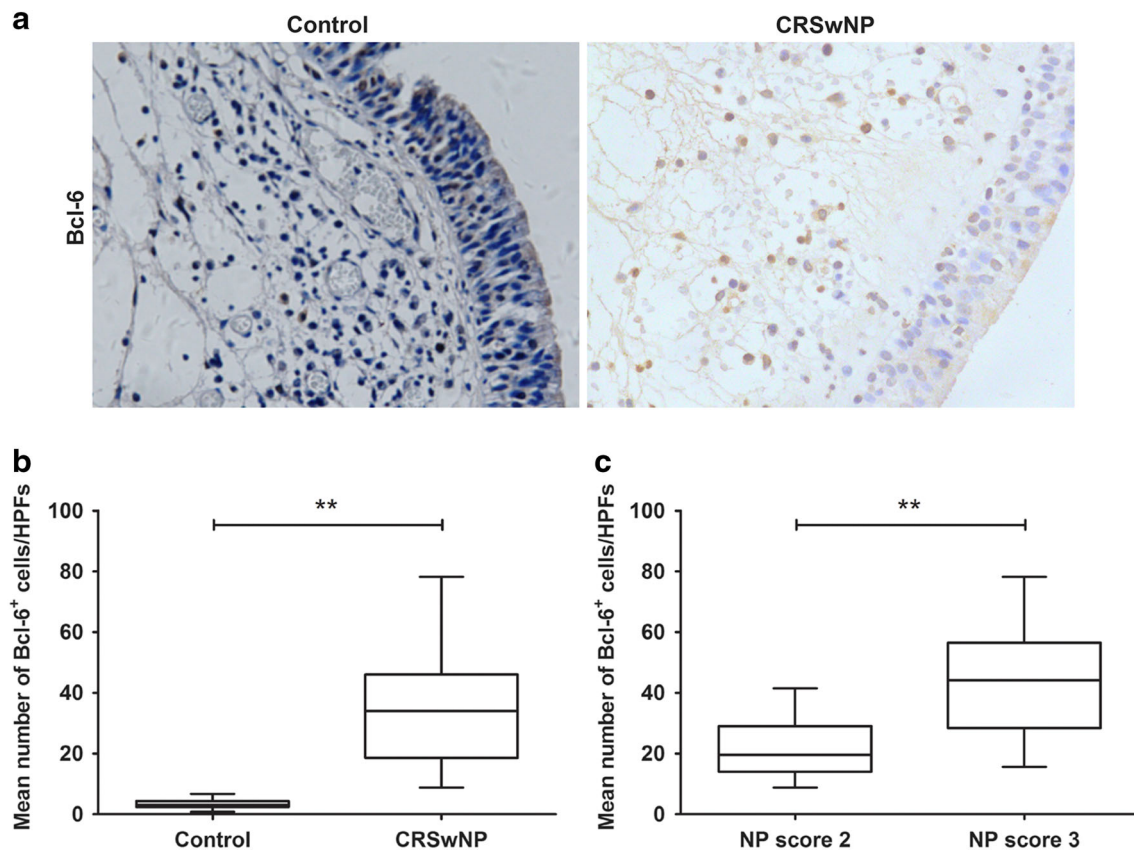


Fig. 1 The immunoreactivity of Bcl-6 in polyp tissues and normal controls. **a** Representative IHC staining of Bcl-6 was shown ($\times 200$); **b** the mean number of Bcl-6⁺ cells in polyp tissues was significant greater than those in normal controls; **c** the mean number of Bcl-6⁺

cells in polyp tissues was significantly associated with disease severity. The data indicated the median (IQR) of CRSwNP patients and normal controls. $**P < 0.01$

and the frequencies of Bcl-6⁺CD4⁺ cells in polyp tissues were significantly higher than those in the normal controls ($P < 0.01$). Next, we examined the distribution of B cells (CD20⁺ cells) in CRSwNP patients and normal controls. As shown in Fig. 4a, we observed that CD20⁺ cells were extensively distributed in the polyp lamina propria of CRSwNP patients as well. The number of CD20⁺ cells in polyp tissues was significantly increased compared to the normal controls ($P < 0.01$, Fig. 4b). Moreover, when evaluated the protein levels of IgA and IgE in polyp tissues, we found both IgA and IgE levels in polyp tissues were significant higher than those in normal controls ($P < 0.01$, Fig. 4c, d).

Association of polyp Tfh cells with clinical severity of CRSwNP patients

To further assess the importance of Tfh cells and B cells in the pathogenesis of CRSwNP, we firstly examined B cell status (proliferation and activation) in polyp tissues by evaluating prominent B cell cluster (GC-like structure) and protein levels of IgA and IgE in different subgroups of

CRSwNP patients. In 27.2 % (6 of 22) of CRSwNP patients, we observed prominent GC-like structure, and the representative GC-like structure in polyp tissues of 2 CRSwNP patients is shown in Fig. 5a. When subdivided these patients into CRSwNP with or without GC-like structure (GC⁺ and GC⁻ NP) subgroups, we found that the IgA and IgE protein levels in GC⁺ polyp tissues were significantly higher than those in GC⁻ polyp tissues ($P < 0.05$, Fig. 5b, c). Next, we evaluated the association of the frequencies of Tfh cells with the clinical phenotype of CRSwNP patients. As shown in Fig. 6a, we found that the frequencies of Bcl-6⁺CD4⁺ cells in GC⁺ polyp tissues were significantly higher than those in GC⁻ polyp tissues ($P < 0.05$). Similarly, the frequencies of Bcl-6⁺CD4⁺ cells in eosinophilic polyp tissues (EOS⁺ NP) or recurrent polyp tissues were significantly higher than those in non-eosinophilic polyp tissues (EOS⁻ NP) or primary polyp tissues, respectively ($P < 0.01$, Fig. 6b, d). Moreover, when compared the CRSwNP patients with or without concomitant asthma (AS⁺ and AS⁻ NP), we found the frequencies of Bcl-6⁺CD4⁺ cells AS⁺ polyp tissues were significantly higher than those in AS⁻ polyp tissues ($P < 0.01$).

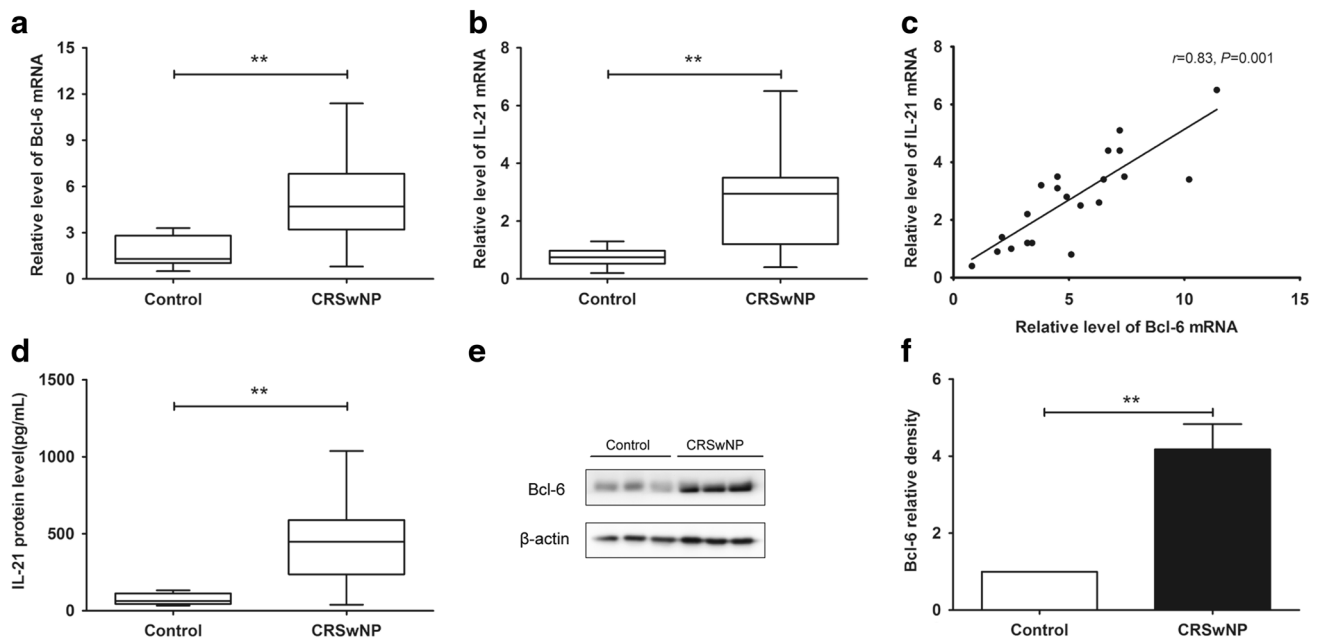


Fig. 2 The mRNA expression of Bcl-6 and IL-21 in polyp tissues and normal controls. **a** The mRNA level of Bcl-6 in polyp tissues was significant higher than that in normal controls, as determined by qPCR; **b** the mRNA level of IL-21 in polyp tissues was significant higher than those in normal controls, as determined by qPCR; **c** the Bcl-6 mRNA level correlated significant with IL-21 mRNA level in polyp tissues; **d** the protein level of IL-21 in polyp tissues was

significant higher than those in normal controls, as determined by ELISA; **e** the protein level of IL-21 in polyp tissues was significant higher than those in normal controls, as determined by ELISA; **f** the representative result of Bcl-6 bands in polyp tissues and normal controls, as shown by Western blot; The data indicated the median (IQR) (**a–d**) and mean (SEM) (**f**) in CRSwNP patients and normal controls, respectively. ** $P < 0.01$

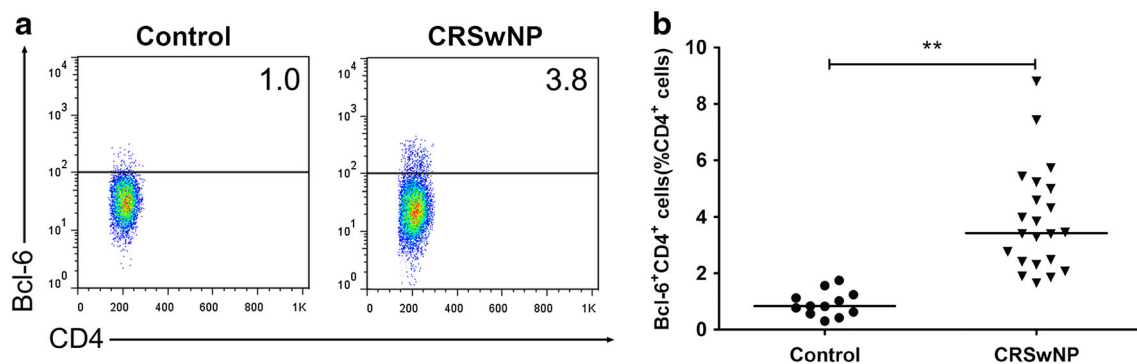


Fig. 3 The frequencies of Tfh cells ($Bcl-6^{+}CD4^{+}$ cells) in polyp tissues and normal controls. **a** The representative histogram of $Bcl-6^{+}CD4^{+}$ cells in polyp tissues and normal controls, as determined by flow cytometry; **b** the frequencies of Tfh cells ($Bcl-6^{+}CD4^{+}$ cells) in

polyp tissues were significant higher than those in normal controls. The data indicated the median (IQR) of CRSwNP patients and normal controls. ** $P < 0.01$

Discussion

By mediating the differentiation of B cells into memory and plasma cells following exposure to T-dependent antigens, Tfh cells have emerged as a subset of $CD4^{+}$ T cells responsible for regulating humoral immunity [11]. However, the studies concerning the role of Tfh cells in CRSwNP patients are still rare. In the present study, we provided the first evidence that Bcl-6 was

significantly upregulated in CRSwNP patients. Moreover, increased frequencies of $Bcl-6^{+}CD4^{+}$ cells (Tfh cells) in polyp tissues were shown to be associated with B cell accumulation, activation and disease severity. These findings therefore expand our understanding on the pathogenesis of CRSwNP and contribute to designing optimal therapeutical strategy for difficult-to-treat CRSwNP patients by targeting Tfh cells and related cytokines.

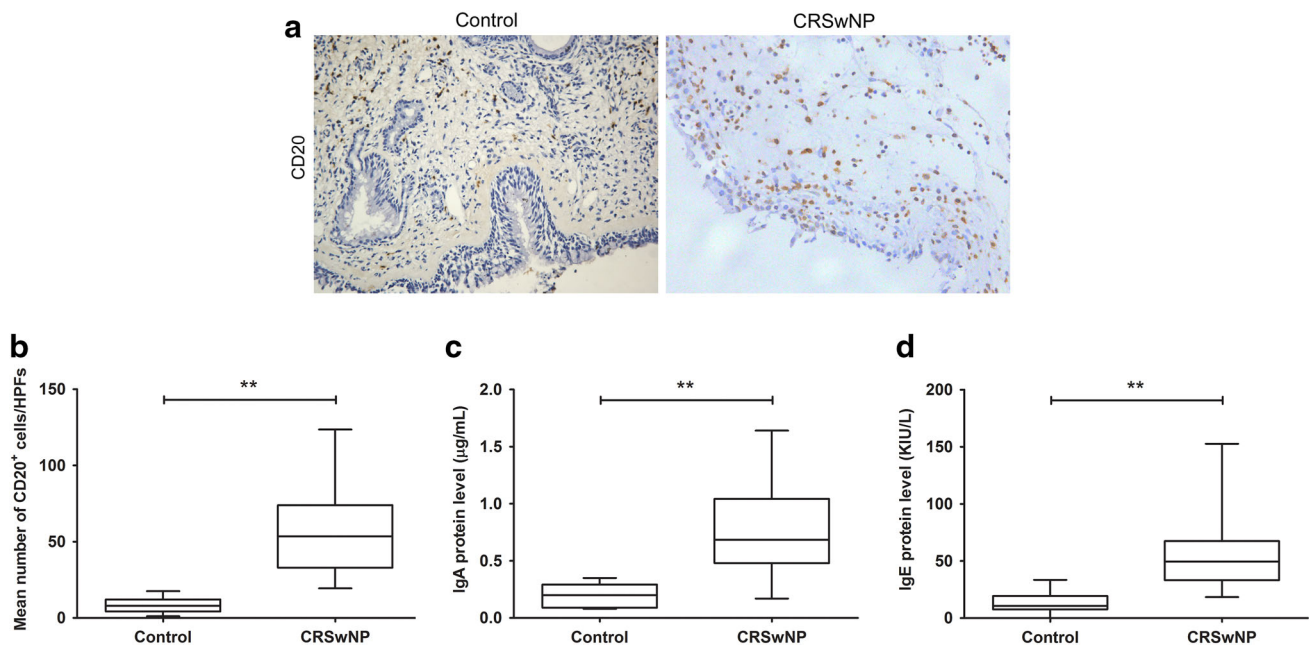


Fig. 4 Distribution of CD20⁺ B cells in polyp tissues and normal controls. **a** Representative IHC staining of CD20 was shown (×200); **b** the mean number of CD20⁺ cells in polyp tissues was significant higher than those in normal controls; **c** the IgA levels in polyp tissues

were significant higher than those in normal controls; **d** the IgE levels in polyp tissues were significant higher than those in normal controls; The data indicated the median (IQR) of CRSwNP patients and normal controls. ** $P < 0.01$

Extensive studies have suggested CRSwNP as a Th2 cell-dominant, eosinophilic inflammatory response with excessive IgA and IgE production [2, 12]. However, the pathogenic mechanisms underlying the B cell activation and Ig production in CRSwNP remain poorly understood. Generally, it has been well recognized that T helper cells provide a critical help to B cells to promote B cell proliferation and maturation and induce Ig production, but the exact nature of the T helper cell subset that provides help to B cells remained poorly understood. Early studies implicated Th2 cells, as they produce IL-4, which induces isotype switching and Ig secretion [13]. However, mice lacking key regulators of Th2 development are still able to form GC and elicit T cell-dependent antibody responses, suggesting Th2 cells may play less important role in B cell activation and Ig production in CRSwNP patients [14]. Recently, several groups identified an additional effector subset, Tfh cells, as the alternative candidate responsible for B cell help, and identified Bcl-6 as a key transcription factor for Tfh differentiation [9, 15–17]. For example, Bcl-6-deficient CD4⁺ T cells are not able to differentiate into Tfh cells in vivo, whereas constitutive Bcl-6 expression induced CD4⁺ T cell differentiation into Tfh cells [9]. In the present study, we reported that Bcl-6⁺ cells were significantly increased in polyp tissues compared with normal controls. Moreover, the increased Bcl-6⁺ cells were significantly associated with NP score, suggesting Bcl-6 may

be useful for evaluating Tfh cell differentiation and CRSwNP severity.

Tfh cells located in the apex of the light zone in GC and expressed CXCR5, ICOS, PD-1 and IL-21, which provide excellent markers for identification of Tfh cells [5]. IL-21 has been found to be significantly increased in human inflammatory diseases, such as rheumatoid arthritis and Sjögren's syndrome [18, 19]. Be consistent with these findings, we observed Bcl-6 and IL-21 mRNA and protein levels were significantly elevated in polyp tissues and established a positive association of Bcl-6 and IL-21 in CRSwNP patients. IL-21 can directly act on B cells and stimulate plasma cell differentiation from memory B cells [20]. The absence of IL-21 signaling profoundly affects GC persistence and function [21, 22]. These results support the pathogenic role of Tfh cells in the development of CRSwNP.

Based on our and other findings, we postulated that Tfh cells may play a critical role in the pathophysiology of CRSwNP. To test our hypothesis, we compared the frequencies of Bcl-6⁺CD4⁺ cells between polyp tissues and normal controls using flow cytometric analysis. As expected, we found the frequencies of Bcl-6⁺CD4⁺ cells were significantly increased in polyp tissues. Be consistent with other reports, we further observed significantly increased CD20⁺ cell (B cells) and IgA and IgE production in polyp tissues. These findings validate a potential role of

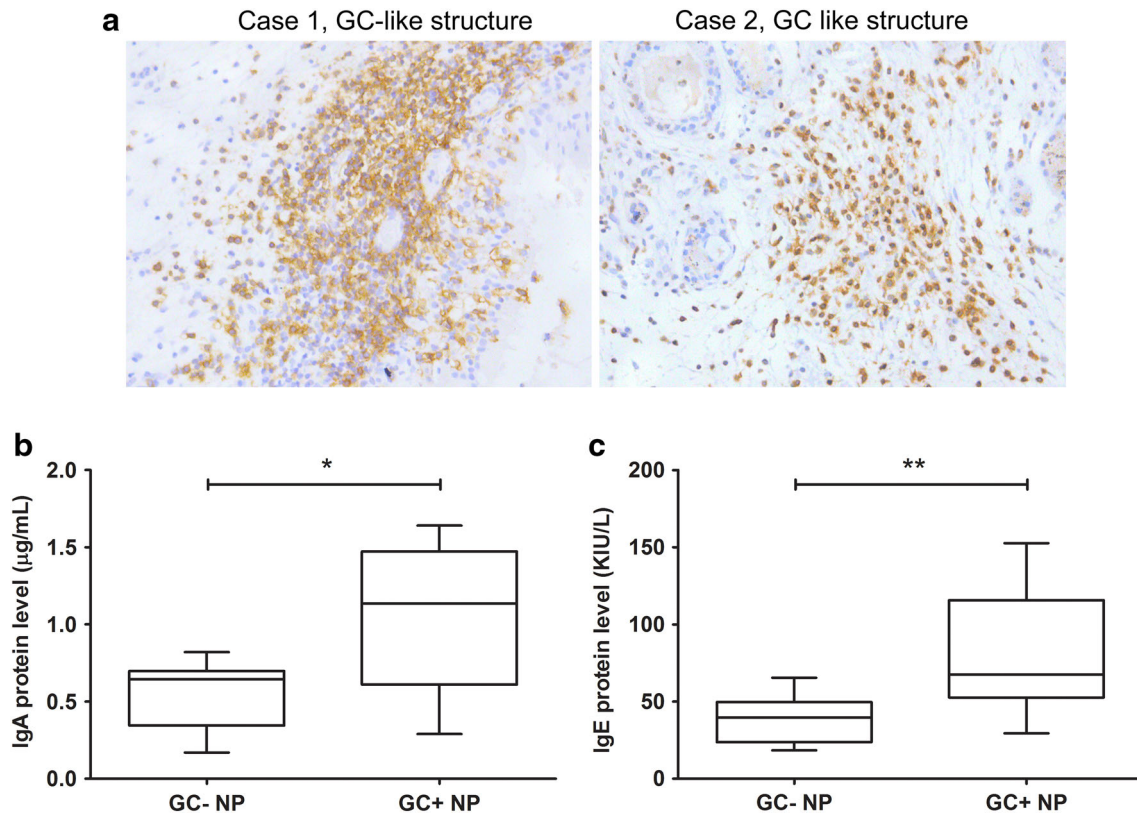
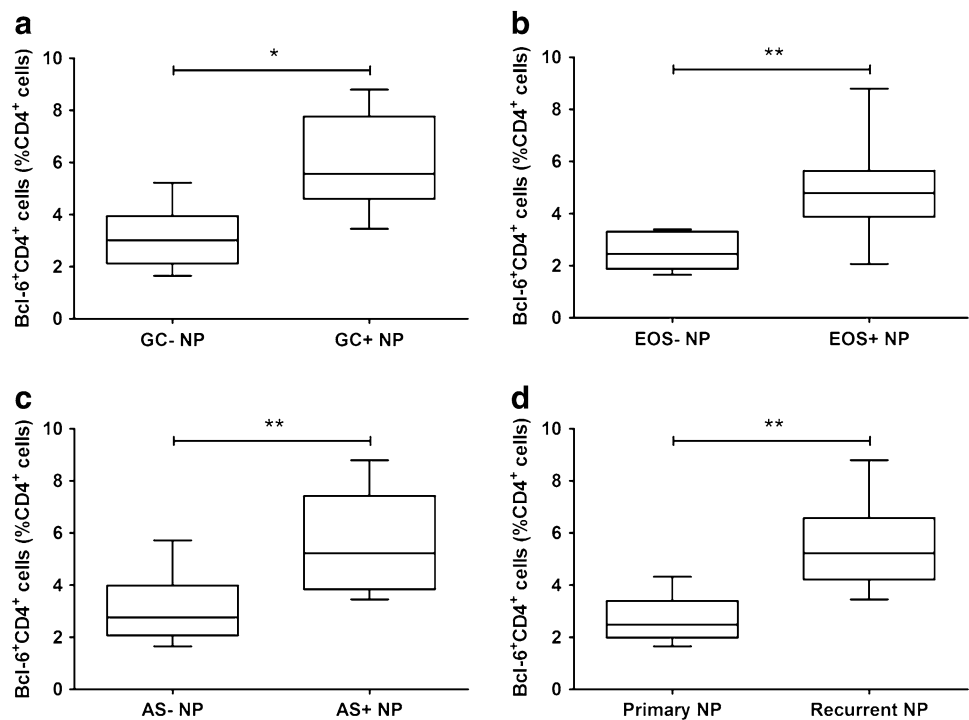


Fig. 5 Germinal center (GC)-like structure in polyp tissues is associated with IgA and IgE levels. **a** Two representative GC-like structure in polyp tissues of 2 CRSwNP patients, as suggested by IHC staining of CD20 ($\times 200$); **b** the IgA level in polyp tissues with or

without GC-like structure (GC⁺ or GC⁻ NP); **c** the IgE level in polyp tissues with or without GC-like structure (GC⁺ or GC⁻ NP). The data indicated the median (IQR) of CRSwNP patients and normal controls. * $P < 0.05$, ** $P < 0.01$

Fig. 6 The frequencies of Tfh cells (Bcl-6⁺CD4⁺ cells) in different subtypes of polyp tissues. **a** The frequencies of Bcl-6⁺CD4⁺ cells in polyp tissues with or without GC-like structure (GC⁺ NP, $n = 6$; or GC⁻ NP, $n = 16$); **b** the frequencies of Bcl-6⁺CD4⁺ cells in polyp tissues with or without tissue eosinophilia (EOS⁺ NP, $n = 12$; or EOS⁻ NP, $n = 10$); **c** the frequencies of Bcl-6⁺CD4⁺ cells in polyp tissues with or without concomitant asthma (AS⁺ NP, $n = 7$; or AS⁻ NP, $n = 15$); **d** the frequencies of Bcl-6⁺CD4⁺ cells in primary or recurrent polyp tissues (primary NP, $n = 13$; or recurrent NP, $n = 9$). * $P < 0.05$, ** $P < 0.01$



Tfh cells in B cell help in polyp tissues. The generation of Tfh cells is a multi-step process involving their initial priming in contact with dendritic cells in the T cell zone, their migration toward the interfollicular region, where they receive additional signals for differentiation from antigen-specific B cells, and their effector phase within the GC [9]. By examining the prominent B cell cluster, we identified GC-like structure in some CRSwNP patients (6/22). Interestingly, we found the IgA and IgE levels, as well as the frequencies of Bcl-6⁺CD4⁺ cells, were significantly higher in CRSwNP patients with GC-like structure (GC⁺NP) compared with those without GC-like structure (GC⁻NP), providing an association of Tfh cell accumulation with the formation of GC-like structure and Ig production in polyp tissues. Previously, tissue eosinophilia, as an indicator of Th2-skewed inflammatory response, has been suggested to be associated with asthma comorbidity and recurrence after surgery in CRSwNP patients, which implied a difficult-to-treat phenotype [1]. In this study, when correlated the frequencies of Bcl-6⁺CD4⁺ cells with the clinical phenotypes, we interestingly found the frequencies of Bcl-6⁺CD4⁺ cells were significantly associated with tissue eosinophilia, asthma comorbidity and recurrence after surgery. These finding collectively suggested that Tfh cells may present a useful surrogate biomarker of disease severity by promoting local B cell activation, Ig production, eosinophil accumulation and concomitant asthma in CRSwNP patients.

Conclusion

Our results showed that Bcl-6 expression was significantly upregulated in CRSwNP patients, and increased frequencies of Bcl-6⁺CD4⁺ cells (Tfh cells) in polyp tissues were associated with enhanced B cell accumulation, IgA and IgE production, and disease severity (tissue eosinophilia, concomitant asthma and polyp recurrence). These findings thus added a new insight into the molecular mechanisms underlying CRSwNP and raise the possibility that Tfh cells could be a novel therapeutic target for difficult-to-treat CRSwNP.

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Conflict of interest No conflicts of interest to declare.

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