

Increased IL-21 expression in chronic rhinosinusitis with nasal polyps

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/cea.12475

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Summary

Background IL-21 is a key cytokine for regulating B cell immunity, which is involved in several inflammatory conditions. This study sought to define a role for IL-21 in activated B lymphocytes and enhanced tissue eosinophilia in NP tissues during the pathogenesis of chronic rhinosinusitis with nasal polyps (CRSwNP).

Methods NP and uncinate process tissues were collected from 64 CRSwNP patients, 25 CRSsNP patients and 29 control subjects. IL-21 expression was examined using IHC staining, qRT-PCR, flow cytometry and ELISA, and its clinical implication was evaluated. Moreover, the effects of IL-21 on B cell differentiation and Ig production in cultured NP cells were examined *in vitro*.

Results The mRNA and protein levels of IL-21 were significantly increased in polyp tissues compared with control tissues ($P < 0.05$). Polyp IL-21 level was significantly associated with polyp size, tissue eosinophilia and asthma comorbidity and recurrence after surgery ($P < 0.05$). Both Th1 and Th17 cells were the main cellular sources of IL-21 in polyp tissues. The percentage of IL-21⁺CD4⁺ cells was significantly higher in polyp tissues compared with control tissues and matched PBMCs ($P < 0.01$). Accordingly, the percentage of CD19⁺CD20^{+/−} CD38^{high} cells was significantly higher in polyp tissues compared with control tissues ($P < 0.01$). Moreover, recombinant IL-21 significantly increased the percentage of CD19⁺CD20^{+/−} CD38^{high} cells (plasmablasts) and IgG and IgA production in cultured NP cells *in vitro* ($P < 0.05$).

Conclusion and Clinical Relevance Increased IL-21 level in polyp tissues was associated with disease severity, local B cell activation and immunoglobulin production, suggesting that IL-21 might play an important role in promoting persistent mucosal inflammation in CRSwNP patients.

Keywords: IL-21, nasal polyp, chronic rhinosinusitis, asthma, B cell, plasma cells, immunoglobulin

Abbreviations CRS, Chronic rhinosinusitis; CRSsNP, CRS without nasal polyps; CRSwNP, CRS with nasal polyps; IL-21, Interleukin-21; Tfh cells, T follicular helper cells; GC, Germinal center; PBMCs Peripheral blood monocytes; IHC, Immunohistochemical ; qRT-PCR, Quantitative reverse transcription

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polymerase chain reaction; ELISA, Enzyme-linked immunosorbent assay; SEM Standard error of the mean; IQR, Interquartile range.

Introduction

Interleukin (IL)-21 is a member of the type I cytokine family and can be secreted by CD4⁺ T cells including T follicular helper (Tfh) cells, Th17 cells and natural killer (NK) T cells [1]. IL-21 regulates activation, proliferation and survival of CD4⁺ T cells, enhances cytotoxic activity of CD8⁺ T cells and NK cells, and contributes to the differentiation of Tfh and Th17 cells [2]. Moreover, IL-21 can directly act on B cells and stimulate B cell immunity. IL-21 costimulation promotes plasma cell differentiation from memory B cells, inducing class switch recombination and stimulating poorly responsive naive cord blood B cells into IgG-secreting plasma cells in humans [3]. The absence of IL-21 signaling profoundly affects germinal center (GC) persistence and function, influencing its proliferation, transition into memory B cells, and affinity maturation [4,5]. These findings support the pathogenic role of IL-21 in human inflammatory diseases, such as inflammatory bowel diseases, diabetes, rheumatoid arthritis and systemic lupus erythematosus [6,7].

Chronic rhinosinusitis (CRS) is characterized by inflammation of the nasal mucosa and paranasal sinuses of at least 12 weeks duration. Based on the presence or absence of nasal polyps (NP), CRS is generally classified into 2 subtypes: CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP) [8]. While CRSsNP has been demonstrated to be a predominantly Th1-polarized inflammation, CRSwNP is characterized by Th2-skewed inflammation and tissue eosinophilia [9,10]. Recent studies have shown B cells and immunoglobulin (Ig) are enriched in polyp tissues as well [11,12], indicating that local B cell immunity may play an important role in perpetuating the nasal inflammatory response in CRSwNP patients [13,14]. However, the expression and function of IL-21 in NP tissues has been largely unknown. For this reason, we hypothesized excessive IL-21 production contributes to the activated B lymphocytes and enhanced tissue eosinophilia in NP tissues during the pathogenesis of chronic rhinosinusitis with nasal polyps (CRSwNP). Thus we examined the expression of IL-21 in NP tissues and evaluated the regulatory role of IL-21 on B cell activation in this study.

Materials and methods

Patients and tissue samples

This study was approved by the local ethics committee boards, and written informed consent was obtained from each subject. Adult patients with CRSwNP (n=64) and CRSsNP (n=25) were recruited from the First Affiliated Hospital of Sun Yat-sen University. The diagnosis of CRSwNP and CRSsNP was made according to the international consensus [8]. As we described elsewhere [15], the skin prick test was performed to evaluate the atopic status, and asthma was diagnosed by a pneumologist based on disease history and airway responsiveness testing. Polyp size was graded 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. Eosinophilic and non-eosinophilic polyps were identified by immunostaining of major basic protein (MBP) as we described elsewhere [15].

All CRSwNP and CRSsNP patients had undergone an unsuccessful standardized course of medical therapy (oral and nasal corticosteroids, antibiotics, antihistamines, antileukotrienes etc.) and consented for tissue collection at the time of surgery. They were asked to stop using all medications for 4 weeks before sample collection. The polyp tissues in CRSwNP patients and uncinate process tissues in CRSsNP patients were sampled during endoscopic surgery. As normal controls, 29 patients undergoing septoplasty due to anatomical variations were enrolled, and the tissues from the uncinate process were sampled during endoscopic surgery. The demographic data of all subjects enrolled in this study are listed in Table 1.

Tissue samples were divided into three portions; the first portion was frozen in liquid nitrogen and stored at -80°C for subsequent RNA isolation, the second portion was used for protein isolation, and the third portion was fixed overnight in a freshly prepared fixative containing 4% paraformaldehyde in PBS (pH 7.4) and embedded in paraffin wax for histological staining. In addition, dispersed NP cells and matched peripheral blood monocytes (PBMCs) were collected for flow cytometry and/or *in vitro* assays.

IHC staining

IHC stainings for IL-21 (Genetex, Irvine, CA, USA), CD38 (Abcam, Cambridge, MA, USA), and MBP (Abd Serotec, Oxford, UK) were performed as described elsewhere [15]. See online supplementary materials for further details.

qPCR and ELISA analysis

IL-21 mRNA levels were detected by qRT-PCR analysis, as we described in the online supplement and elsewhere [15]. The levels of IL-21, IgA and IgG in the nasal tissue extracts and/or supernatants of cultured NP cells were performed using ELISA. See online supplementary materials for further details.

Multiparameter flow cytometric staining and analysis

Cell isolation and staining were provided in the online supplementary materials. Cells were isolated and washed and then incubated in the LIVE/DEAD Fixable Red Dead cell stain kit (Invitrogen, Carlsbad, CA, USA) for 30 minutes for dead cell discrimination (as presented in Fig S1). The cells were stained with the antibodies listed elsewhere (as presented in Table S1). Stained cells were washed twice before analysis using BD FACSCalibur or FACS Aria II cytometer. The gating strategy for lymphocytes in nasal tissues and peripheral blood is indicated in each figure. To obtain a reliable number of cells for analysis, $1-5 \times 10^5$ dispersed nasal tissue cells or PBMCs were used for flow cytometry staining, and more than 5000 events were collected on the flow cytometer using forward scatter/side scatter and analyzed using FlowJo software (Treestar, San Carlos, CA, USA). All incubation steps were performed on ice.

Cell culture and stimulation

To determine intracellular IL-21 expression levels, dispersed NP cells and matched PBMCs were cultured and stimulated and applied for ELISApot and flow cytometric analyses. See online supplementary materials for further details.

Statistical analysis

The data are expressed as the medians and interquartile ranges (IQRs) and were analyzed using the Kruskal-Wallis *H* test followed by nonparametric Mann-Whitney *U* test. Spearman's rank test was used for correlation analysis. For *in vitro* assays, the data are expressed as the mean $\pm$ SEM. Comparisons between
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multiple groups were performed using one-way Analysis of Variance (ANOVA) followed by Student's t test. P -values < 0.05 were considered to be significant.

Elevated IL-21 level in polyp tissue is associated with clinical severity

It is well known that CRSwNP is characterized by Th2-skewed, eosinophilic chronic inflammation of the paranasal sinuses; however, the potential implication of IL-21 expression in CRSwNP has not been characterized. This study enrolled 64 CRSwNP patients, 25 CRSsNP patients and 29 normal controls to evaluate the importance of IL-21 expression in the disease severity of CRSwNP. The demographic data of all subjects included in this study are listed in table 1. Our results showed that the mRNA and protein levels of IL-21 were significantly elevated in CRSwNP patients compared with CRSsNP patients and normal controls ($P<0.05$, Fig. 1a and b). When the relationship of IL-21 production and disease severity of CRSwNP was evaluated, we found that IL-21 level in polyp tissues was significantly associated with polyp size, tissue eosinophilia, asthma comorbidity and polyp recurrence after endoscopic surgery ($P<0.05$, Fig.1c-f). Our findings provided the clue that IL-21 may play a critical role in persistent mucosal inflammation in CRSwNP patients.

The characteristics of IL-21-producing cells in polyp tissues compared with normal nasal mucosa

To determine the characteristics of IL-21-producing cells in polyp tissues, we examined the distribution of IL-21 and CD38 (a putative surface marker of activated B cells, plasmablasts, etc.) in CRSwNP and CRSsNP patients and normal controls using IHC staining. As shown in Fig. 2, IL-21⁺ cells and CD38⁺ cells were extensively distributed in the subepithelial area of polyp and control tissues. The mean numbers of IL-21⁺ cells, as well as CD38⁺ cells, were significantly increased in CRSwNP patients compared with CRSsNP patients and normal controls ($P<0.05$), and the number of IL-21⁺ cells significantly correlated with the number of CD38⁺ cells in polyp tissues ($P<0.05$). To characterize the cellular sources of IL-21 in polyp tissues, we next examined intracellular IL-21 expression in 3 groups of T cell subsets (CD4⁺ Th cells, CD8⁺ Tc cells and CD56⁺ NKT cells) in polyp tissues using flow cytometric analysis. As shown in Fig 3, the mean frequencies of IL-21⁺CD3⁺ T cells within the CD3⁺CD4⁺ Th cells, CD3⁺CD8⁺ Tc cells and CD3⁺CD56⁺ NKT in polyp tissues were $55.4\% \pm 7.92\%$, $22.1\% \pm 4.55\%$ and $13.9\% \pm 2.18\%$, respectively, suggesting that CD4⁺ Th cells were the main cellular sources of IL-21 in polyp tissues. Compared with the normal controls, the percentage of IL-21⁺CD4⁺ Th cells was significantly

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increased in polyp tissues ($P < 0.01$, Fig 4a, b), and similarly higher percentages of IL-21⁺CD8⁺ Tc cells and IL-21⁺CD56⁺ NKT cells were observed in polyp tissues compared with normal controls (as presented in Fig S2). When further characterized the cellular sources of IL-21 in different Th subsets in polyp tissues, we found the mean percentages of IFN- γ ⁺CD4⁺ cells, IL-17A⁺CD4⁺ cells and Bcl-6⁺CD4⁺ T cells within IL-21⁺CD4⁺ cells were 76.3% $\pm$ 5.36%, 19.9% $\pm$ 6.09% and 8.6% $\pm$ 3.21%, respectively, suggesting Th1 and Th17 cell populations were the main cellular sources of IL-21 in polyp tissues (Fig. 4c). Additionally, the percentage of CD19⁺CD20⁺ CD38^{high} plasmablasts was significantly higher in polyp tissues than in normal controls ($P < 0.01$, Fig. 5a, b), indicating that IL-21-producing cells may contribute to the maturation of B cells in polyp tissues.

The characteristics of IL-21-producing cells in polyp tissues compared with PBMCs

The characteristics of IL-21⁺CD4⁺ Th cells in polyp tissues were further compared with those of matched PBMCs. The percentage of IL-21⁺CD4⁺ Th cells was significantly higher in NP cells than in matched PBMCs ($P < 0.01$, as presented in Fig. S3), suggesting that certain IL-21⁺CD4⁺ Th cells may have migrated into the inflammatory site from circulating blood cells. The IL-21-producing cells in polyp tissues and PBMCs were evaluated after stimulation as well. In the presence or absence of anti-CD3 and anti-CD28 stimulation, cultured NP cells showed more IL-21⁺ cells than did the matched PBMCs ($P < 0.01$), and IL-21 levels were significantly increased in the supernatants of NP cells than those in the supernatants of PBMCs in response to different stimuli (PMA plus ionomycin or anti-CD3 plus anti-CD28) ($P < 0.01$, see presented in Fig. S4), indicating that the polyp tissues contained more IL-21-producing cells than did the PBMCs. On the other hand, we found that the frequencies of IL-21R⁺CD20⁺ cells and CD40L⁺CD20⁺ cells were significantly higher in NP cells compared with the matched PBMCs ($P < 0.01$, see presented in Fig. S5), indicating an potentially enhanced IL-21-dependent B cell immunity in polyp tissues.

Recombinant IL-21 promoted B cell differentiation in NP cells *in vitro*.

To test the promotive effect of recombinant IL-21 on B cell differentiation, we stimulated NP cells with CD40L or IL-21 alone and with the combination of CD40L and IL-21 *in vitro*. After stimulation for 10 days, we found that recombinant IL-21 significantly increased the percentage of CD19⁺CD20⁺ CD38^{high} plasmablasts in cultured NP cells ($P < 0.01$, Fig. 6a), and a synergistic effect of IL-21 and CD40L. This article is protected by copyright. All rights reserved.

on induction of CD19⁺CD20⁺ CD38^{high} plasmablasts was observed ($P<0.01$, Fig. 6b). Consistently, recombinant IL-21 or CD40L alone increased the levels of IgG and IgA in cultured NP cells (Fig. 6c and d). These *in vitro* findings confirmed that IL-21 was capable of promoting B cell maturation and Ig production in polyp tissues.

Discussion

From previous reports, the increased number of plasma cells and the ratio of plasma cells to B cells in polyp tissues compared with normal controls indicates the excessive differentiation of B cells into Ig-secreting plasma cells in CRSwNP patients [11,16]; however, the mechanisms underlying this dysfunction remain poorly understood. In the present study, we hypothesized that elevated IL-21 may contribute to the pathogenesis of CRSwNP by modulating local B cell immunity. Consequently, we demonstrated that IL-21 expression was significantly increased in NP tissues compared with control tissues and matched PBMCs, and we established a clear relationship between IL-21 expression and disease severity in CRSwNP patients. Moreover, we showed that recombinant IL-21 is able to exert effect on polyp B cell differentiation and Ig production *in vitro*. These findings, therefore, expand our knowledge on the pathogenesis of CRSwNP and may contribute to the establishment of optimal therapy strategy by targeting IL-21.

Because IL-21 is critical for the generation of effective, long-lived, protective antibodies, in cases of dysregulation, the excessive adaptive immune response mediated by B cells and plasma cells confers persistent inflammation in human subjects. For example, elevated IL-21 levels have been found in some patients with systemic lupus erythematosus [17], rheumatoid arthritis and multiple sclerosis [18,19], which are autoimmune conditions characterized by the production of pathogenic autoantibodies. Similarly, in a study by Tan et al, the authors showed evidence for elevated levels of IgG production and antinuclear autoantibodies in polyp tissues of CRSwNP patients [6], which were found to be associated with disease severity (polyp recurrence after surgery). However, the possible role of IL-21 in the pathogenesis of CRSwNP has been largely unknown. To our knowledge, the current study is the first to investigate IL-21 expression and its clinical implication in CRSwNP patients. In doing so, we have shown an enhanced infiltration of IL-21⁺ cells and CD38⁺ cells in polyp tissues and have provided evidence that elevated IL-21 level was significantly associated with polyp size, tissue eosinophilia, asthma and polyp recurrence. The

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association of tissue eosinophilia with asthma, polyp size and recurrence has previously been somewhat addressed by our and other groups [15, 20, 21]. This study suggests that tissue IL-21 level in this population is strongly associated with tissue eosinophilia, concomitant asthma, polyp size and recurrence, providing a valuable biomarker for disease activity evaluation and a potential therapeutic target for the management of severe CRSwNP. Unfortunately, we failed to detect any eosinophil promoting cytokines, such as IL-5 or IL-13, in activated B cells in polyp tissues by flow cytometric analysis (data not shown), so we can't make the conclusion that activated B cells can promote tissue eosinophilia by directly producing eosinophil promoting cytokines, such as IL-5 or IL-13 in NP tissues in this study. As a reference, Kato et al. recently showed increased BAFF and IgA levels in NP tissues and concluded the increased IgA production in NP tissues may contribute to the local activation of eosinophils [22], which was supportive of our finding in this study. However, the molecular mechanisms underlying the association between tissue eosinophilia and increased IL-21 producing B cells are still need further investigation.

Previously, it has been proposed that CD4⁺ T cells are required for the formation of productive GC, as well as for Ig-secreting plasma cells [23]; however, the exact nature of the CD4⁺ T cell subset that helps B cells have remained elusive. Early studies implicated Th2 cells, as they produce IL-4 and may induce isotype switching and Ig secretion [24,25]. For example, CRSwNP is characterized by an excessive Th2 response and Ig production. Thus, the enhanced Ig production and class switch recombination have generally been ascribed to elevated IL-4 level [16]. However, mice lacking key regulators of Th2 development are still able to form GC and elicit the T cell-dependent antigen response; therefore, it is necessary to seek a novel cellular target to improve understanding [26]. In recent years, IL-21-producing or T follicular helper (Tfh) cells have emerged as the candidate cell type required for local B cell immunity [27]. The high concentrations of IL-21 production may suggest the presence of Tfh cells in polyp tissues, which was further validated by the increased Bcl-6 mRNA and protein expression levels in nasal polyps (data not shown). However, we failed to observe a strong clustering of CD38 cells into clusters by IHC staining. Thus, elucidating the characteristics of IL-21-producing cells in polyp tissues might provide novel insight on the pathophysiology of CRSwNP and a potential therapeutic strategy in severe CRSwNP patients.

Although few studies have addressed the roles of IL-21-producing cells in CRSwNP and other airway diseases, the importance of IL-21 in the pathogenesis of CRSwNP and asthma should not be neglected. The upper airway and lung represent an important mucosal immune site that is in constant contact with airborne antigens and microbial organisms [28]. Many studies have focused on elucidating the mechanisms involved in the induction and maintenance of B cell responses within the gut mucosa in animal models and human subjects [29]; however, there are few data regarding these mechanisms in the airway mucosa, especially in human subjects. Unlike lung tissues in asthmatics, polyp tissue is relatively easily accessible under endoscopic surgical conditions, which has provided us with a unique opportunity to investigate the regulation of B cell-mediated inflammatory response in human tissues. To determine the characteristics of IL-21-producing cells in polyp tissues, we next examined intracellular IL-21 expression in 3 groups of T cell subsets ($CD4^+$ Th cells, $CD8^+$ Tc cells and $CD56^+$ NKT cells), and observed that $CD4^+$ Th cells were the main cellular sources of IL-21 in polyp tissues.

While CRSwNP is characterized by Th2-skewed inflammatory responses, other $CD4^+$ Th cells, such as the Th1 and Th17 subsets, have been proposed to play an important role in the pathogenesis of CRSwNP in China and other Asian regions [15,30,31]. In the present study, we surprisingly found that the percentage of IL-21⁺ cells within the Th1 cells ($IFN-\gamma^+CD4^+$ cells) and the Th17 cells ($IL-17A^+CD4^+$ cells) was 76.3% and 19.9%, respectively. Previously, we have shown an increase in Th1/Th17 cells in the NP tissues of Chinese CRSwNP patients [15,30]. In the present study, we expanded our earlier work and found that Th1 and Th17 cells were the main cellular sources of IL-21 in polyp tissues. With regard to the Tfh cell population ($Bcl-6^+CD4^+$ T cells), we found that the percentage of IL-21⁺ cells within them was rather low (8.6%), which may explain why we did not observe a strong GC-like B cell cluster in this cohort. Because the characteristics of these IL-21⁺ $CD4^+$ T cells in the local microenvironment are not yet clear, we next characterized the properties of IL-21-producing cells in polyp tissues by compared them with those in matched PBMCs. In agreement with aforementioned results, we found that the percentage of IL-21⁺ $CD4^+$ Th cells was significantly higher in polyp tissues than in matched PBMCs, and IL-21 levels were significantly increased in the supernatants of cultured NP cells compared with the supernatants of PBMCs in response to different stimuli, indicating that IL-21-producing cells in polyp tissues, which may migrate from the blood, possessed a higher amount and activity than those in PBMCs.

NP tissues not only contained increased numbers of inflammatory cells, but also contained significantly more B cells, plasma cells and antibodies (11). In this study, we expanded these findings by demonstrating that the percentages of IL-21⁺CD4⁺ Th cells and CD19⁺CD20⁺ CD38^{high} plasmablasts were significantly higher in polyp tissues than in normal controls. It has been speculated that B cells that have migrated into polyp tissues may have a favorable microenvironment for activation and differentiation, but the underlying mechanisms are still under investigation. In the present study, we found that B cells in NP cells contained a higher ratio of memory B cells (Bim5, IgD⁻CD38⁺) and GC-B cells (Bim3+4, IgD⁻CD38⁺; data not shown) by determining the phenotypes of B cells in NP cells, as Sanz et al. described elsewhere [32]. Another novel finding was that the surface expression of IL-21R and CD40L in B cells was significantly increased in NP cells compared with PBMCs. Accordingly, by establishing a cultured system of NP cells, we obtained a key finding that recombinant IL-21 and CD40L significantly increased the percentage of plasmablasts in NP cells and IgG and IgA production *in vitro*. These findings collectively indicated that enhanced B cell activation and differentiation might be associated with increased IL-21-producing cells in NP tissues, highlighting the potentially critical role of IL-21 in the differentiation of plasma cells and the pathogenesis of CRSwNP. Further studies are required to elucidate other mechanisms used by plasma cells in CRSwNP patients.

In summary, we have shown that increased IL-21 expression is associated with disease severity in CRSwNP patients and have provided evidence that IL-21 is capable of promoting B cell differentiation and IgG and IgA production in NP cells *in vitro*. Our findings suggest that IL-21 might play an important role as a valuable biomarker for disease activity by promoting persistent mucosal inflammation in CRSwNP patients, which may be considered a potential therapeutic target.

Acknowledgement

This study was supported by grants from the National Natural Science Committee of China (No. 81072403, 81271054, 81400451, 81470673) and Programs from Ministry of Hygiene (No. 201202005) and Ministry of Education (No. 20130171110064)

Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Methods

Table S1. The antibodies used for flow cytometric staining

Figure S1. FACS gating used in the analysis of cells from polyp tissues and normal controls.

Figure S2. Intracellular IL-21 expression in Tc cells and NKT cells in polyp tissues.

Figure S3. Intracellular expression of IL-21 in CD4⁺ T cells in NP cells and matched PBMCs.

Figure S4. The expression of IL-21 in cultured NP cells and matched PBMCs.

Figure S5. The expression of IL-21R and CD40L in CD20⁺ cells in NP cells and matched PBMCs.

Figure legends

Fig. 1. Increased IL-21 mRNA and protein levels in polyp tissues of CRSwNP patients.

IL-21 mRNA (a) and protein (b) levels in normal controls, CRSsNP and CRSwNP patients. IL-21 protein level in polyp tissue was significantly associated with polyp size (c), tissue eosinophilia (d), asthma comorbidity (e) and recurrence after endoscopic surgery (f). *, $P < 0.05$; ***, $P < 0.001$ as determined by Mann-Whitney U test.

Fig. 2. Distribution of IL-21 and CD38 in polyp tissues of CRSwNP patients.

The representative IHC stainings of IL-21 and CD38 (a) in normal controls, CRSsNP and CRSwNP patients are shown (magnification, 200×). The mean numbers of IL-21⁺ cells (b) and CD38⁺ cells (c) were significantly increased in CRSwNP patients compared with CRSsNP patients and normal controls; The mean number of IL-21⁺ cells significantly correlated with the mean number of CD38⁺ cells in polyp tissues (d). *, $P < 0.05$; ***, $P < 0.001$ as determined by Mann-Whitney U test.

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Fig. 3. The cellular source of IL-21 in polyp tissues of CRSwNP patients.

(a) Representative flow cytometric dot-plots showing intracellular IL-21 expression in CD3⁺CD4⁺ Th cells, CD3⁺CD8⁺ Tc cells and CD3⁺CD56⁺ NKT in polyp tissues; (b) The mean percentages of IL-21⁺ T cells within the Th cell population, the Tc cell population, the NKT cell population and others in polyp tissues are shown (n=3).

Fig. 4. Intracellular expression of IL-21 in different Th cell populations in polyp tissues of CRSwNP patients.

(a) Representative flow cytometric dot-plots showing the intracellular expression of IL-21 in Th cells in normal controls and polyp tissues; (b) The frequencies of IL-21⁺CD4⁺ T cells in polyp tissues were significantly higher than in the normal controls; (c) Representative flow cytometric dot-plots showing the intracellular expression of IL-21 in Th1, Th17 and Tfh cells in polyp tissues. ***, $P < 0.001$ as determined by Mann-Whitney U test.

Fig. 5. Percentages of CD19⁺CD20⁺CD38^{high} plasmablasts in polyp tissues of CRSwNP patients.

(a) Representative flow cytometric dot-plots showing the percentages of CD19⁺CD20⁺CD38^{high} plasmablasts in polyp tissues and normal controls; (b) The percentages of CD19⁺CD20⁺CD38^{high} plasmablasts were significantly higher in polyp tissues than in normal controls; ***, $P < 0.001$ as determined by Mann-Whitney U test.

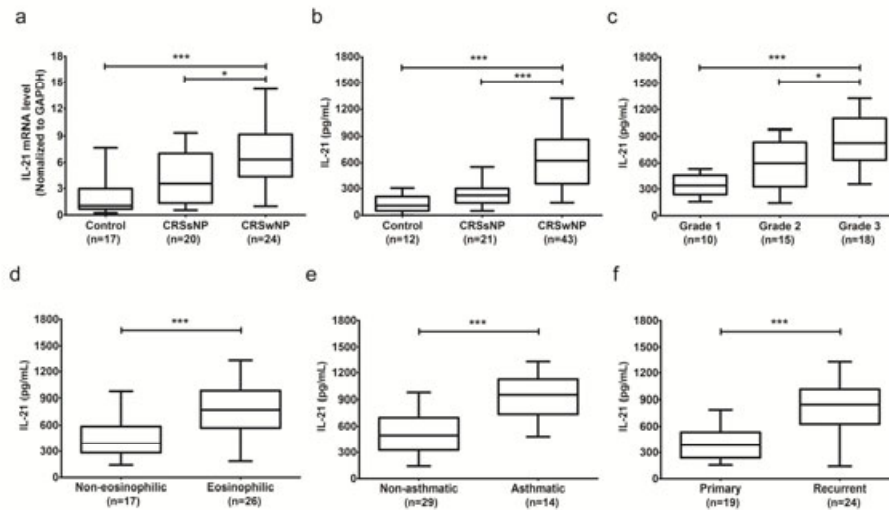
Fig. 6. Recombinant IL-21 promoted differentiation of plasmablasts and Ig production in cultured NP cells *in vitro*.

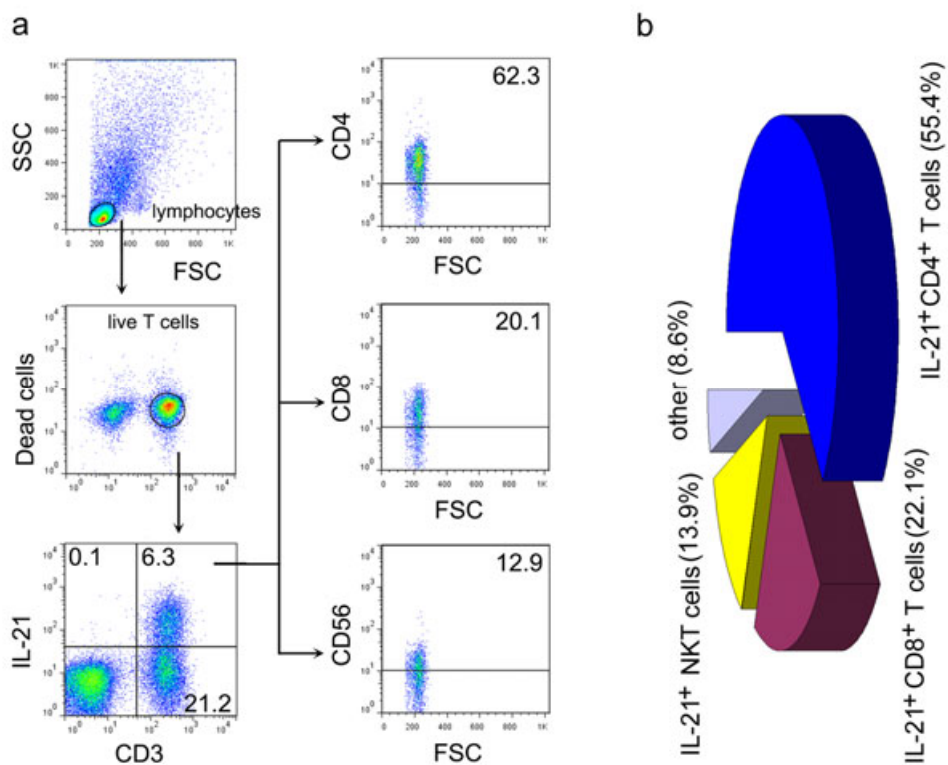
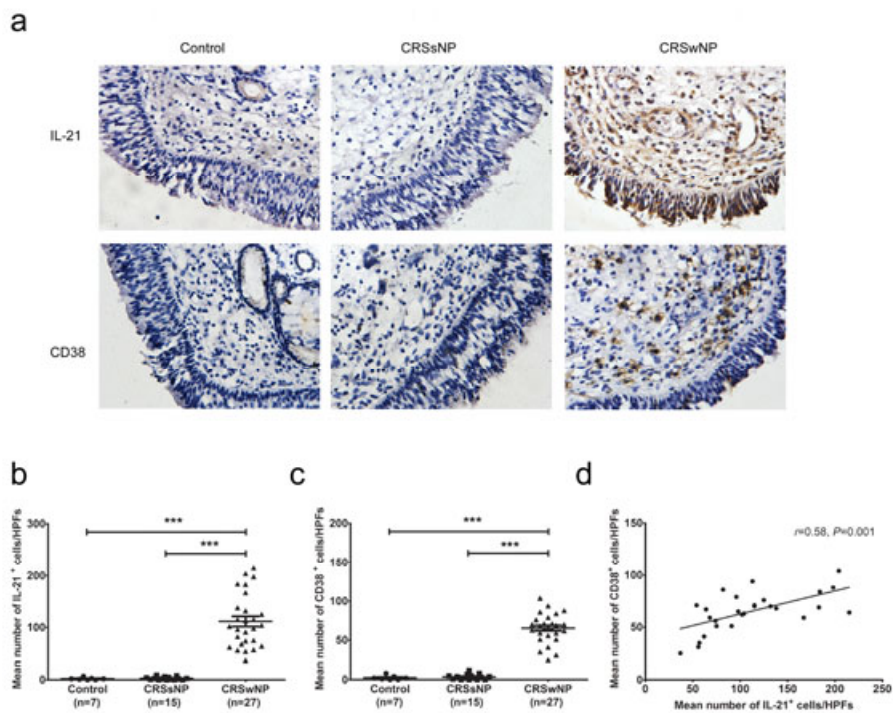
(a) Representative flow cytometric dot-plots of CD19⁺CD20⁺CD38^{high} plasmablasts in cultured NP cells are shown; (b) The percentages of CD19⁺CD20⁺CD38^{high} plasmablasts were significantly increased in response to CD40L and/or IL-21 compared with the control group; (c,d) The levels of IgG and IgA were significantly increased in the supernatants of cultured NP cells stimulated with CD40L and/or IL-21 compared with the control group. The data are expressed as the means (SEM) of 3 independent experiments. * $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$ as determined by Student's t test.

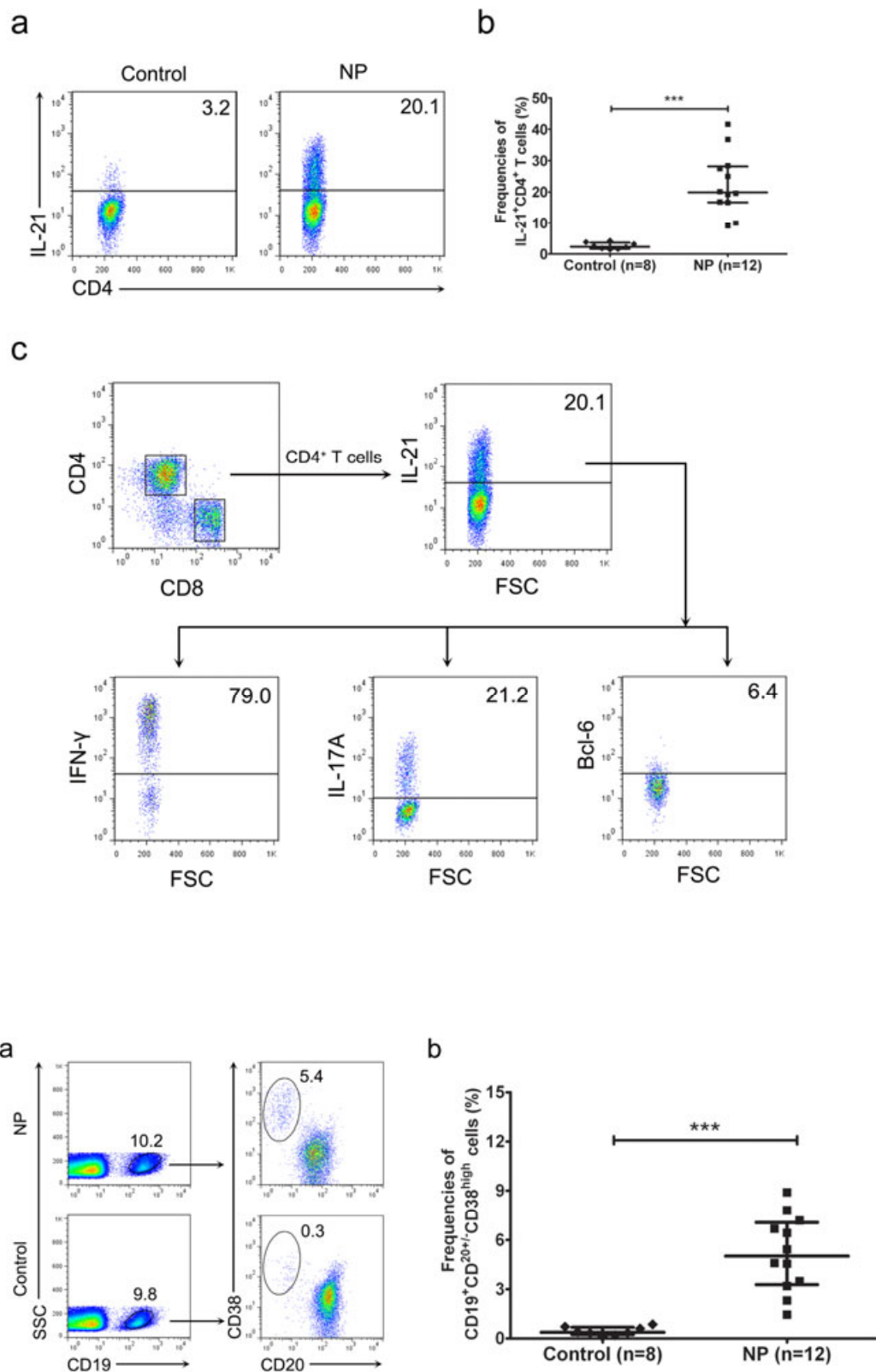
Table 1. Subjects' characteristics

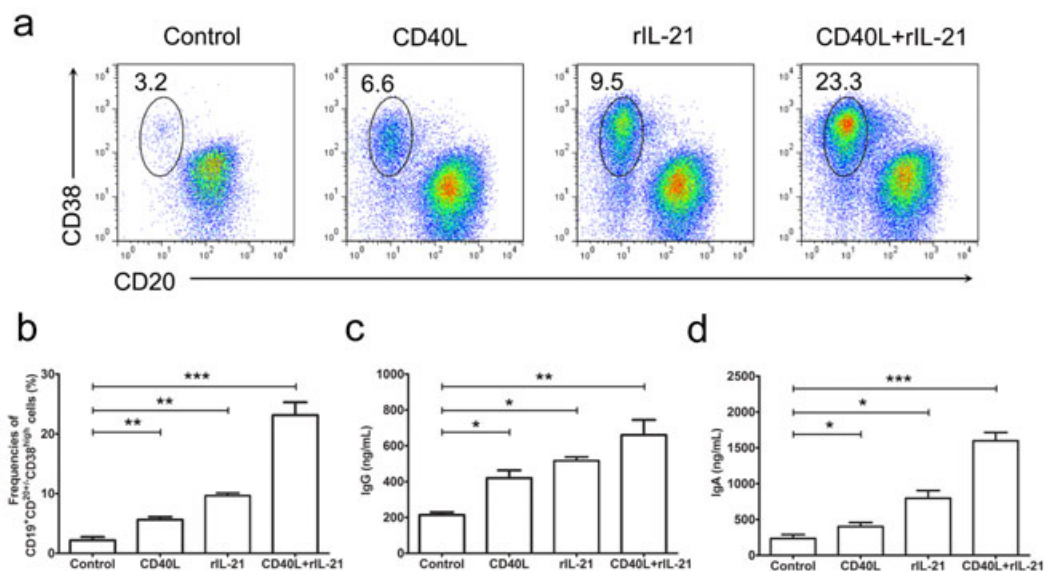
Characteristics	Normal controls	CRSsNP patients	CRSwNP patients
Subject no.	n=29	n=25	n=64
Gender (M/F)	16/13	14/11	38/26
Age (range)	21-38	24-48	23-58
Atopy	NA	n=10	n=22
Asthma history	NA	n=9	n=16
AERD history	NA	NA	NA
Sinus surgery history	NA	n=6	n=28
Methodologies used			
IHC	n=7 (4 M)	n=15 (11 M)	n=27 (17 M)
qRT-PCR	n=17 (9 M)	n=20 (11 M)	n=24 (16 M)
ELISA	n=12 (8 M)	n=21 (14 M)	n=43 (25 M)
Flow cytometry	n=7 (4 M)	NA	n=21 (14 M)
<i>In vitro</i> NP cells culture assay			n=32 (24 M)

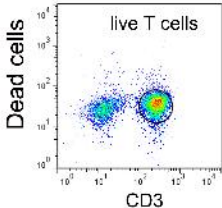
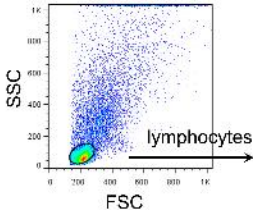
CRS, Chronic rhinosinusitis; NP, Nasal polyps; AERD, Aspirin-exacerbated respiratory disease



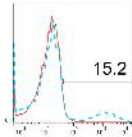
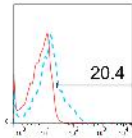
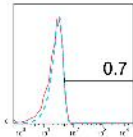
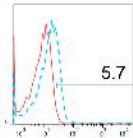








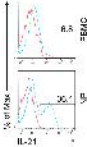
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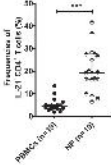
IL-21+NKT T cells

IL-21+CD8+ T cells

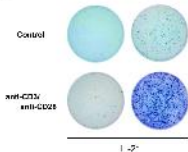
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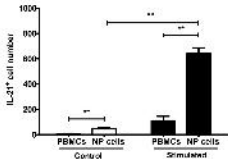
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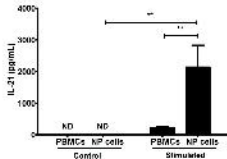
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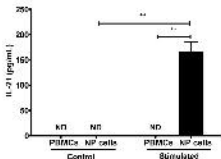
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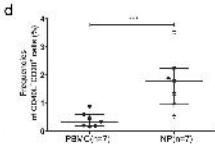
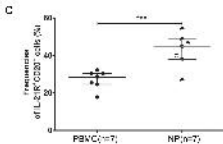
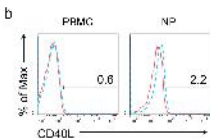
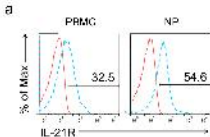


c



d





Supporting Information

Increased IL-21 expression in chronic rhinosinusitis with nasal polyps

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Methods

IHC staining

Paraffin sections (4 μm) were used, and IHC staining was performed using the peroxidase-labeled streptavidin-biotin technique. Briefly, 4- μm -thick sections were deparaffinized by serial treatment. After blocking the endogenous peroxidase in 3% hydrogen peroxide and with 1% bovine serum albumin, the sections were incubated overnight at 4°C in the presence of the primary antibodies IL-21 (1:100, Genetex, Irvine, CA, USA), CD38 (1:100, Abcam, Cambridge, MA, USA), and MBP (1:50, Abd Serotec, Oxford, UK). Thereafter, each section was incubated with a secondary antibody and then with horseradish peroxidase-labeled streptavidin complex (Zhongshanjinqiao, Beijing, China). Peroxidase distribution was revealed by incubating the sections in a solution containing 3% 3,3-diaminobenzidine tetrahydrochloride before being counterstained with hematoxylin and coverslipped. Negative control studies were performed by replacing the primary antibodies with normal IgG in appropriate concentrations.

The sections were blindly examined and coded with no awareness of the clinical data with an Olympus CX40 Microscope (Olympus Optical Co, Hamburg, Germany). The number of positive cells was counted in 10 high-power fields (HPFs, 400 $\times$) and averaged.

qRT-PCR

IL-21 mRNA levels were detected through real-time qPCR analysis. Briefly, total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Reverse transcription was performed, and cDNA was synthesized from 2 μg of total RNA using an oligo (dT) 18 primer and M-MLV reverse transcriptase (TAKARA, Syuzou, Shiga, Japan) for quantitative PCR. RNA integrity and the success of the reverse transcription reaction were monitored by PCR amplification of β -actin transcripts. mRNA expression was determined using ABI PRISM 7500 Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR Premix TaqTM (TAKARA). The primer sequences were as follows: 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'; and GAPDH reverse, 5'-GAA GAT GGT GAT GGG ATT TC-3'. PRISM samples contained 1 $\times$ SYBR Green Master Mix, 1.5 μL of 5 μM primers, and 25 ng of synthesized cDNA in a 25- μL volume. Reactions were heated to 95°C for 10 min followed by 40 cycles of denaturation at 95°C

for 10 s and annealing extension at 60°C for 60 s. Melting curve analysis was used to control for amplification specificity. The mean threshold cycle (Ct) values were normalized to GAPDH, and the relative mRNA levels of target genes were analyzed with $2^{-\Delta\Delta C_t}$. Experiments were performed in triplicate for each data point.

Cell isolation

Tissue samples were cut into pieces of approximately 1 to 2 mm, digested in incomplete RPMI-1640 medium with endotoxin-free collagenase I (2 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) and DNase I (5 U/mL, Takara) for 30~60 min at 37°C. The digested fragments were filtered through a 100-mm cell nylon mesh (BD Biosciences) to disperse the cells. The mononuclear cells were then obtained from the dispersed cells by Ficoll-Hypaque (Haoyang Bio., Tianjin, China) density gradient centrifugation at 2200 rpm for 20 min and then washed twice with Hank's solution. The cells were suspended at a concentration of 1×10^6 /ml in complete RPMI-1640 medium containing 10% FCS (Sijiqing, Hangzhou, China), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L L-glutamine (all from Gibco, Grand Island, NY, USA).

Peripheral blood mononuclear cells (PBMCs) of CRSwNP patients were isolated by Ficoll-Hypaque density gradient centrifugation.

Multiparameter flow cytometric staining and analysis

For surface staining, cells were incubated with the respective mAbs at 4°C in the dark for 30 min. For the staining of cytokine, cells were then fixed with 4% paraformaldehyde and permeabilized in PBS buffer containing 0.1% saponin (Sigma-Aldrich), 0.1% BSA and 0.05% NaN_3 for 2 h and stained with conjugated mAbs for cytokines. For the staining of transcription factors, the cells were first stained for surface antigens. Following fixation and permeabilization with Permeabilization/Fixation buffer (BD Biosciences), these cells were stained with conjugated mAbs for transcription factors and cytokines 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.

Cell culture and stimulation

To determine the expression of cytokines, dispersed NP cells, control nasal cells and matched PBMCs were stimulated for 5 h with PMA (20 ng/mL, Sigma-Aldrich) and ionomycin (1 µg/mL, Sigma-Aldrich) at 37°C with 5% CO₂ in the presence of brefeldin A (10 µg/mL, Sigma-Aldrich, St. Louis, MO, USA). IL-21 expression was analyzed by flow cytometry. To examine the effect of IL-21 on B cell differentiation, NP cells were stimulated with recombinant IL-21 (50 ng/mL, PeproTech, Rocky Hill, NJ, USA) alone or in the presence of CD40L (10 µg/ml, eBioscience, Inc. San Diego, CA, USA) for 10 days. Then, the cells were collected, and the percentage of plasmablast cells was detected by flow cytometry. Cell-free supernatants were harvested and stored at -80°C for ELISA.

ELISA

IL-21 level in the nasal tissue extracts and the levels of IL-21, IL-17A, IgA and IgG in the supernatants of cultured NP cells and PBMCs were measured using ELISA. For nasal tissue, one milliliter of PBS containing a protease-inhibitor cocktail (Keygentec Inc., Nanjing, China) was added for every 100 mg of tissue. The sample was homogenized for 1 min on ice. After homogenization, the suspension was centrifuged at 4000 rpm for 20 min at 4°C, and the supernatants were stored at -80°C for ELISA. A commercially available ELISA kit for IL-21 (eBioscience) was used according to the manufacturer's protocols. The detection limit for IL-21 was 39.0 pg/mL. For cell culture, NP cells and matched PBMCs were stimulated with PMA plus ionomycin (or soluble anti-CD3 plus anti-CD28) for 48 h or LPS (1 µg/mL, Sigma-Aldrich) for 24 h. Cell-free culture supernatants were harvested, and commercially available ELISA kits were used to measure IL-21 levels (eBioscience) according to the manufacturer's instructions. In addition, the levels of IgA and IgG in the supernatants of NP cells in response to CD40L and/or IL-21 were measured using commercially available ELISA kits (IgA, Bethyl, Montgomery, TX, USA; IgG, eBioscience). The detection limits for the assays were as follows: IgA, 7.8 ng/mL and IgG, 1.6 ng/mL. For convenient analysis, all values below the detectable limit were considered 0.

Table S1. The antibodies used for flow cytometric staining

Antigen-Fluorochrome	Manufacturer	Clone	Dilution
Anti-CD20-FITC	eBioscience	2H7	1:50
Anti-IL-17A-FITC	eBioscience	eBio64DEC17	1:50
Anti-IL-21-Alexa Fluor647	BD Pharmingen	3A3-N2.1	1:20
Anti-CD8-APC	eBioscience	sk1	1:50
Anti-CD40L-APC	eBioscience	24-31	1:50
Anti-CD38-APC	eBioscience	HIT2	1:50
Anti-CD3-PE-CF594	BD Pharmingen	SP34-2	1:100
Anti-CD4-APC-Cy7	BD Pharmingen	GK1.5	1:100
Anti-CD56-Pecy7	eBioscience	CMSSB	1:50
Anti-IFN- γ -Pecy7	eBioscience	4S.B	1:50
Anti-IL-21R-APC	BD Pharmingen	17A12	1:50
Anti-CD19-Alexa Fluor700	eBioscience	HIB19	1:50

Figure S1. FACS gating used in the analysis of cells from polyp tissues and normal controls.

The gating strategy for lymphocytes in nasal tissues and peripheral blood. To obtain a reliable number of cells for analysis, $1-5 \times 10^5$ dispersed nasal tissue cells or PBMCs were used for flow cytometry staining, and more than 5000 events were collected on the flow cytometer using forward scatter/side scatter. The LIVE/DEAD Fixable Red Dead cell stain kit (Invitrogen) was used to for dead cell discrimination. FSC, forward scatter; SSC, side scatter.

Figure S2. Intracellular IL-21 expression in Tc cells and NKT cells in polyp tissues.

Representative flow cytometric histograms showing the frequencies of IL-21 in CD3⁺CD8⁺ T cells and CD3⁺CD56⁺ NKT cells in normal controls and polyp tissues.

Figure S3. Intracellular expression of IL-21 in CD4⁺ T cells in NP cells and matched PBMCs.

(a) Representative flow cytometric dot-plots showing IL-21 expression in CD4⁺ T cells in NP cells and matched PBMCs; (B) The percentages of IL-21⁺CD4⁺ cells were significantly higher in NP cells than in matched PBMCs (n=19). ***, $P < 0.001$ as determined by Mann-Whitney U test.

Figure S4. The expression of IL-21 in cultured NP cells and matched PBMCs.

(a) Representative ELISpot results for IL-21 expression in NP cells and matched PBMCs in the presence or absence of anti-CD3 and anti-CD28 for 24 h (1×10^5 cells/well); (b) The number of IL-21⁺ cells was significantly increased in NP cells compared with PBMCs in the presence or absence of anti-CD3 and anti-CD28 stimulation; (c) The level of IL-21 in cell-free supernatants, as measured by ELISA, was significantly increased in the supernatants of NP cells than in the supernatants of PBMCs in response to PMA plus ionomycin for 48 h; (d) The level of IL-21, as measured by ELISA, was significantly increased in the supernatants of NP cells than in the supernatants of PBMCs in response to anti-CD3 plus anti-CD28 for 48 h. **, $P < 0.01$ as determined by Student's t test.

Figure S5. The expression of IL-21R and CD40L in CD20⁺ cells in NP cells and matched PBMCs.

(a) Representative flow cytometric histograms of the expression of IL-21R on CD20⁺ cells in NP cells and matched PBMCs are shown; (b) The frequencies of IL-21R⁺CD20⁺ cells were significantly higher in NP cells compared with the matched PBMCs (n=7); (c) Representative flow cytometric histograms of the expression of CD40L on CD20⁺ T cells in NP cells are shown; (d) The frequencies of CD40L⁺CD20⁺ cells were significantly higher in NP cells compared with the matched PBMCs (n=7). *** $P < 0.001$ as determined by Mann-Whitney U test.