

IL-21 Is Increased in Nasal Polyposis and after Stimulation with *Staphylococcus aureus* Enterotoxin B

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Keywords

Chronic rhinosinusitis · Germinal center · Interleukin-21 · Nasal polyposis · T-follicular helper cell

Abstract

Background: Chronic rhinosinusitis with nasal polyposis (CRSwNP) is an inflammatory disease associated with lymphoid aggregates and local IgE production related to *Staphylococcus aureus* enterotoxins. T-follicular helper cells and their effector cytokine interleukin (IL)-21 play an important role in germinal center proliferation. **Methods:** IL-21 was determined on the mRNA level by qPCR in nasal tissue of 3 groups of patients: control ($n = 17$), chronic rhinosinusitis without nasal polyposis (CRSsNP; $n = 23$), and CRSwNP ($n = 35$). The expression of IL-21 by CD4⁺ T cells was analyzed in tissue at baseline and after 24-h stimulation of tissue fragments with *S. aureus* enterotoxin B (SEB) using flow cytometry. Finally, human nasal IL-21+CXCR5+CD4⁺ T cells were isolated and cocultured with human blood naive B cells to investigate their functionality. **Results:** IL-21 mRNA expression was increased in the CRSwNP group ($p < 0.05$) compared to the control group, and B-cell lymphoma-6 and

B-lymphocyte-induced maturation protein-1 were upregulated in CRSwNP versus CRSsNP. Furthermore, SEB was able to increase IL-21 mRNA expression significantly ($p < 0.01$) in nasal polyps. Flow cytometry revealed that the source of IL-21 was predominantly CD4⁺ T cells and that IL-21+CD4⁺ T cells were significantly increased in polyp tissue and further increased after SEB stimulation. Finally, tissue CXCR5+CD4⁺ T cells derived from nasal polyp tissue were able to induce maturation of human naive B cells. **Conclusions:** IL-21- and IL-21-producing CD4⁺ T cells were increased in CRSwNP. In addition, SEB induced an increase in IL-21 and IL-21+CD4⁺ T cells, suggesting that *S. aureus* can modulate the function of Tfh cells in nasal polyps. We speculate that T-follicular helper cells and IL-21 are important in the pathophysiology of CRSwNP.

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Introduction

Chronic rhinosinusitis with nasal polyposis (CRSwNP) is a chronic inflammation of the nose and the paranasal cavities and this disease constitutes a large burden for the

society. Based on symptoms and nasal endoscopy the prevalence varies between 0.5 and 4.7% in the general population [1]. Asthma and aspirin sensitivity are more common in patients with CRSwNP [2]. The basic concept of inflammation in CRSwNP includes infiltration of eosinophils and T helper (Th) 2 cells with the production of interleukin (IL)-5 in 85% of Caucasian patients [3, 4]. CRSwNP is also characterized by a local polyclonal immunoglobulin E (IgE) production associated with the presence of specific IgE to *Staphylococcus aureus* enterotoxins (SAE-IgE) [2, 5]. A recent study proved the clinical efficacy of anti-IgE treatment in patients with CRSwNP and comorbid asthma [6].

Previous research has shown the organization of secondary lymphoid tissue and lymphoid aggregates in patients with CRSwNP [7]. In nasal polyp patients, lymphoid aggregates were identified with accumulation of ample T cells and B cells. Lymphoid aggregates resemble germinal centers (GC), which are well-organized structures where T cells communicate with B cells to select high-affinity B cells and where the development of B-cell memory occurs. Within the unique milieu of the GC, follicular B cells undergo somatic hypermutation and affinity maturation resulting in the diversification and selection of B cells and in differentiation into antibody-secreting plasma cells/memory B cells [8]. However, in lymphoid aggregates there is no characteristic division into 2 distinct zones, i.e., the light zone and the dark zone. Next to the presence of lymphoid aggregates, CRSwNP has increased levels of B cells and plasma cells [9, 10] and an increase in key markers of class switch recombination and receptor revision, like RAG₁ and RAG₂ [11].

A specialized subset of Th cells has recently been identified, i.e., T-follicular helper cells (Tfh), which are characterized by C-X-C motif receptor 5 (CXCR5) and capable of migrating to these GC and interacting with B cells [12, 13]. This colocalization of CD4⁺T cells with B cells is critical for T-B cell interactions and the function of GC. Furthermore, Tfh cells express molecules, such as B-cell lymphoma-6 (Bcl-6), and they lack B-lymphocyte-induced maturation protein-1 (Blimp-1). Bcl-6 is expressed on Tfh cells and it is identified as a master regulator of Tfh differentiation [14]. Blimp-1 is an antagonist of Bcl-6 and suppresses the generation of Tfh cells. Moreover, Tfh cells secrete IL-4 and IL-21, cytokines that promote B cell growth, differentiation, and class switch.

IL-21 is part of the common γ chain receptor family [15]. This cytokine has broad effects on innate and adaptive immune responses. Its receptor can be expressed on various cell types including T cells, natural killer cells, B

cells, and dendritic cells [16]. The pathogenic effect is strictly linked to the ability of IL-21 to control the functional activities of multiple immune and nonimmune cells. For instance, ligation of IL-21 to its receptor promotes differentiation of CD4⁺ Th cells, activation, proliferation, and survival of B cells, and cytotoxic activity of CD8⁺ T cells and NK cells [15]. Therefore it becomes clear that IL-21 produced by Tfh cells serves as an important regulator of humoral responses by directly regulating B-cell proliferation and class switching [17].

Although lymphoid aggregates are known to be present in polyp tissue, the role of Tfh cells and IL-21 in CRSwNP is uncertain. Therefore, our primary goal was to detect and quantify IL-21 and IL-21+CD4⁺ T cells both in healthy and in inflamed human nasal tissue. Secondly, we investigated the influence of *S. aureus* enterotoxins on the production of IL-21 and the presence of IL-21+CD4⁺ T cells in nasal tissue. Finally, we aimed to investigate the functionality of Tfh cells in nasal polyp tissue.

Patients and Methods

Patients

Seventy-five patients aged 18 years or older were eligible to participate in this study and 3 different groups of patients were selected: CRSwNP, chronic rhinosinusitis without nasal polyps (CRSsNP), and control. The diagnosis of sinus disease was based on the history, clinical examination, nasal endoscopy, and CT scan according to the current EPOS guidelines [1]. Samples from patients suffering from CRSsNP ($n = 23$) and CRSwNP ($n = 35$), respectively sinus mucosa and polyp tissue, were obtained at the ethmoid during endoscopic sinus surgery. Inferior turbinate samples from healthy patients without sinus disease, who were undergoing septoplasty or rhinoseptoplasty, were collected as controls ($n = 17$). The use of nasal corticosteroids was not allowed for 2 weeks prior to surgery, and systemic corticosteroids were not allowed 1 month prior to surgery. All patients underwent a skin prick test for common inhalant allergens [18]. The diagnosis of asthma was based on the GINA guidelines [19]. The local ethics committee of Ghent University Hospital, Belgium, approved this study. Written informed consent was obtained from each patient before inclusion in this study.

mRNA Expression Using Real-Time PCR

In order to measure mRNA levels of the genes coding for IL-21, Blimp-1, and Bcl-6, real-time PCR was used. The data are shown as normalized relative quantities (NRQ). A list of the primers used is given in online supplementary Table E1 (for all online suppl. material, see www.karger.com/doi/10.1159/000481435). More detailed information can be found in the online supplementary material.

Cytokine Measurements in Tissue Homogenates

Cytokine measurements (IL-5, IL-21, ECP, total IgE, and specific SAE-IgE) were performed on tissue homogenates as previously described [20]. More detailed information can be found in the online supplementary material.

Table 1. Baseline demographic and clinical characteristics of the control, CRSsNP, and CRSwNP patients

	Control (n = 17)	p value (control vs. CRSsNP)	CRSsNP (n = 23)	p value (CRSsNP vs. CRSwNP)	CRSwNP (n = 35)	p value (CRSwNP vs. control)
Age ^b , years	32.0 (27.0–49.0)	0.42	40.0 (28.0–54.0)	0.07	51.0 (34.0–62.0)	<0.05
Male/female ratio ^a	9/8	0.61	14/9	0.55	24/11	0.27
Allergy ^a	0	<0.05	8 (34.8)	0.54	15 (43.9)	<0.01
Asthma ^a	0	0.23	2 (8.7)	<0.01	17 (48.6)	<0.01
Aspirin intolerance ^a	0	ND	0	<0.05	6 (17.1)	0.07
IgE ^b , kU/L	15.60 (5.45–29.83)	<0.01	124.60 (26.48–223.90)	<0.01	303.10 (113.30–990.00)	<0.001
ECP ^b , µg/L	576.40 (265.93–856.80)	<0.05	1,812.25 (434.95–8,566.25)	<0.01	13,585.00 (4,040.75–25,080.00)	<0.001
IL-5 ^b , pg/mL	1.20 (1.20–1.20)	<0.01	23.65 (1.20–118.28)	<0.05	103.15 (24.13–451.85)	<0.001
SAE-IgE positive/negative ratio ^a	0/17	0.25	3/20	<0.05	15/20	<0.05

Values are presented as medians (IQR) or numbers (%) unless otherwise stated. Differences between groups were analyzed for each parameter and corresponding *p* values are indicated. ^aFisher's exact test. ^bMann-Whitney *U* test. *p* < 0.05 (bold values) was considered statistically significant. CRSsNP, chronic rhinosinusitis without nasal polyps; CRSwNP, chronic rhinosinusitis with nasal polyps; ND, not done.

Stimulation of T Cells

Preparation of the tissue single cell suspension from fresh human nasal mucosa was performed as previously described [20]. To check T-cell cytokine expression, nasal mucosa single cells were resuspended in RPMI (Roswell Park Memorial Institute) 1640 supplemented with 10% fetal calf serum, 50 U/mL penicillin, and 50 µg/mL streptomycin (Invitrogen Life Technologies, Belgium). T cells were activated with 50 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich, Bornem, Belgium) and 1 µg/mL Ionomycin (Sigma) for 6 h and incubated at 37°C and 5% CO₂. After 1 h, Brefeldin A (eBioscience) was added to the cell culture to prevent cytokine transport. Cells were harvested, centrifuged, washed, and readied for intracellular cytokine staining. Detailed information about intracellular cytokine staining and flow cytometry can be found in the online supplementary material.

Ex vivo Human Sinonasal Tissue Fragment Stimulation

We performed an in-house designed “human sinonasal tissue fragment stimulation” assay as described by Patou et al. [21]. Wells received 0.5 µg/mL *S. aureus* enterotoxin B (SEB) or tissue culture medium for 24 h, supernatant was collected to analyze IL-21 protein, and tissue pellets were snap frozen and further processed for RNA isolation and IL-21 mRNA determination. More detailed information can be found in the online supplementary material.

Stimulation of Naive B Cells with Different Nasal Polyp Tissue T-Cell Subsets

Different CD4⁺ T cells were sorted from the total single mucosa cell population via FACS sorting as described in detail in the online supplementary material. Labeled naive B cells were cocultured with the different types of sorted CD4⁺ T cells (ratio 1/1) in the presence of SEB (1 µg/mL) in RPMI 1640 complete medium supplemented with 10% heat-inactivated FBS for 7 days. Proliferation (CSFE expression) and differentiation (CD38) of the naive B cells was checked after 7 days using FACS analysis.

Statistical Analysis

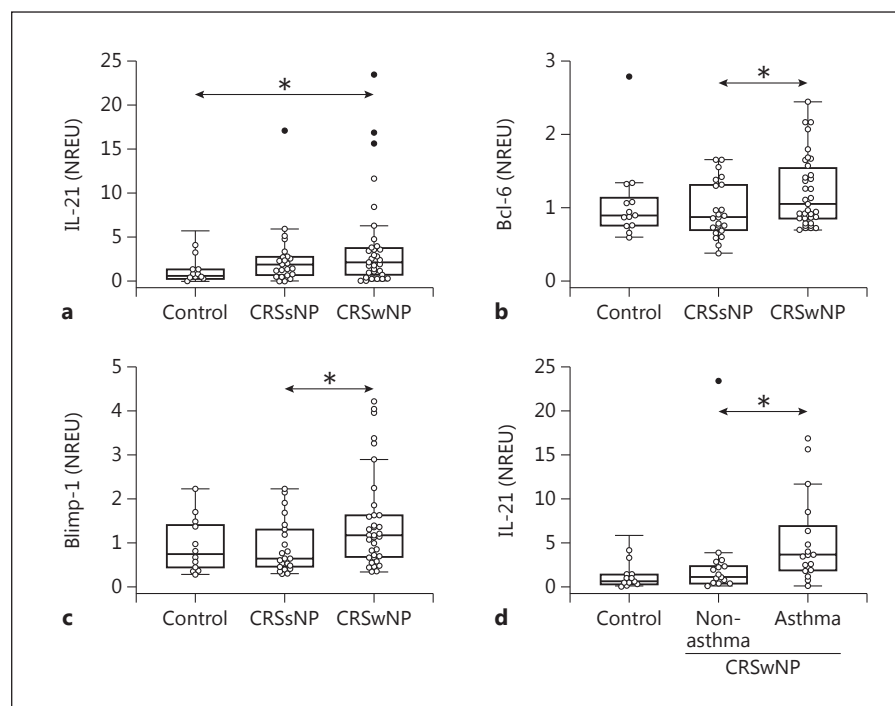
All statistics were performed using SPSS version 21.0 software. Data are presented as medians (IQR). The mRNA data were used as logarithmic data. The distributions of baseline characteristics between disease groups were compared according to Fisher's exact test for dichotomous data and the Mann-Whitney *U* test for continuous data. Comparisons between diseases were performed using the Mann-Whitney *U* test for continuous data. Two-sided *p* values < 0.05 were considered statistically significant.

Results

Patients and Baseline Characteristics

Thirty-five patients with CRSwNP, 23 patients with CRSsNP, and 17 control patients were included in this study. Baseline characteristics are shown in Table 1. The clinical characteristics showed a higher incidence of asthma and allergy in the CRSwNP group compared to control and CRSsNP groups. The tissue of the different disease groups was characterized based on IgE, ECP, and IL-5 concentrations. Patients with CRSwNP had a significant higher amount of IgE, ECP, and IL-5 compared to CRSsNP (IgE, *p* < 0.01; ECP, *p* < 0.01; and IL-5, *p* < 0.05) and control turbinates (IgE, *p* < 0.001; ECP, *p* < 0.001; IL-5, *p* < 0.001). SAE-IgE was present in 60% of the patients with CRSwNP, which is significantly higher than the rate in CRSsNP patients (15%, *p* < 0.05) and in the control group (0%, *p* < 0.05).

Fig. 1. Real-time PCR analysis of interleukin (IL)-21 mRNA (**a**), B-cell lymphoma-6 (Bcl-6) mRNA (**b**), and B-lymphocyte-induced maturation protein-1 (Blimp-1) mRNA (**c**) expression was performed in 3 disease groups (control, chronic rhinosinusitis without nasal polyps [CRSsNP], and chronic rhinosinusitis with nasal polyps [CRSwNP]). The presence of asthma comorbidity provoked a significant increase in IL-21 mRNA expression in patients with CRSwNP (**d**). Open circles represent individual measurements, and closed circles represent outliers. * $p < 0.05$.



IL-21 mRNA Is Increased in Nasal Polyp Tissue Compared to Control Tissue

The expression of IL-21 mRNA in nasal polyp tissue (2.12 NRQ, IQR 0.74–3.80 [22]) was significantly higher than in control nasal tissue (0.06 NRQ, IQR 0.25–1.36; $p < 0.05$; Fig. 1a). Transcript levels of Bcl-6 were significantly higher in polyp tissue (1.05 NRQ, IQR 0.85–1.57) compared to CRSsNP (0.87 NRQ, IQR 0.68–1.31, $p < 0.05$; Fig. 1b). In addition, Blimp-1, a transcription factor expressed by B and T cells, showed a higher expression in CRSwNP (1.17 NRQ, IQR 0.66–1.64) than in CRSsNP (0.64 NRQ, IQR 0.45–1.34, $p < 0.05$; Fig. 1c). We found that CRSwNP with asthma (3.58 NRQ, IQR 1.80–7.39) had a significantly higher amount of IL-21 mRNA compared to CRSwNP without asthma (1.04 NRQ, IQR 0.32–2.40, $p < 0.05$; Fig. 1d). The protein levels of IL-21 measured in the 3 groups did not differ significantly.

Identification of IL-21-Producing Tfh-Like Cells in Nasal Polyp Tissue

We were successful in preparing a single cell suspension that had a good survival after processing (80–90% living cells). Moreover, specific cell subsets could be sorted out because surface markers were not affected during processing. The cellular source of IL-21 was analyzed by flow cytometry on nasal tissue of control patients ($n = 3$;

Fig. 2a) and CRSwNP patients ($n = 6$; Fig. 2b). The percentage of IL-21+CD4+ cells of the total living cells was higher in CRSwNP compared to the control group (Fig. 2c; $p < 0.05$). Even after correction for the frequency of CD4+ T cells in the 2 groups, we still observed a significantly higher rate of IL-21+CD4+ T cells in CRSwNP patients compared to the control group (Fig. 2d; $p < 0.05$). These IL-21-positive cells mirror in part the number of Tfh cells, as part of these cells were CXCR5 positive (between 18 and 35% of the Tfh cells; Fig. 2e). We also checked for coexpression of IL-21 with IFN γ , IL-4, or IL-17 and identified IL-21+ cells with Th1, Th2, or Th17 characteristics (Fig. 2f).

S. aureus Enterotoxin Induces IL-21 in Nasal Polyp Tissue

Within the CRSwNP group, the presence of SAE-IgE was associated with a significantly higher amount of IL-21 protein (2.42 pg/mL, IQR 1.12–3.58) compared to SAE-IgE-negative samples (1.61 pg/mL, IQR 0.50–3.92, $p < 0.05$; Fig. 3a). Also a higher amount of IL-21 mRNA was found in CRSwNP patients with SAE-IgE expression compared to those without SE-IgE, but this did not reach statistical significance (Fig. 3b).

In a functional experiment we incubated tissue fragments derived from nasal polyp tissue with SEB. After

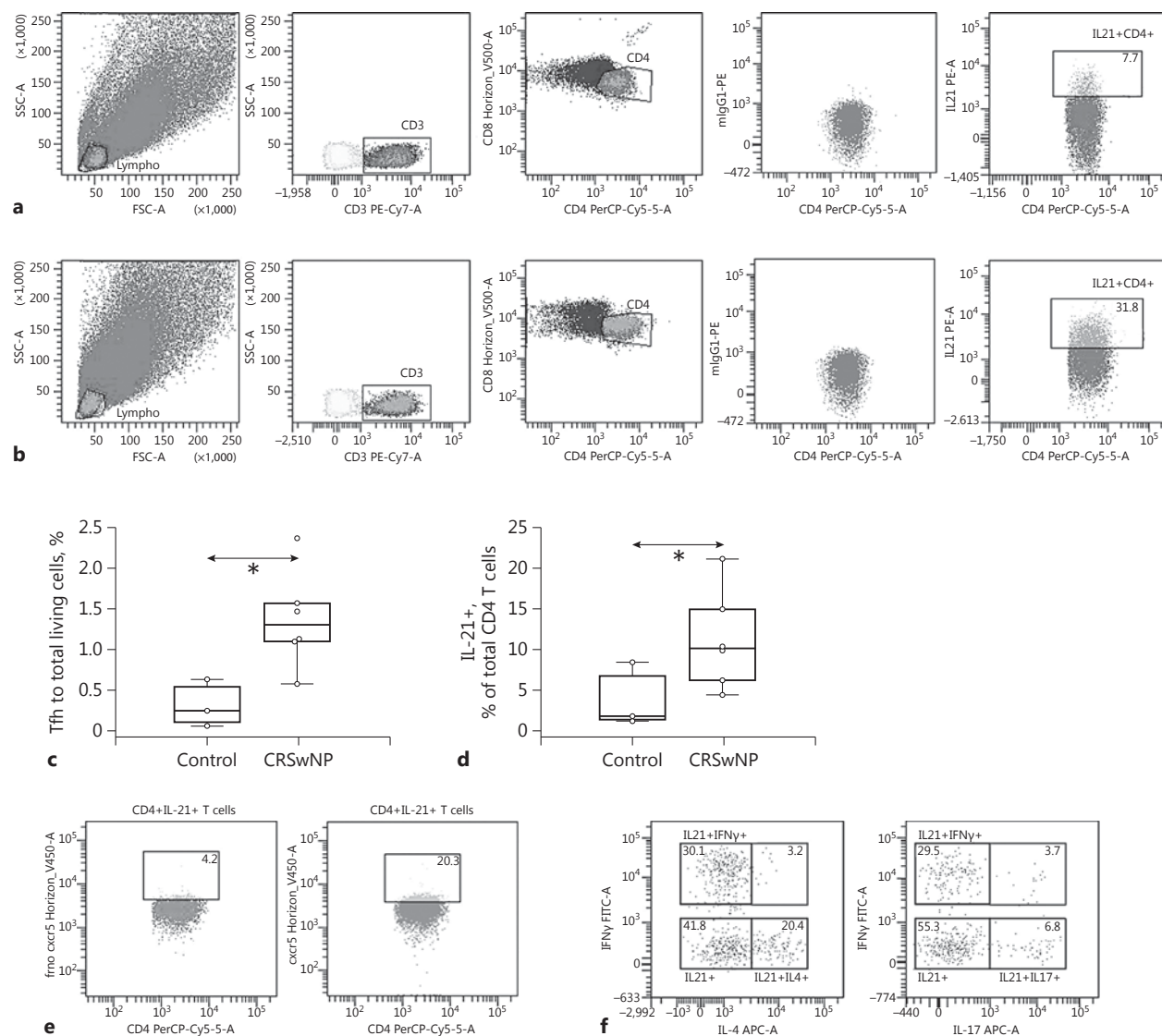


Fig. 2. Fluorescence-activated cell sorting of CD3+CD4+IL-21+ cells in nasal tissue of control patients ($n = 3$; **a**) and patients with chronic rhinosinusitis with nasal polyps (CRSwNP) ($n = 6$; **b**). The percentage of IL-21+ cells in relation to the total living cells was significantly higher in CRSwNP than in controls (**c**), even after correction for CD4+ T cells (**d**). * $p < 0.05$. **e** Expression of CXCR5

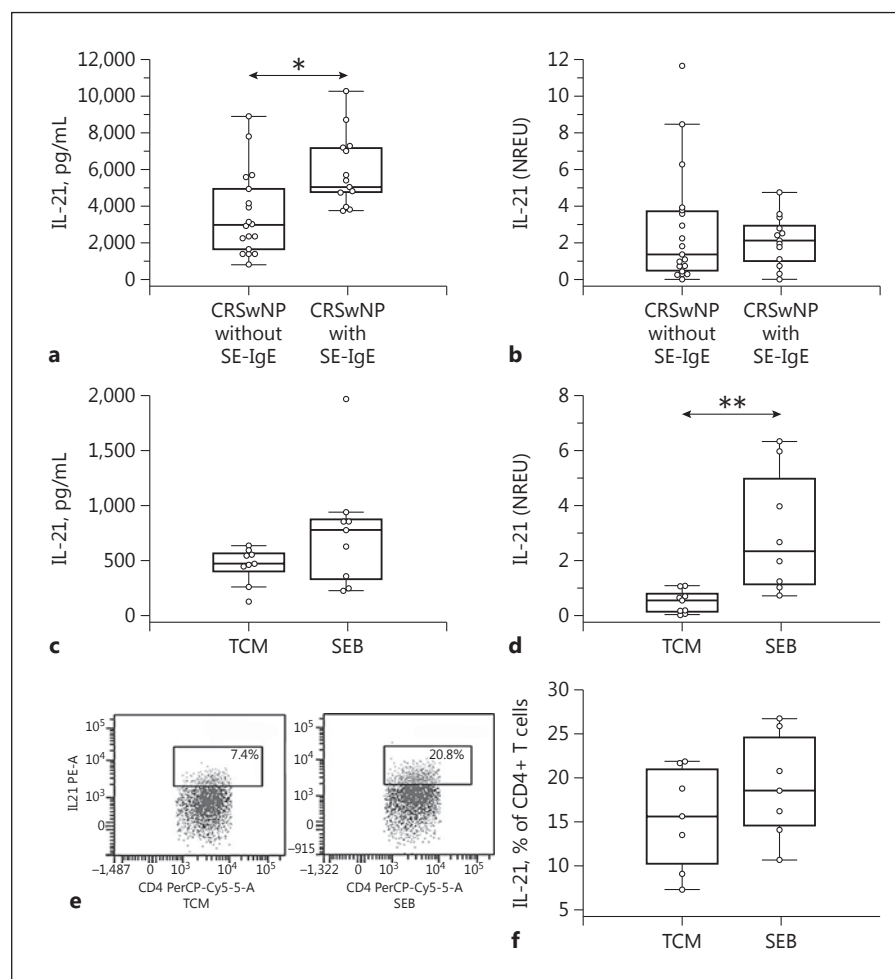
on CD3+CD4+IL-21+ gated cells in nasal polyp tissue, with the isotype control. Coexpression of IFN γ , IL-4, and IL-17 in the CD3+CD4 IL-21+ gated cells is shown in the dot plots (**f**). **e, f** Representative of the samples analyzed for IL-21 in CRSwNP. IL, interleukin.

stimulation with SEB for 24 h, a significant increase in IL-21 mRNA (Fig. 3d; $p < 0.01$) and a nonsignificant increase in IL-21 protein was observed in CRSwNP (Fig. 3c). Supporting these findings, we documented an increase in IL-21 producing T cells after SEB stimulation (Fig. 3e, f).

Nasal Polyp Tfh Cells Promote Proliferation and Differentiation of Naive B Cells in vitro

CXCR5+ T cells (Tfh), CRTH2+ T cells (Th2), and CXCR5–CRTH2– T cells (non-Tfh-nonTh2) were successfully sorted and isolated. The sorted cells were cocultured with CFSE-labeled naive blood B cells. After 7 days

Fig. 3. Protein analysis shows a significant increase in interleukin (IL)-21 when SAE-IgE is present in chronic rhinosinusitis with nasal polyps (CRSwNP) (a). * $p < 0.05$. b Amount of IL-21 mRNA in CRSwNP with or without specific IgE to *Staphylococcus aureus* enterotoxins (SAE-IgE). After tissue stimulation with *S. aureus* enterotoxin B (SEB), the amount of IL-21 protein (c) and IL-21 mRNA (d) in nasal polyp tissue increased. ** $p < 0.01$. The number of IL-21+CD4+ cells increased after stimulation with tissue culture medium or SEB (e). f Increase in the percent of IL-21-producing CD4+ T cells after SEB stimulation.



75% of the isolated CD4+T cells were still alive; naive B cells proliferated in the presence of all 3 T-cell subsets (Fig. 4a). Moreover, the differentiation of B cells into plasmablasts (CD38 expression) was present in the CXCR5+ T cells; however, CRTH2+ T cells and CXCR5–CRTH2– T cells also induced strong maturation of B cells (CD38 expression) (Fig. 4b).

Discussion

This study investigated Tfh cells and IL-21 both in healthy and in diseased human nasal tissue, more specifically in CRSwNP. We demonstrated that IL-21 expression and the number of IL-21-positive T cells are increased in nasal polyp tissue versus control tissue. This is the first study to show that IL-21 is clearly responsive to SEB in patients with CRSwNP. In addition, Tfh cells are

capable of inducing maturation and proliferation of naive B cells.

Tfh cells are known to have a specific role in GC; they are specialized in providing help to B cells [12]. Interestingly, our research identified IL-21+CD4+ T cells in nasal tissue and found that the number was significantly higher in nasal polyp tissue compared to nasal control tissue, as did a previous studies [22, 23]. Those IL-21+CD4+ T cells were further characterized by CXCR5 in the current study. We identified a group of CXCR5-positive or Tfh cells but also a group of CXCR5-negative IL21+CD4+ T cells. IL-21 is also produced by other CD4+ T cells, which could explain these IL-21+CXCR5– cells. So far it is not clear whether Tfh cells represent a separate lineage of CD4+ T cells or whether they originate from typical Th1, Th2, and Th17 cells that further differentiate and acquire Tfh cell features [14, 24–26]. Our research identified IL-21+CD4+ T cells with and

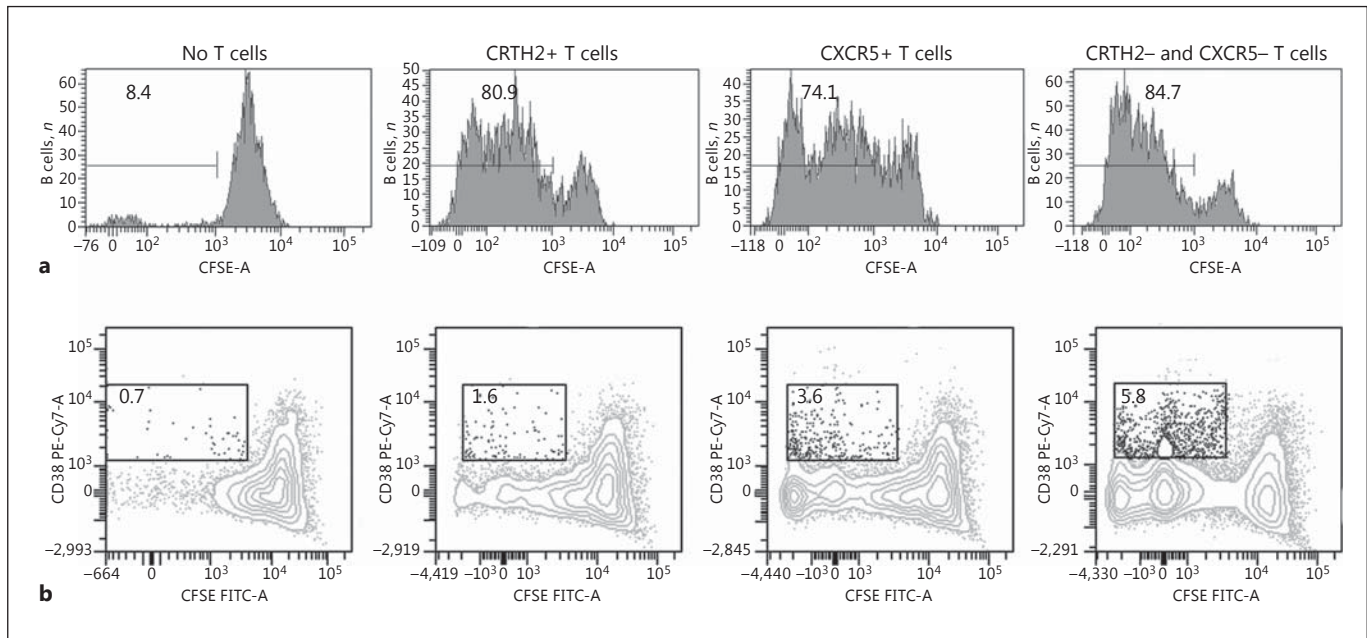


Fig. 4. Proliferation and maturation of naive B cells after incubation with no T cells, CRTH2+ T cells, CXCR5+ T cells, and CRTH2–CXCR5– T cells. **a** B cell proliferation (CFSE) after incubation with the different T cell populations. **b** Maturation (CD38) of proliferated B cells after incubation with the different T-cell populations.

without clear Th1, Th2, or Th17 characteristics based on cytokine coexpression.

Human Tfh cell differentiation has is not yet fully understood [26]. However, Kroenke et al. [27] found evidence that Bcl-6 controls Tfh cell differentiation by inducing migration genes and proteins critical for T-B cell interaction. Our analysis showed that Bcl-6 and Blimp-1 mRNA is significantly increased in polyp tissue. Blimp-1 and Bcl-6 are known as antagonistic transcription factors with effects on the differentiation of T and B cells [28]. Bcl-6 expression is confined to Tfh and B cells but B and plasma cells express Blimp-1. Both B cells and plasma cells are highly expressed in CRSwNP, which could have influenced our results. Our findings strongly suggest that Bcl-6 and Blimp-1 have a regulatory function on T- and B-cell differentiation in nasal polyp tissue.

The function of Tfh cells is based on expression of IL-21, the most prominent cytokine produced by Tfh [12]. Our results showed that in nasal polyp tissue the amount of IL-21 mRNA was significantly higher compared to control tissue. Tfh cells also express CD40 and IL-4, which are also found in CRSwNP [29, 30]. The amount of IL-21 mRNA could be influenced by the fact that activated CD4+ T cells and natural killer T cells also produce IL-21.

In addition, *S. aureus* is an important disease modifier in CRSwNP [31]. This was confirmed by the observation that the level of IL-21 protein was correlated with SAE-IgE. Additionally, by incubating nasal polyp tissue with SEB, a significant increase in IL-21 mRNA and in IL-21+ CD4+ T cells was found. These data suggest that the influence of *S. aureus* and its enterotoxins on nasal polyps is pleiotropic, with effects on IL-5, IgE [2] but also on IL-21 production. To identify the exact role of *S. aureus* on lymphoid aggregate formation and function, further research is necessary.

In nasal polyp tissue, functional polyclonal IgE is another cardinal feature [31, 32]. This polyclonal IgE is believed to be the result of a close T-B cell interaction in lymphoid aggregates nasal polyp tissue. Tfh cells and their main effector cytokine IL-21 have a known effect on induction of differentiation of B cells and thereby immunoglobulin production [33]. Previous research has shown that B-cell and plasma cell numbers as well as IgE and IgA concentrations are increased in polypoid sinonasal mucosal tissue from patients with CRSwNP compared to controls [9, 34]. Moreover, Gevaert et al. [11] recently showed the presence of local receptor revision and class switching to IgE in CRSwNP [35].

Our study achieved the sorting of 3 T-cell subsets (CXCR5+, CRTH2+, and CXCR5–CRTH2–) from nasal polyp tissue. Moreover, by incubating CXCR5+CD4+ T cells with naive B cells, proliferation of those naive B cells was observed. This proves that Tfh cells derived from nasal polyp tissue show potential to induce B-cell proliferation and maturation. However, CRTH2+ or Th2 cells, which are important in the pathophysiology of CRSwNP, also induced proliferation of B cells. CXCR5–CRTH2– T cells contain other non-Th2 and non-Tfh cells and also showed potential to induce B-cell proliferation. Therefore, the process of induction of naive B cells is a complex one which can be induced by the 3 selected populations and different Th cells can contribute to the inflammatory profile of CRSwNP.

Previous research has shown that in humans IL-21 is produced when CXCR5+CD4+ T cells are incubated with naive B cells, and removal of IL-21 during coculture resulted in a dose-dependent inhibition of immunoglobulin secretion and B-cell recovery [36]. However, these results were measured in blood and are not comparable to ones obtained from tissue. Research on tonsil tissue showed differentiation of Tfh cells based on low, intermediate, and high CXCR5 expression [37]. This would in the future be a valuable approach; the only drawback is the lower amount of CXCR5 expression in nasal polyp tissue compared to tonsil tissue. So, our findings prove the functionality of Tfh cells in nasal polyp tissue. Zhang et al. [23] conducted a study that proved that IL-4 and IL-21 are involved in IgE production in nasal polyp tissue.

By way of conclusion, we can state that we found an increased presence of IL-21-producing cells in nasal polyp tissue compared to control nasal tissue, which in part are CXCR5+ Tfh cells. In addition, SEB induced an in-

crease in IL-21 mRNA levels and IL-21-positive T cells, suggesting the importance of *S. aureus* in nasal polyps. Since these CXCR5+ Tfh cells are capable of inducing proliferation and differentiation in naive B cells to plasma cells, we presume that they could play a role in the pathophysiology of nasal polyps.

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Author Contributions

Lien Calus and Lara Derycke were involved in data collection, statistical analysis, and interpretation of the data as well as in the writing of this paper. Natalie De Ruyck, Gabriele Holtappels, Claudina Pérez-Novó, and Melissa Dullaers contributed to the experimental analyses. Philippe Gevaert, Thibaut Van Zele, and Claus Bachert created the concept, contributed to sample collection, interpretation of the data, and development of this paper. Bart Lambrecht contributed to interpretation of the data and read this paper. All authors critically revised this paper and approved the final version. The corresponding author had full access to all of the data in this study and he had the final responsibility for the decision to submit it for publication.

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Online Supplementary Material

mRNA expression analysis of IL-21, Blimp-1 and Bcl-6 in human nasal tissues

Real-time quantitative PCR (qPCR) was used to determine the mRNA levels of the genes coding for interleukine-21 (IL-21), B lymphocyte-induced maturation protein-1 (Blimp-1) and the B-cell lymphoma-6 (Bcl-6). For that, total RNA was isolated using the RNeasy Mini Kit (Qiagen) from snap frozen nasal tissue (25 mg) from patients with CRSsNP, CRSwNP and controls. An extra DNase I treatment, to remove genomic DNA contamination, was done with the RNase-Free DNase Set (Qiagen). Concentration of each RNA sample was measured using the Nanodrop 2000 Spectrophotometer (Thermo Scientific). The RNA integrity was analysed using the Experion Automated Electrophoresis System (Bio-Rad Laboratories). 2 µg of RNA was transcribed to cDNA with the iScript Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad Laboratories, CA, USA) following the manufacturer's instructions. Real time PCR was performed on a LightCycler 480 System (Roche) using specific primer pairs (see this article's Table E1). PCR reactions contained 2 µl cDNA (5 ng total RNA equivalents or 20 ng for IL-21), 0.125 µl forward and reverse primer (10 µM each) and 2.5 µl SsoAdvanced SYBR Green Supermix (Bio-Rad Laboratories) in a final volume of 5 µl. The cycling conditions were comprised of 2 min polymerase activation at 95°C and 44 cycles of 5s at 95°C, 30s at 60°C and 1s at 72°C followed by a dissociation curve analysis from 60°C to 95°C. The amplification data obtained as quantification cycles (Cq) were then transformed to normalized relative quantities (NRQ) that were subsequently log transformed using the qBasePlus software (Biogazelle NV, Zwijnaarde, Belgium).³ The expression of the genes eukaryotic translation elongation factor 1 (EF-1), Hydroxymethyl-bilane synthase (HMBS) and the succinate dehydrogenase complex, subunit A (SDHA) were used to normalize for transcription and amplification variations among samples. The geNorm M value was less than 0.5 confirming their value as reference genes.⁴

Cytokine measurements in tissue homogenates

These measurements were assayed by using the Luminex xMAP suspension array technology in a BioPlex 200 system (BioRad, MI, USA). For this, IL-5 kits were purchased from R&D Systems (Minneapolis, USA). IL-21 concentration was determined with the commercially available ELISA kit (eBioscience, Vienna, Austria). ECP, total IgE and specific SE-IgE were measured by the UNICAP system according to the manufacturer's guidelines (Phadia, Thermo Fisher, Sweden).

Intracellular cytokine staining and flow cytometry

Tissue single cells were first stained with LIVE/DEAD® Fixable Near-IR dye (Invitrogen) (1µl/10⁶ cells). Then, cells were washed, centrifuged and the cell pellet was dissolved in FACS buffer (1X phosphate buffered saline, 2% fetal bovine serum, 0.05% Na-azide) and stained with different antibodies against cell surface molecules (see this article's Table E2 in this supplemental information) for 15 min at room temperature and followed by a washing step. After fixation and permeabilization, cells were stained with antibodies against IL-21. The cells were analyzed by BD FACS Canto II with 3 lasers using FACS Diva software, version 6 (BD Bioscience, Erembodegem, Belgium). Compensation was done with beads and with cells for live/dead staining. We measured 100000 CD3+ lymphocytes in each samples. As gating strategy, living cells were first gated based on the live/dead stain. Further the lymphocyte population was gated based on FSC and SSC. On the lymphocyte population gating was performed for different surface markers and intracellular markers.

mRNA expression analysis of IL-21 in human sinonasal tissue fragments after SEB stimulation

Real-time quantitative PCR was used to determine the mRNA level of IL-21 in 9 nasal polyp tissues after stimulation with TCM or SEB for 24 hours. For that, total RNA was isolated from snap frozen tissue pellets (20 mg) using the Aurum Total RNA Mini Kit (Bio-Rad Laboratories) following the manufacturer's instructions. A DNase I treatment, to remove genomic DNA contamination, is included in the protocol. Concentration of each RNA sample was measured using the Nanodrop 2000 Spectrophotometer (Thermo Scientific). The RNA integrity was analysed using the Experion Automated Electrophoresis System (Bio-Rad Laboratories). 500 ng of RNA was transcribed to cDNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) according to the manufacturer's instructions and subsequently diluted with nuclease-free water to 10 ng/µl cDNA (total RNA equivalents) and stored at -20°C. Real time PCR was performed on an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories) using specific primer pairs (see this article's Table E1). PCR reactions contained 3 µl cDNA (30 ng total RNA equivalents), 0.5 µl forward and reverse primer (10 µM each) and 10 µl iQTM SYBR Green Supermix (Bio-Rad Laboratories) in a final volume of 20 µl. The cycling conditions were comprised of 10 min polymerase activation at 95°C and 40 cycles of 15s at 95°C and 1 min at 60°C (for RT² qPCR Primer Assays) or 40 cycles of 30s at 95°C and 1 min at 62°C (for reference genes) followed by a dissociation curve analysis from 60°C to 95°C. The amplification data obtained as quantification cycles (Cq) were then transformed to normalized relative quantities (NRQ) that were subsequently log transformed using the qBasePlus software (Biogazelle NV, Zwijnaarde, Belgium).³ The expression of 2 reference genes beta-actin (ACTB) and elongation factor 1 (EF-1) was

used to normalize for transcription and amplification variations among samples. The geNorm M value was less than 0.5 confirming their value as reference genes.⁴

Stimulation of naïve B cells with different nasal polyp tissue T cell subsets

Cells were stained with the anti-CD3-V500 (UCHT1), anti-CD4-PerCpCy5.5 (RPA-T4), anti-CXCR5-PE (RF8B2), anti-CD294(CRTH2)-APC (BM16), anti-CCR6-PECy7 (11A9) and anti-CXCR3-AF488 (1C6). Three different CD4⁺ T cell populations were sorted: CXCR5+CD294-CCR6-CXCR3-, CXCR5-CD294+CCR6-CXCR3- and CXCR5-CD294-CCR6+CXCR3+. Next, PBMC were isolated from allogeneic buffy coats followed by enrichment of human B cells by using a negative selection BD Bioscience kit. To isolate naïve B cells from the isolated pool of B cells, staining was performed with the antibodies anti-IgD FITC (IA6-2), anti-CD27 PE (L128), and anti-CD3 APC. Naïve B cells were sorted as CD3-CD19+CD27-IgD⁺ and then labeled with carboxyfluorescein diacetate, succinimidyl ester (CFSE, Invitrogen).

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Table E1: Primers used for Real time PCR

PCR primers	Sequence 5' → 3'	Amplicon Size	Accession number
ACTB - Forward	CTGGAACGGTGAAGGTGACA	139 bp	NM_001101
ACTB - Reverse	AAGGGACTTCCTGTAACAATGCA		
IL-21*	RT ² qPCR Primer Assay PPH01684A	119 bp	NM_021803.1
EF1 - Forward	CTGAACCATCCAGGCCAAAT	59 bp	NM_001402
EF1- Reverse	GCCGTGTGGCAATCCAAT		
IL-21- Forward	AGCTCCCAAGGTCAAGATCGCCA	108 bp	NM_021803.3
IL-21- Reverse	GCTGGCAGAAATTCAGGGACCAAGT		
HMBS - Forward	GGCAATGCGGCTGCAA	154 bp	NM_00319
HMBS - Reverse	GGGTACCCACGCGAATCAC		
SDHA - Forward	TGGGAACAAGAGGGCATCTG	86 bp	NM_004168.2
SDHA - Reverse	CCACCACTGCATCAAATTCATG		
BCL6*	RT ² qPCR Primer Assay PPH00080E	91 bp	NM_001706.3
PRDM1/Blimp-1*	(This primer can also generate amplicons from the splice variants NM_138931, NM_001130845, NM_001134738) RT ² qPCR Primer Assay PPH18652A	97 bp	NM_182907.1
	(This primer can also generate amplicons from the splice variant NM_001198)		

* These primers were purchased from SABiosciences (Qiagen, CA, USA)

Table E2: Antibodies used for flow cytometry

Antigen	Clone	Fluorochrome	Catalog number	Isotype	Concentrations	Company
CD3	SK7	PECY7	557851	Mouse IgG1	3,75pg/10 ⁶ cells	BD bioscience
CD4	RPA-T4	PERCPCY5.5	45-0049	Mouse IgG1	125pg/10 ⁶ cells	eBioscience
CD8	RPA-T8	V500	560775	Mouse IgG1	200 µg/ml	BD bioscience
IL-21	3A3-N2	PE	12-7219	Mouse IgG1	500pg/10 ⁶ cells	eBioscience