Nasal IL-4$^+$CXCR5$^+$CD4$^+$ T follicular helper cell counts correlate with local IgE production in eosinophilic nasal polyps

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Background: Locally produced IgE contributes to the initiation and development of eosinophilic inflammation in eosinophilic nasal polyps independent of systemic atopy. However, whether CXCR5$^+$CD4$^+$ T follicular helper (T_{FH}) cells are involved in local IgE production at mucosal sites remains unexplored.

Objective: We sought to explore the presence, phenotype, and function of CXCR5$^+$CD4$^+$ T_{FH} cells in eosinophilic nasal polyp tissues compared with noneosinophilic nasal polyp and control normal nasal tissues.

Methods: T_{FH} cell-surface phenotypes and subsets and B-cell subsets in nasal tissues and peripheral blood were studied by means of flow cytometry. Immunohistochemistry was used to detect the tissue location of T_{FH} cells. Sorted nasal T_{FH} cells and CXCR5$^-$ T cells were cultured with autologous naive B cells purified from blood.

Results: Nasal T_{FH} cells expressed inducible costimulator, programmed cell death protein 1, and the transcription factor B-cell lymphoma 6 (Bcl-6) at an intermediate level when compared with *bona fide* T_{FH} cells in tonsils and circulating T_{FH} cells. Although counts of total T_{FH} cells and IL-21$^+$, IFN-γ^+, and IL-17$^+$ T_{FH} cells were increased in both eosinophilic and noneosinophilic nasal polyp tissues compared with those in normal nasal tissues, IL-4$^+$ T_{FH} cell counts were only increased in eosinophilic polyp tissues. IL-4 and IL-21 were involved in polyp T_{FH} cell–induced IgE production from naive B cells, and nasal IL-4$^+$ T_{FH} cell counts correlated highly with local IgE levels *in vivo*. IL-4$^+$Bcl-6$^+$CD4$^+$ T_{FH} cells were identified in ectopic lymphoid structures in eosinophilic nasal polyps. T_{FH} cells also positively correlated with germinal center B cells and plasma cells in nasal tissues.

Conclusion: Nasal IL-4$^+$ T_{FH} cells might be involved in local IgE production in eosinophilic nasal polyps. (J Allergy Clin Immunol 2016;137:462-73.)

Key words: *B cell, ectopic lymphoid structure, eosinophil, IgE, IL-4, nasal polyp, T follicular helper cell*

Despite advances in medical and surgical therapy, chronic rhinosinusitis remains difficult to treat, particularly for patients with chronic rhinosinusitis with nasal polyps (CRSwNP).[1] A great obstacle in improving the treatment of chronic rhinosinusitis is our limited understanding of the mechanisms of this complex and heterogeneous disease. Eosinophilic inflammation has commonly been considered a cardinal feature of CRSwNP in white subjects. In Asian subjects half of CRSwNP cases also present with eosinophilic inflammation.[2] The ultimate factors in inducing this mucosal eosinophilia remain uncertain; however, increased local IgE production in polyp tissues might contribute to mucosal mast cell activation and eosinophilic inflammation independent of systemic atopy.[3,4] Although B-cell class-switch recombination (CSR) to IgE has been generally assumed to be restricted to the germinal centers (GCs) of lymphoid organs, the presence of follicle-like structures and the expression of CSR markers, including ε germline gene transcript and ε circle transcripts in polyp tissues, strongly suggest local CSR to IgE in patients with eosinophilic CRSwNP.[4-6]

The B-lymphocyte CSR to IgE is initiated by the cytokines IL-4 or IL-13, which have long been believed to be produced by T_H2 cells.[7,8] Recently, it has become clear that a distinct subset of T_H cells beyond the T_H1/T_H2 paradigm, termed T follicular helper (T_{FH}) cells largely on the basis of their localization in B-cell follicles, plays a crucial role in B-cell response induction.[7,8] T_{FH} cells are also distinguishable from other T_H cells by increased expression of CXCR5, inducible costimulator (ICOS), and programmed cell death protein 1 (PD1) and the transcription factor B-cell lymphoma 6 (Bcl-6) and v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog *(c-Maf)*; downregulation of CCR7, CD127, and B lymphocyte-induced maturation protein 1 (Blimp1); and production of the canonical cytokine IL-21.[7,8] The fundamental role of T_{FH} cells

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Abbreviations used
Bcl-6: Transcription factor B-cell lymphoma 6
Bm: B mature
CRSwNP: Chronic rhinosinusitis with nasal polyps
CSR: Class-switch recombination
FACS: Fluorescence-activated cell sorting
Foxp3: Forkhead box P3
GC: Germinal center
ICOS: Inducible costimulator
NMC: Dispersed nasal mucosal mononuclear cell
PD1: Programmed cell death protein 1
T_{FH}: T follicular helper

in humoral immunity has resulted in many studies designed to understand their roles in, for example, human infections, autoimmune diseases, and vaccination.[9-11] In addition to secondary lymphoid organs, human $CXCR5^+CD4^+$ T_{FH} cells have also been identified in peripheral blood, sharing similar functional properties with *bona fide* GC T_{FH} cells in secondary lymphoid organs and possibly representing a circulating memory compartment of T_{FH} lineage cells.[12-15] Recently, the presence of $CXCR5^+CD4^+$ T_{FH} cells has also been documented in ectopic lymphoid structures in lesional tissues, such as breast cancer and rheumatoid arthritis synovium[16,17]; nevertheless, the phenotypes and functions of these $CXCR5^+CD4^+$ T_{FH} cells in nonlymphoid lesional tissues have yet to be defined.

Given the local CSR to IgE in patients with eosinophilic CRSwNP and the pivotal role of T_{FH} cells in immunoglobulin production, we hypothesized that $CXCR5^+CD4^+$ T_{FH} cells might be present in nasal polyp tissues and involved in local IgE production. In this study we comprehensively evaluated nasal mucosal $CXCR5^+CD4^+$ T_{FH} cell numbers, phenotype, and function in patients with eosinophilic and noneosinophilic CRSwNP. We reported that increased nasal mucosal $IL\text{-}4^+CXCR5^+CD4^+$ T_{FH} cell counts correlate with local IgE production in eosinophilic polyps. Nasal mucosal $CXCR5^+CD4^+$ T_{FH} cells manifest different phenotypic characteristics compared with circulating and *bona fide* T_{FH} cells.

METHODS

Patient population and clinical samples

This study was approved by the Ethics Committee of Tongji Hospital and conducted with written informed consent from each patient. The diagnosis of CRSwNP was made according to the current European Academy of Allergy and Clinical Immunology "European position paper on rhinosinusitis and nasal polyps 2012."[1] CRSwNP was defined as eosinophilic when the percentage of tissue eosinophils exceeded 10% of total infiltrating cells, as reported by our previous study.[2] Subjects undergoing septoplasty because of anatomic variations and without other sinonasal diseases were enrolled as control subjects.[2,4] Patient characteristics are summarized in Table E1 and other additional information is provided in the Methods section in this article's Online Repository at www.jacionline.org.

Histologic study

Hematoxylin and eosin and immunohistochemical staining were conducted, as previously described.[2] Consecutive sections were stained to study the relationship between CD4, IL-4, and Bcl-6 expression. Antibodies used are listed in Table E2 and other additional information is provided in the Methods section in this article's Online Repository.

Nasal mucosal mononuclear cell isolation

Sinonasal mucosa were dissociated mechanically with the GentleMACS Dissociator (Miltenyi Biotec Technology & Trading [Shanghai] Co, Shanghai, China).[16] The resulting cell suspension was filtered 2 times through a mesh of 40 μm, and then the dispersed nasal mucosal mononuclear cells (NMCs) were isolated by means of density gradient centrifugation on Lymphoprep (AXIS-SHIELD PoC AS, Oslo, Norway), as previously described.[18]

Flow cytometry

NMCs were obtained, as described above. PBMCs were also isolated by using Lymphoprep (AXIS-SHIELD PoC AS), as previously described.[18] The stained cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, Calif). Antibodies used are listed in Table E3 and other additional information is provided in the Methods section in this article's Online Repository.

Isolation and purification of naive B cells and T_{FH} cells

Naive $CD19^+IgD^+$ B cells, $CXCR5^-CD4^+$ T cells, and $CXCR5^+CD4^+$ T_{FH} cells were isolated by means of immunomagnetic cell sorting from peripheral blood and nasal polyp tissues, respectively.[18] The representative results of purification of B and T cells are shown in Fig E1 and more information is provided in the Methods section in this article's Online Repository.

Coculture of $CD4^+$ T cells and autologous naive B cells

Sorted T_{FH} cells or $CXCR5^-$ T cells (30×10^3 cells per well) were cocultured with autologous naive B cells (25×10^3 cells per well) for 8 days in U-bottom, 96-well plates, as previously described.[19] More information is provided in the Methods section in this article's Online Repository.

Immunoglobulin measurement

Protein levels of immunoglobulins in tissue homogenates and cell-culture supernatants were detected by using Bio-Plex suspension chip technology (Bio-Rad Laboratories, Hercules, Calif).[4,20] Total IgG was calculated as the sum of the 4 subclasses, as previously mentioned.[11] Specific IgE to Der p 1 was detected by using the ImmunoCAP system (Phadia, Uppsala, Sweden).[4] Detection limit for Bio-Plex assay is listed in Table E4 and other additional information is provided in the Methods section in this article's Online Repository.

Quantitative real-time PCR

Quantitative RT-PCR was performed with specific primers, as stated elsewhere.[2,4] More information is provided in the Methods section and Table E5 in this article's Online Repository.

Statistics

Statistical analysis was performed with SPSS 13.0 software (SPSS, Chicago, Ill). Expression data are presented in dot plots. Symbols represent individual samples; horizontal bars represent medians, and error bars show interquartile ranges. Cell-culture data are expressed as means ± SDs. When comparisons were made between groups, the Kruskal-Wallis H test was used to assess significant intergroup variability. The 2-tailed Mann-Whitney U test was used for between-group comparison. The Spearman rank test was used for correlations. Significance was accepted at a P value of less than .05. For multiple comparisons among 3 study groups, Bonferroni correction was used to adjust the significance level by using an α value of $.05/3 = 0.017$ for each comparison.

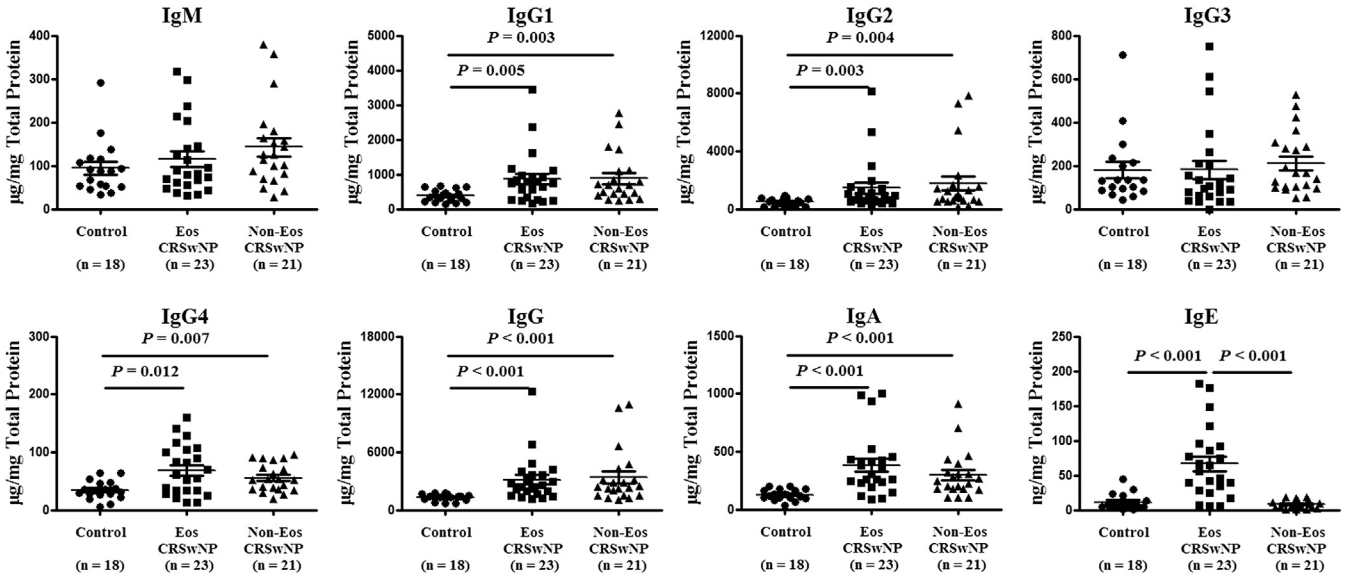


FIG 1. Increased local immunoglobulin levels in nasal polyp tissues. Protein levels of IgM, IgG and IgG subclasses, IgA, and IgE in tissue homogenates from control tissue, eosinophilic chronic rhinosinusitis with nasal polyps *(Eos CRSwNP)*, and noneosinophilic CRSwNP *(Non-Eos CRSwNP)* are detected by using the Bio-Plex Assay.

RESULTS

Enhanced local immunoglobulin levels in nasal polyp tissues

We first confirmed increased local IgE levels in eosinophilic polyps in comparison with that seen in noneosinophilic polyps and control nasal tissues (Fig 1).[4] Moreover, we found that levels of total IgG, IgG_1, IgG_2, IgG_4, and IgA in nasal tissues were upregulated similarly in both patients with eosinophilic and those with noneosinophilic CRSwNP compared with those in control subjects (Fig 1).

Enrichment of $CXCR5^+CD4^+$ T_{FH} cells in nasal polyp tissues

Given the enhanced local immunoglobulin levels in nasal polyps, we next explored the presence of T_{FH} cells in nasal polyps. As shown in Fig E2 in this article's Online Repository at www.jacionline.org, the mRNA expression of the T_{FH} cell transcription factor genes B-cell lymphoma 6 *(BCL6)* and v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog and a transcription factor gene critical for plasma cell differentiation, B lymphocyte–induced maturation protein 1 *(BLIMP1)*, was increased in both eosinophilic and noneosinophilic polyps compared with that seen in control nasal tissues. CD40 ligand and neuropilin 1 (Nrp1) are the cell-surface molecules expressed on activated T_{FH} cells. The mRNA expression of *CD40L* and neuropilin 1 *(NRP1)* has been found to be upregulated in eosinophilic and noneosinophilic polyps. In addition, the mRNA expression of *IL21* and IL-21 receptor *(IL21R)*, which are critical in regulating T_{FH} cell and B-cell differentiation, were markedly enhanced in polyp tissues. There was also a significant upregulation of the expression of B-cell and T_{FH} cell chemokines and corresponding chemokine receptor genes in polyps, including *CXCL13-CXCR5* and *CXCL12-CXCR4*, compared with that seen in control nasal tissues.

Our previous study demonstrated a similar increase in NMC counts in eosinophilic and noneosinophilic polyps compared with those in control nasal tissues.[2] Current flow cytometric analysis revealed a higher frequency of $CD4^+$ T cells within NMCs from both patients with eosinophilic and those with noneosinophilic CRSwNP compared with that seen in control subjects (Fig 2, *B*). Importantly, for the first time, we discovered that the frequencies of $CXCR5^+CD4^+$ T_{FH} cells in both NMCs and $CD4^+$ T cells were increased similarly in eosinophilic and noneosinophilic polyp tissues when compared with those in control tissue (Fig 2, *B*). Further analysis revealed that these nasal mucosal $CXCR5^+CD4^+$ T_{FH} cells can also express ICOS and PD1. The percentages of $ICOS^+$, $PD1^+$, and $ICOS^+PD1^+$ T_{FH} cells among NMCs and total $CXCR5^+CD4^+$ T_{FH} cells were higher either in both eosinophilic and noneosinophilic polyps or only in eosinophilic polyps than in control tissues (Fig 2, *C* and *D*). In contrast, in peripheral blood no significant difference in the frequencies of the major types of T_{FH} cells could be found among different study groups (see Fig E3 in this article's Online Repository at www.jacionline.org). Representative fluorescence-activated cell sorting (FACS) plots showing the frequencies of T_{FH} cells in tissue and blood in different study groups are shown in Figs E4 and E5, respectively, in this article's Online Repository at www.jacionline.org.

Increased $IL\text{-}4^+$ T_{FH} cell subsets in eosinophilic nasal polyp tissues

Despite enhanced local IgE production in patients with eosinophilic CRSwNP compared with that seen in patients with noneosinophilic CRSwNP, we did not find a significant difference in total, $ICOS^+$, and $PD1^+$ T_{FH} cell counts between eosinophilic and noneosinophilic polyps. This prompted us to analyze the polarization of T_{FH} cells toward T_H1, T_H2, and T_H17 phenotypes in nasal polyp tissues. First, we found that the frequencies of $IL\text{-}21^+$, $IFN\text{-}\gamma^+$, and $IL\text{-}17A^+$ cells within the NMCs were increased in both eosinophilic and noneosinophilic polyps compared with control tissues; however, the percentages of $IL\text{-}4^+$

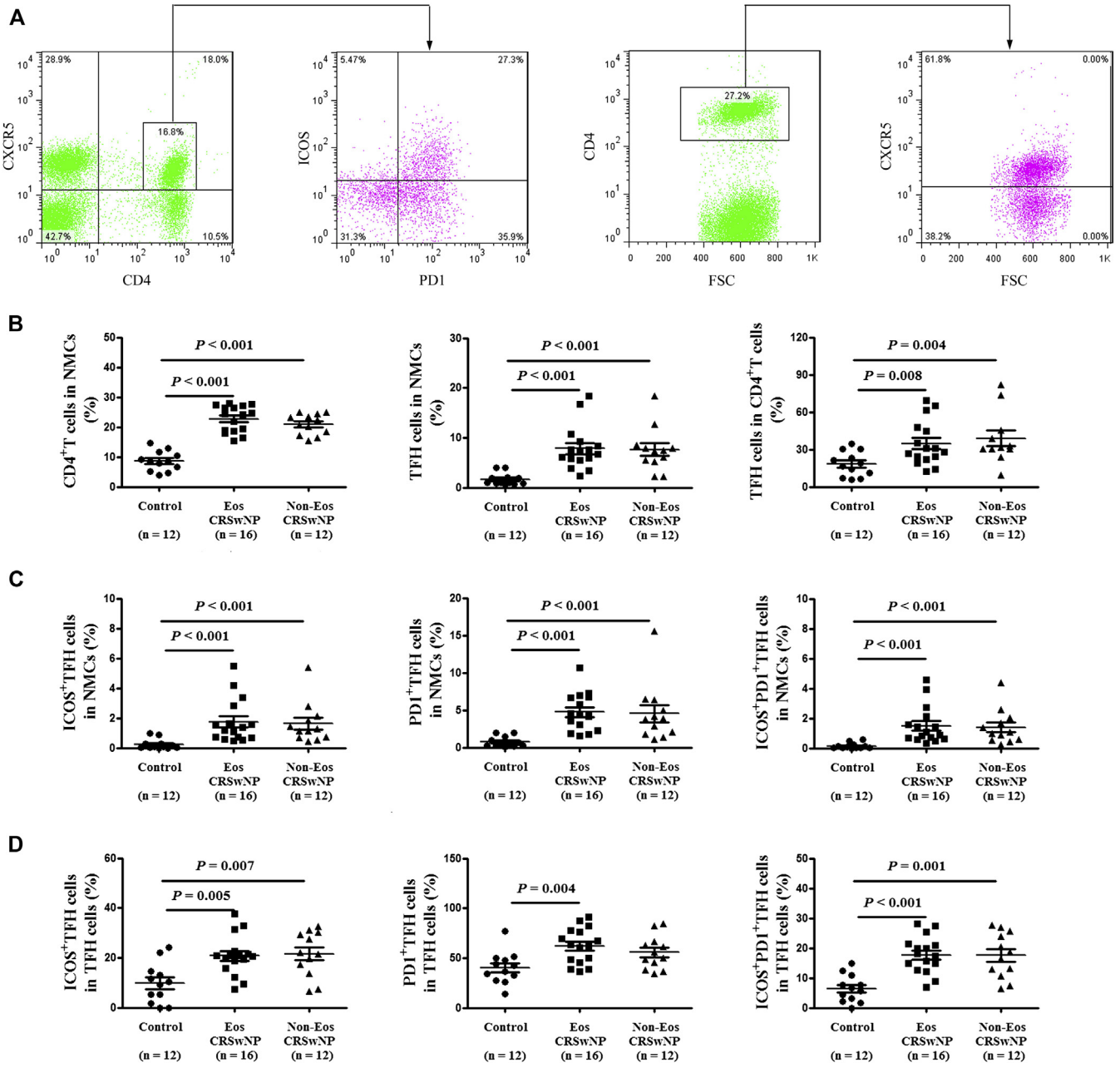


FIG 2. Expansion of CXCR5$^+$CD4$^+$ T_{FH} cells in nasal polyp tissues. **A,** Gating strategy and representative flow plots. T_{FH} cells were defined as CXCR5$^+$CD4$^+$ and further characterized based on ICOS and PD1 expression. **B,** Frequencies of CD4$^+$ T cells and T_{FH} cells in NMCs and percentages of T_{FH} cells in total CD4$^+$ T cells. **C** and **D**, Frequencies of ICOS$^+$, PD1$^+$, and ICOS$^+$PD1$^+$ T_{FH} cells in NMCs (Fig 2, *C*) and total T_{FH} cells (Fig 2, *D*). *Eos CRSwNP*, Eosinophilic CRSwNP; *Non-Eos CRSwNP*, noneosinophilic CRSwNP.

and IL-4$^+$IL-21$^+$ cells within NMCs were only increased in eosinophilic polyps compared with those in noneosinophilic polyps and control tissues (Fig 3, *B*). Furthermore, we discovered that the percentages of IL-21$^+$, IFN-γ$^+$, and IL-17A$^+$ T_{FH} cells in both NMCs and total CXCR5$^+$CD4$^+$ T_{FH} cells were increased similarly in eosinophilic and noneosinophilic polyps compared with those of control tissue, although the percentages of IL-4$^+$ and IL-4$^+$IL-21$^+$ T_{FH} cells among both NMCs and total T_{FH} cells were only increased in eosinophilic polyps in comparison with those in noneosinophilic polyps and control tissues (Fig 3, *C* and *D*).

We next assessed whether the increase in T_{FH} cell counts in eosinophilic polyp tissues might also be reflected in an antigen-specific expansion or was independent of antigen specificity. Our previous study has shown that dust mite allergens are one of the major antigens driving local IgE production in Chinese patients with eosinophilic CRSwNP.[4] Therefore we selected eosinophilic polyps with local IgE against *Dermatophagoides pteronyssinus* group 1 (Der p 1) allergen and compared them with noneosinophilic polyps without obvious local IgE against Der p 1 (see Table E6 in this article's Online Repository at

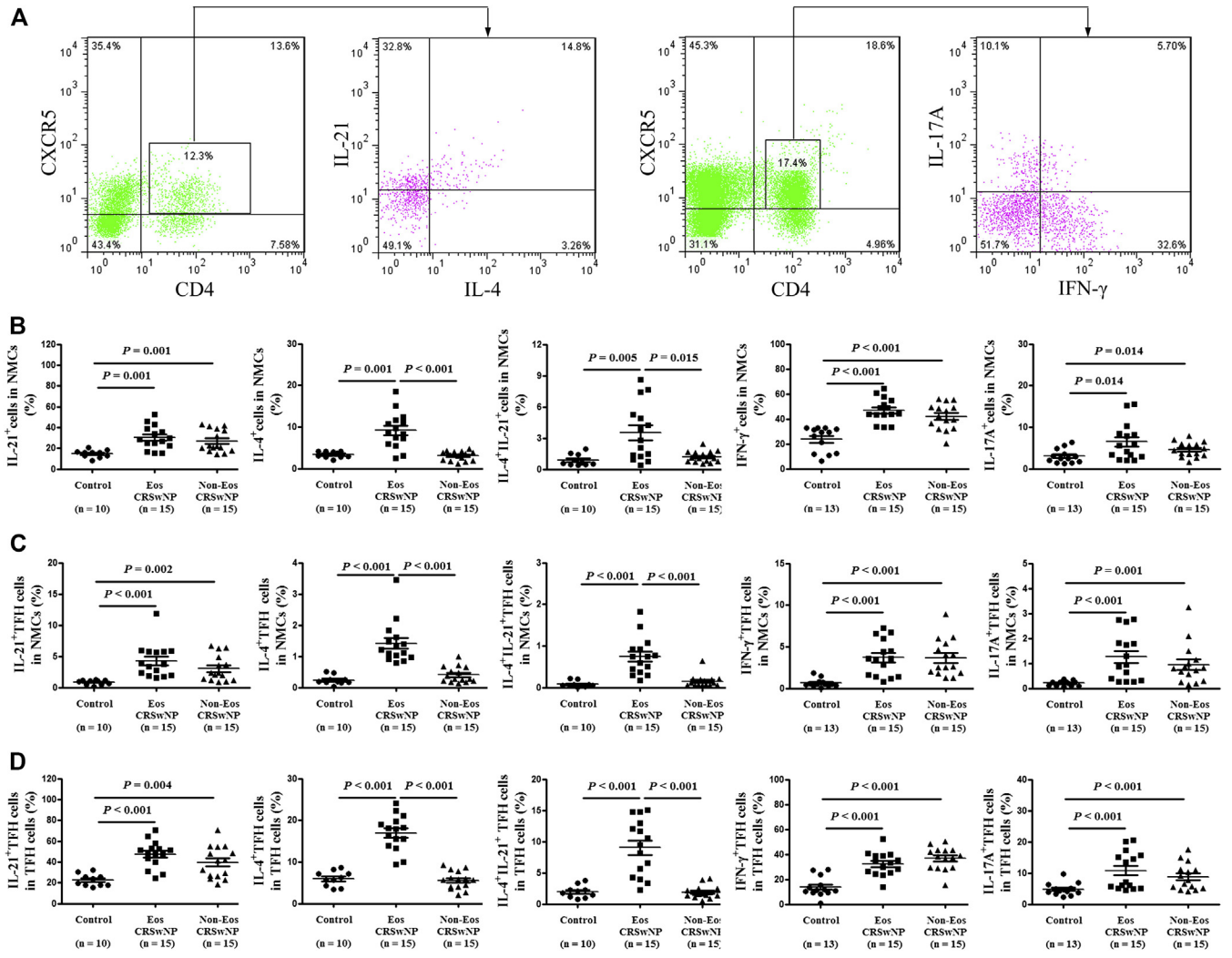


FIG 3. Skewed IL-4$^+$ T_{FH} cells in eosinophilic nasal polyp tissues. **A,** Gating strategy and representative flow plots. T_{FH} cells were defined as CXCR5$^+$CD4$^+$ cells and further characterized based on the cytokine production of IL-21 and IL-4 or IFN-γ and IL-17A. Analysis of IL-21 and IL-4 and IL-17A and IFN-γ expression was conducted on different sample sets because of a limited amount of tissue samples. **B,** Percentages of IL-21$^+$, IL-4$^+$, IL-4$^+$IL-21$^+$, IL-17A$^+$, and IFN-γ$^+$ cells in NMCs. **C** and **D**, Percentages of IL-21$^+$, IL-4$^+$, IL-4$^+$IL-21$^+$, IL-17A$^+$, and IFN-γ$^+$ T_{FH} cells in NMCs (Fig 3, *C*) and total T_{FH} cells (Fig 3, *D*). *Eos CRSwNP,* Eosinophilic CRSwNP; *Non-Eos CRSwNP,* noneosinophilic CRSwNP.

www.jacionline.org). We stimulated NMCs extracted from polyp tissues with Der p 1 allergen. We found that the percentages of IL-4$^+$, IL-21$^+$, and IL-4$^+$IL-21$^+$ T_{FH} cells were significantly increased in patients with eosinophilic CRSwNP but not in patients with noneosinophilic CRSwNP after stimulation (see Fig E6 in this article's Online Repository at www.jacionline.org), indicating an antigen-specific T_{FH} cell response in patients with eosinophilic CRSwNP.

We also investigated the distribution of nasal mucosal T_{FH} cells using immunohistochemical staining. Consistent with our previous report,[4] a lymphoid follicle-like structure with T/B-cell aggregation could be found in both eosinophilic and noneosinophilic polyp tissues. Bcl-6$^+$CD4$^+$ T_{FH} cells could be identified in these ectopic lymphoid structures in both eosinophilic and noneosinophilic polyps, whereas IL-4$^+$Bcl-6$^+$CD4$^+$ T_{FH} cells were mainly found in ectopic lymphoid structures in eosinophilic polyp tissues (Fig 4).

Again, there was no significant difference in the percentage of IL-21$^+$, IL-4$^+$, IFN-γ$^+$, IL-17A$^+$ or IL-21$^+$IL-4$^+$ cells or the corresponding T_{FH} cell subset in the blood among different study groups (see Fig E7 in this article's Online Repository at www.jacionline.org). Representative FACS plots showing the frequencies of these cells in tissue and blood in different study groups are shown in Figs E8 and E9, respectively, in this article's Online Repository at www.jacionline.org.

Distinct phenotype of nasal polyp CXCR5$^+$CD4$^+$ T_{FH} cells

Previous studies demonstrated a distinct phenotype of blood CXCR5$^+$CD4$^+$ T_{FH} cells compared with *bona fide* GC T_{FH} cells.[13,15] Consistent with those previous reports,[13,15] no obvious Bcl-6 expression and low intensity of ICOS and PD1 expression were found in blood T_{FH} cells in this study (Fig 5

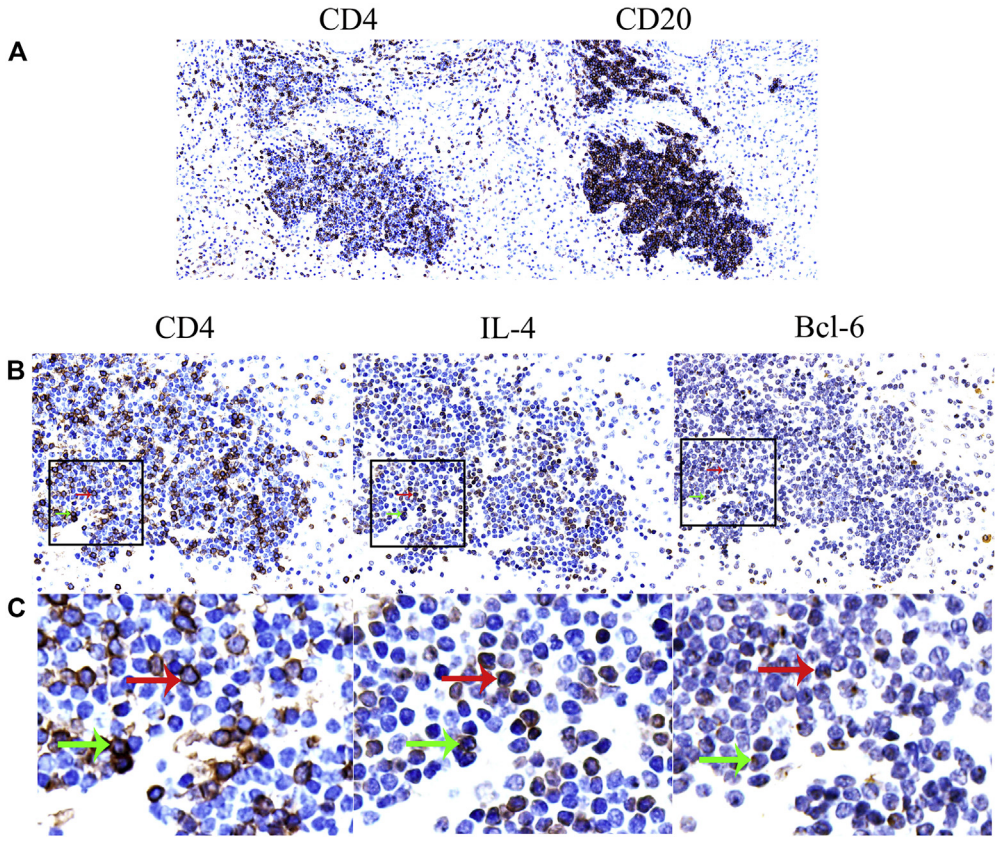


FIG 4. Distribution of IL-4$^+$ T_{FH} cells in ectopic lymphoid structures in eosinophilic nasal polyp tissues. **A** and **B**, Representative photomicrographs showing successive serial sections of paraffin-embedded nasal polyp tissue from a patient with eosinophilic nasal polyps stained by using immunohistochemistry for CD20, CD4, IL-4, and Bcl-6. Fig 4, *A*, Original magnification ×200. Fig 4, *B*, Original magnification ×400. **C,** Higher magnification of the outlined area of Fig 4, *B*. *Arrows* of the same color indicate the same cell in serial sections.

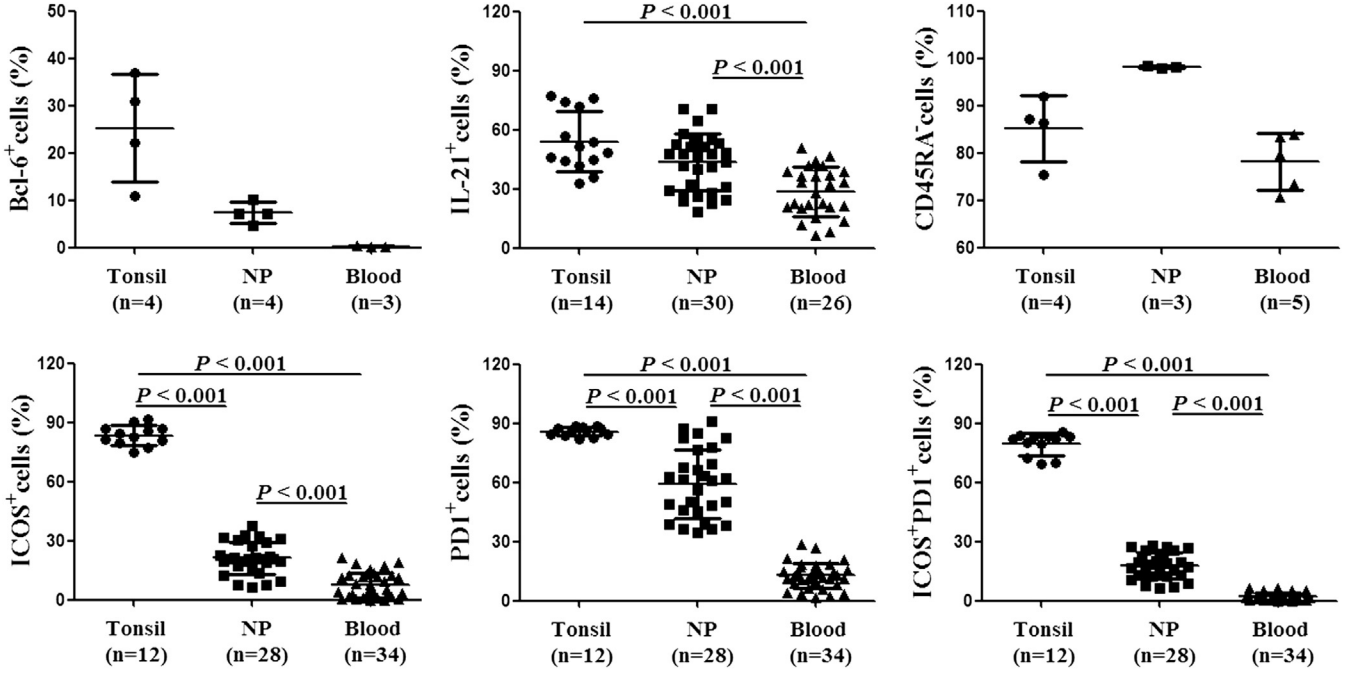
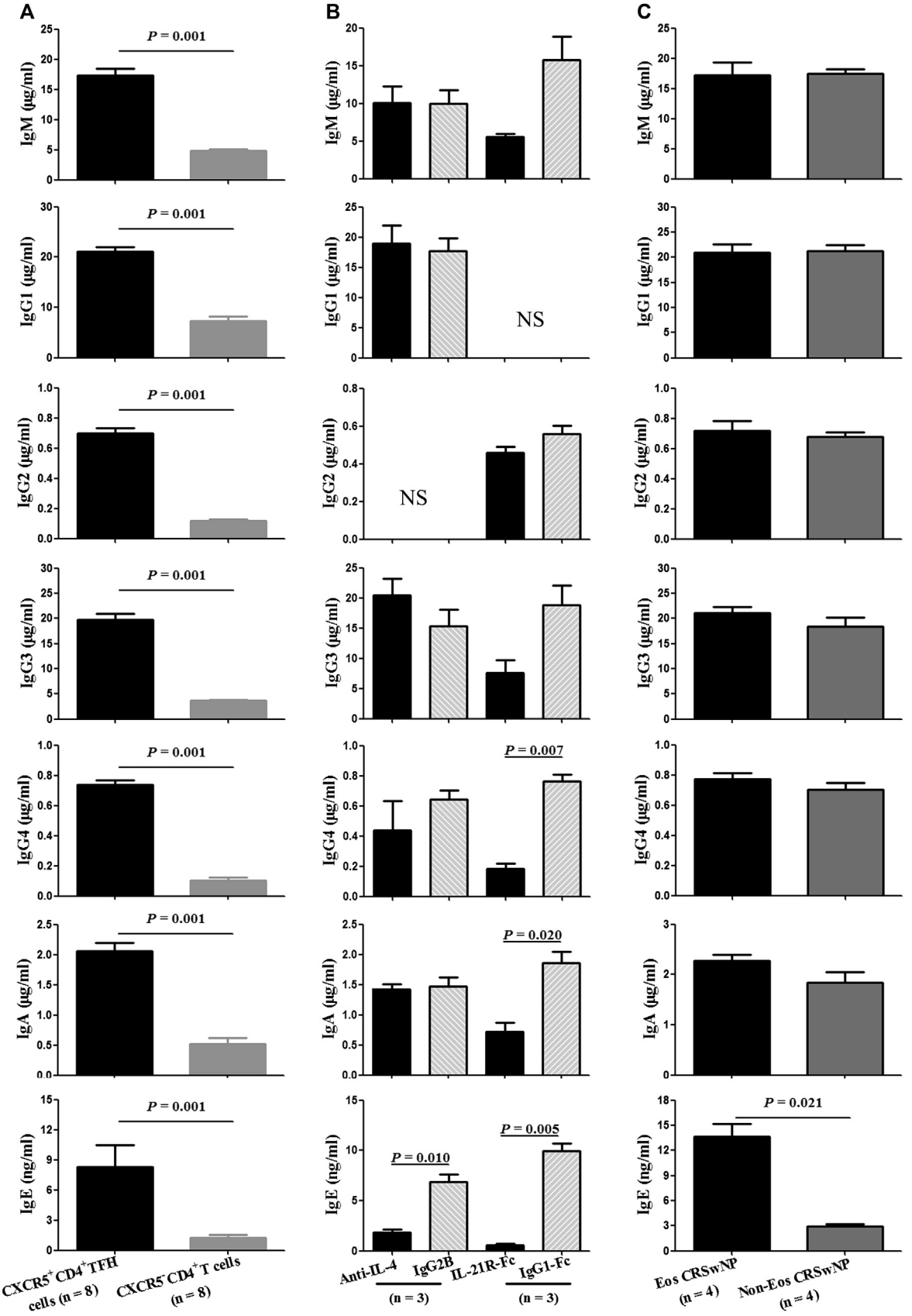


FIG 5. Phenotypic characteristics of nasal polyp *(NP)* CXCR5$^+$CD4$^+$ T_{FH} cells. Compared with those of tonsillar and circulating T_{FH} cells, the frequencies of Bcl-6$^+$, IL-21$^+$, ICOS$^+$, PD1$^+$, and ICOS$^+$PD1$^+$ T_{FH} cells within total CXCR5$^+$CD4$^+$ T_{FH} cells were presented at intermediate levels in eosinophilic and noneosinophilic nasal polyp tissues. Similar to tonsillar and circulating T_{FH} cells, most nasal polyp T_{FH} cells were CD45RA$^-$ memory cells.


A
B
C
IgM (μg/ml)
IgG1 (μg/ml)
IgG2 (μg/ml)
IgG3 (μg/ml)
IgG4 (μg/ml)
IgA (μg/ml)
IgE (ng/ml)
P = 0.001
NS
P = 0.007
P = 0.020
P = 0.010
P = 0.005
P = 0.021
CXCR5+ CD4+ TFH cells (n = 8)
CXCR5- CD4+ T cells (n = 8)
Anti-IL-4
IgG2B
IL-21R-Fc
IgG1-Fc
(n = 3)
(n = 3)
Eos CRSwNP (n = 4)
Non-Eos CRSwNP (n = 4)

and see Fig E10 in this article's Online Repository at www.jacionline.org). Similar to *bona fide* GC T_{FH} cells in tonsils, circulating T_{FH} cells in blood, or both, we found that most nasal polyp T_{FH} cells were $CD45RA^-$ memory cells and expressed higher levels of ICOS and Bcl-6 than $CXCR5^-$ T cells (see Fig E10, *A*). Moreover, we found that polyp T_{FH} cells expressed T_{FH} cell markers, including Bcl-6, IL-21, ICOS, and PD1, at an intermediate level compared with tonsillar GC T_{FH} cells and circulating T_{FH} cells (Fig 5). In addition, we found that polyp T_{FH} cells produced more IL-21 and IL-4 than $CXCR5^-$ T cells (see Fig E10, *B*). In contrast, polyp $CXCR5^-$ T cells expressed higher levels of IFN-γ and IL-17A than T_{FH} cells (see Fig E10, *B*). Representative FACS plots showing expression of Bcl-6, IL-21, ICOS, PD1, and CD45RA in $CXCR5^+CD4^+$ and $CXCR5^-CD4^+$ cells from tonsils, nasal polyps, and blood are shown in Fig E11 in this article's Online Repository at www.jacionline.org.

Nasal polyp IL-4^+ T_{FH} cells participate in IgE production

Sequentially, we investigated the helper function of nasal polyp $CXCR5^+CD4^+$ T_{FH} cells on naive B cells. We found that polyp $CXCR5^+$ T_{FH} cells presented potent capacity to promote IgM, IgG_1, IgG_2, IgG_3, IgG_4, IgA, and IgE production, whereas polyp $CXCR5^-$ T cells induced production of these immunoglobulins at very low levels (Fig 6, *A*). Consistent with previous reports,[13] we did not find a significant difference in T-cell viability between $CXCR5^+$ and $CXCR5^-$ T cells after coculture (data not shown), suggesting that the inability of $CXCR5^-$ T cells to induce immunoglobulin production was not caused by their survival status. In coculture of $CXCR5^+CD4^+$ T_{FH} cells sorted from eosinophilic polyps with naive B cells, IL-21 blockade resulted in decreased IgG, IgA, and IgE production (Fig 6, *B*), suggesting that similar to circulating and GC T_{FH} cells, the capacity of nasal polyp $CXCR5^+CD4^+$ T_{FH} cells to promote immunoglobulin production is dependent to some extent on IL-21. However, blocking IL-4 resulted in a substantial inhibition of IgE production but not of other immunoglobulins (Fig 6, *B*). In a blocking experiment IgG_1 or IgG_2 could not be detected because of cross-reactivity with a blocking experiment reagent. In addition, we found that $CXCR5^+CD4^+$ T_{FH} cells from eosinophilic polyps induced significantly upregulated production of IgE from B cells than those from patients with noneosinophilic CRSwNP, despite inducing comparable production of other immunoglobulins (Fig 6, *C*).

Skewed B-cell compartments are associated with T_{FH} cell counts in nasal tissues

Given the presence of functional T_{FH} cells in nasal mucosa, we studied the changes of B-cell compartments in nasal polyps. We referred to the populations according to the B mature (Bm; Bm1-Bm5) nomenclature.[11] We observed an obvious skewing of nasal B-cell populations toward GC (Bm3/4) and memory (Bm5) B cells and plasma cells accompanied by a loss of naive (Bm1) and activated naive (Bm2) B cells in both eosinophilic and noneosinophilic polyps in comparison with control tissues (Fig 7). A strong positive correlation between the frequency of nasal GC (Bm3/4) B cells and T_{FH} cells ($R = 0.875$, $P < .001$) was discovered (Fig 7). In addition, a modest positive correlation between the percentage of plasma cells and T_{FH} cells ($R = 0.396$, $P = .030$) and a modest negative association between the percentage of naive (Bm1) B cells and T_{FH} cells ($R = -0.373$, $P = .042$) were revealed (Fig 7). Similar to circulating T_{FH} cells, we observed no significant difference in the percentages of different B-cell populations in the blood among the different study groups (see Fig E12 in this article's Online Repository at www.jacionline.org).

Nasal IL-4^+ T_{FH} cell counts correlate with local IgE levels

We analyzed the relationship between different T_{FH} cell subsets and local immunoglobulin levels in nasal mucosa. We discovered that the frequencies of local IL-4^+, IL-21^+, and IL-4^+IL-21^+ T_{FH} cells, but not total T_{FH} cells, IFN-γ^+ T_{FH} cells, or IL-$17A^+$ T_{FH} cells, were positively correlated with IgE levels in nasal tissues (Fig 8). In addition, percentages of IL-21^+ and IL-$17A^+$ T_{FH} cells were positively correlated with IgG levels (see Fig E13 in this article's Online Repository at www.jacionline.org), and percentages of total T_{FH} cells and IL-4^+, IL-21^+, and IL-4^+IL-21^+ T_{FH} cells were positively correlated with IgA levels in nasal tissues (Fig 8 and see Fig E13). No significant correlation between total T_{FH} cells or T_{FH} cell subsets and IgM levels in nasal tissue was discovered (Fig 8 and see Fig E13).

DISCUSSION

Local IgE production might play an important role in the development of mucosal eosinophilia not only in nasal polyps but also in patients with allergic rhinitis, asthma, and eosinophilic esophagitis, yet the mechanisms underlying this mucosal IgE overproduction remain poorly understood.[3,21,22] Recently, the

FIG 6. Nasal polyp IL-4^+ T_{FH} cells are able to induce IgE production. Immunoglobulin concentrations at day 8 in the coculture of $CXCR5^+CD4^+$ T_{FH} cells or $CXCR5^-CD4^+$ T cells with autologous naive B cells were measured by using the Bio-Plex Assay. Data are presented as means ± SDs. **A,** $CXCR5^+$ T_{FH} cells and $CXCR5^-$ T cells sorted from 8 nasal polyp samples (4 eosinophilic and 4 noneosinophilic) were compared. $CXCR5^+$ T_{FH} cells, but not $CXCR5^-$ T cells, could efficiently induce immunoglobulin production from B cells. **B,** $CXCR5^+$ T_{FH} cells purified from 3 eosinophilic nasal polyp samples were studied. In coculture of those T_{FH} cells with autologous naive B cells, IL-21 blockage suppressed the production of all types of immunoglobulins; however, IL-4 blockage only diminished IgE production. IgG_1 and IgG_2 levels were not shown because of cross-reactivity with blocking experiment reagents. **C,** $CXCR5^+$ T_{FH} cells purified from 4 eosinophilic and 4 noneosinophilic nasal polyp samples were compared. T_{FH} cells isolated from eosinophilic nasal polyp tissues, but not from noneosinophilic nasal polyp tissue, could efficiently induce IgE production from B cells, although T_{FH} cells from both types of polyp tissues were able to promote production of other immunoglobulins. *Eos CRSwNP*, Eosinophilic CRSwNP; *Non-Eos CRSwNP*, noneosinophilic CRSwNP; *NS*, not shown.

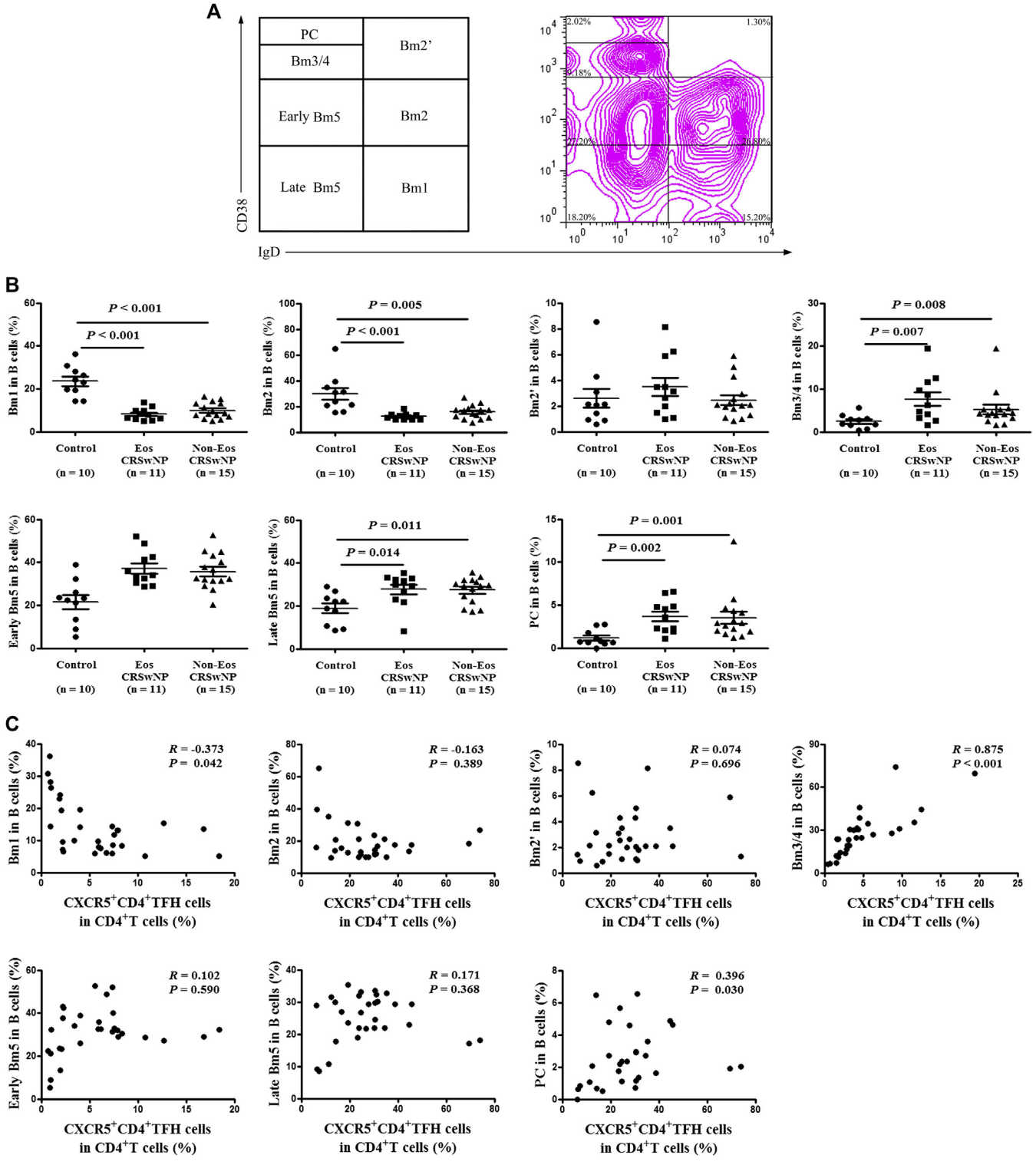


FIG 7. Skewing within the B-cell compartment in nasal polyp tissues correlates with local $CXCR5^+CD4^+$ T_{FH} cell expansion. **A,** Gating strategy and representative flow plots. B cells were defined as $CD3^-CD19^+$ and further characterized based on the Bm classification: *Bm1* (naive), $CD38^-IgD^+$; *Bm2* (activated naive), $CD38^+IgD^+$; *Bm2'* (pre-GC), $CD38^{++}IgD^+$; *Bm3/4* (GC), $CD38^{++}IgD^-$; *Early Bm5* (early memory), $CD38^+IgD^-$; *Late Bm5* (late memory), $CD38^-IgD^-$; and plasma cells *(PCs)*, $CD38^{+++}IgD^-$. **B,** Frequencies of different B-cell compartments within total B cells in nasal tissues from control subjects, patients with eosinophilic CRSwNP *(Eos CRSwNP)*, and patients with noneosinophilic CRSwNP *(Non-Eos CRSwNP)*. **C,** Significant positive correlations between T_{FH} cell frequency and GC (Bm3/4) B-cell and PC frequency and a significant negative correlation between T_{FH} cell frequency and naive (Bm1) B-cell frequency in the sinonasal mucosa from all study groups (n = 30).

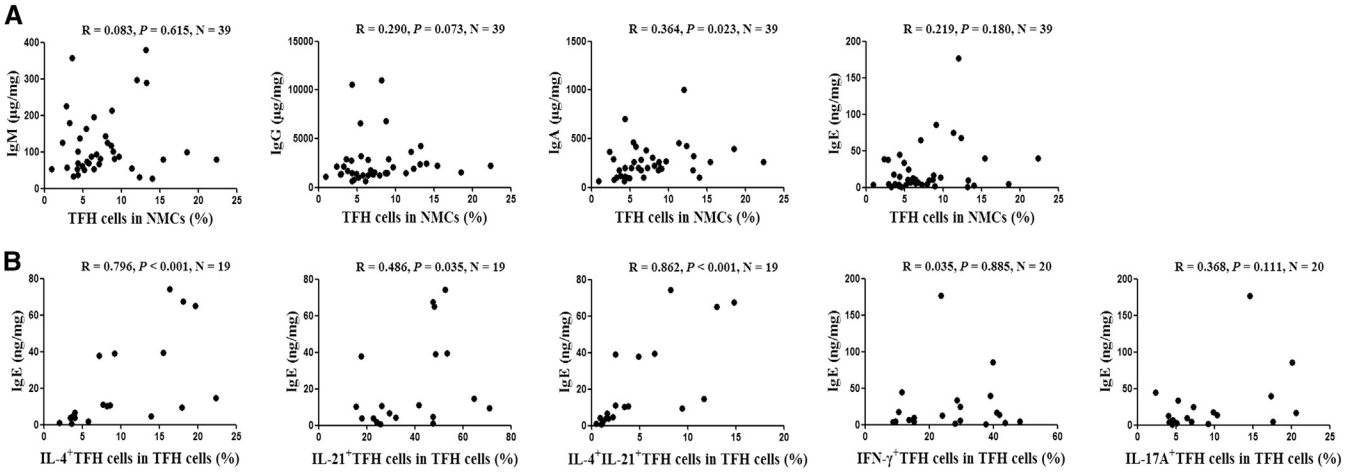


FIG 8. Correlations between immunoglobulin levels and frequencies of total T_{FH} cells and between IgE levels and frequencies of T_{FH} cell subsets in sinonasal mucosa. **A,** No significant correlation between the frequency of total $CXCR5^+CD4^+$ T_{FH} cells and levels of immunoglobulins, except IgA, in the sinonasal mucosa from all study groups. **B,** Significant positive correlations between IgE and IL-4^+, IL-21^+, and IL-4^+IL-21^+ T_{FH} cells were demonstrated in the sinonasal mucosa from all study groups.

percentages of circulating T_{FH} cells have been found to be increased in patients with autoimmune diseases and correlated with serum concentrations of IgG autoantibodies[9,10]; however, the role of T_{FH} cells in patients with IgE-mediated diseases has been barely studied.

In the present study we provided evidence of accumulation and expansion of $CXCR5^+CD4^+$ T_{FH} cells in both eosinophilic and noneosinophilic nasal polyp tissues. These mucosal T_{FH} cells demonstrated phenotypic differences compared with *bona fide* GC T_{FH} cells and blood T_{FH} cells, with the expression of ICOS, PD1, and Bcl-6 at an intermediate level. These T_{FH} cells could be identified in ectopic lymphoid structures in nasal polyp tissues and could efficiently induce immunoglobulin production from naive B cells. These features of nasal T_{FH} cells are partly similar to those of $CXCR5^{lo}ICOS^{lo}CD4^+$ T cells residing outside of GCs, which are possible precursors of GC T_{FH} cells in human tonsils, indicating an immature phenotype of T_{FH} cells in ectopic lymphoid structures.[19] To clarify the ontogeny of these human T_{FH} cells will be of great interest. In addition, we found that the frequency and numbers of $ICOS^+$, $PD1^+$, and $ICOS^+PD1^+$ T_{FH} cell subsets that represent more activated subpopulations in T_{FH} cells with superior capacity to help B cells[12-15] were also increased in eosinophilic and noneosinophilic nasal polyps, further suggesting a dysfunction of T_{FH} cells in diseased mucosa of patients with CRSwNP.

Increased numbers of B cells have been reported in polyp tissues[3,20]; nevertheless, the phenotypes of B cells have never been delineated in patients with CRSwNP. In this study a significant skewing of B-cell populations toward GC B cells and plasma cells was observed in polyp tissues, which was correlated with the expansion of local T_{FH} cells, suggesting a T_{FH} cell–dependent process of B-cell phenotype perturbation.[23] Our findings also underscore a potential role of dysregulated lesional T_{FH} cells in the formation and maintenance of the ectopic lymphoid structures in nasal polyp tissues.

In the present study we did not find significant differences in local total $CXCR5^+CD4^+$ T_{FH} cell counts and phenotypes of B cells between eosinophilic and noneosinophilic nasal polyps. Recent studies have demonstrated that blood memory T_{FH} cells are composed of heterogeneous cell populations with discrete capabilities to support B cells to produce antibodies.[12-15] $CXCR3^-CCR6^-$ (T_H2) and $CXCR3^-CCR6^+$ (T_H17) T_{FH} cells can induce B cells to produce IgG and IgE and IgG and IgA, respectively, whereas $CXCR3^+CCR6^-$ (T_H1) T_{FH} cells do not produce IL-21 and lack the ability to help naive B cells.[13] The enhanced local IgE production in eosinophilic but not noneosinophilic nasal polyps prompted us to explore whether polyp T_{FH} cells encompass distinct subsets and whether the skewing of polyp T_{FH} cell subsets might underlie the IgE overproduction in eosinophilic polyps. We found that the frequency and numbers of IL-21^+, IL-17^+, and IFN-γ^+ T_{FH} cells were increased comparably in eosinophilic and noneosinophilic polyps; in contrast, IL-4^+ and IL-4^+IL-21^+ T_{FH} cells were uniquely increased in eosinophilic polyps, depicting distinct profiles of T_{FH} cell subsets in nasal polyps with different inflammation patterns. We also tested the relationship between $CXCR3^-CCR6^-$ T_{FH} cells and IL-4^+ T_{FH} cells in nasal polyps. Consistent with the findings in circulating $CXCR5^+CD4^+$ T_{FH} cells,[13,24] we found here that nasal IL-4^+ T_{FH} cells were not definitely $CXCR3^-CCR6^-$ cells because some of them could be found in $CXCR3^-CCR6^+$ and $CXCR3^+CCR6^-$ T_{FH} cells, and $CXCR3^-CCR6^-$ T_{FH} cells were not necessarily IL-4^+ cells (data was not shown). Therefore given the importance of the cytokines in inducing immunoglobulin class-switching, we believe that it might be more suitable to define the T_{FH} cell subset based on cytokine expression in our current study. Importantly, we revealed that only $CXCR5^+CD4^+$ T_{FH} cells from eosinophilic polyps, but not those from noneosinophilic polyps, promoted IgE production, which could be suppressed by IL-21 and IL-4 blockage. Moreover, we found that the frequency of IL-4^+ cells was higher in $CXCR5^+$ T_{FH} cells than in $CXCR5^-$ T cells in nasal polyp tissues and that IL-4^+Bcl-6^+CD4^+ T_{FH} cells existed in ectopic lymphoid structure in eosinophilic polyps. These results underscore a critical role of nasal mucosal IL-4^+ T_{FH} cells in local IgE induction, which was further confirmed *in vivo* by the

finding that the number of nasal mucosal IL-4$^+$, IL-21$^+$, and IL-4$^+$IL-21$^+$ T_{FH} cells, but not total CXCR5$^+$CD4$^+$ T_{FH} cells or other T_{FH} cell subsets, correlated with local IgE levels. Our previous study has shown that dust mite allergens are the major antigens driving local IgE production in Chinese patients with eosinophilic CRSwNP.[4] Here we demonstrated the presence of dust mite–specific T_{FH} cells in eosinophilic polyps with local IgE against dust mites, reflecting an antigen-specific expansion of lesional IL-4$^+$ T_{FH} cells in patients with eosinophilic CRSwNP. In contrast to IgE, we found that IgG and IgA levels were similarly enhanced in eosinophilic and noneosinophilic polyps and that CXCR5$^+$CD4$^+$ T_{FH} cells from eosinophilic and noneosinophilic polyps induced comparable amounts of IgG and IgA production from naive B cells. Moreover, we found that local IgG and IgA levels might be related to IL-17A$^+$ and IL-21$^+$ T_{FH} cells and IL-4$^+$, IL-21$^+$, and total T_{FH} cells in nasal tissues, respectively. Therefore the distinct T_{FH} cell subset enrichment might contribute to the difference in immunoglobulin switching in polyp tissues. Interestingly, the local IgM levels did not correlate with nasal mucosal total T_{FH} cells or any T_{FH} cell subset, potentially suggesting a systemic origination rather than local production of IgM in nasal mucosa. Several previous reports have also demonstrated increased IgG and IgA levels in nasal polyps, suggesting that not only IgE but also other immunoglobulins might participate in CRS pathogenesis.[25,26]

In contrast to nasal polyp tissues, we did not find a marked difference in the frequency and numbers of total CXCR5$^+$CD4$^+$ T_{FH} cells, the majority of T_{FH} cell subsets, and B-cell compartments in peripheral blood among the different study groups, which is in line with our previous observation of T_H1/T_H2/T_H17 cell subsets and dendritic cells and further indicates a predominately localized immune response in patients with CRSwNP.[27]

There are several limitations associated with the present study. First, although we investigated PD1$^+$ and ICOS$^+$ T_{FH} cells and IFN-γ^+, IL-4$^+$, and IL-17$^+$ T_{FH} cells, respectively, further defining the polyp T_{FH} cell phenotypes by the combination of surface markers and cytokine expression would deepen our understanding of T_{FH} cells in inflamed tissues. Second, recently, CXCR5$^+$Bcl-6$^+$ forkhead box P3 (Foxp3)$^+$ follicular regulatory T cells have been identified in GCs with the capacity to suppress GC reactions.[28,29] It is critical to add Foxp3 expression to distinguish T_{FH} cells from Foxp3$^+$ follicular regulatory T cells in future researches.[30] Third, the ontogeny and mechanisms of polarization of T_{FH} cells in the tertiary lymphoid tissues remain to be elucidated.[31]

These comments notwithstanding, for the first time, our results reflect the existence of expanded T_{FH} cells in inflamed mucosal tissues from patients with CRSwNP. We suggest that tissue T_{FH} cells might promote localized B-cell differentiation and proliferation and thus induce local immunoglobulin production in nasal polyps. Mucosal IL-4$^+$ T_{FH} cells are critical for B-lymphocyte CSR to IgE in nasal polyps. These data not only extend our understanding of the mechanisms of CRSwNP and provide a potential new therapeutic target but also have broad implications for our understanding of the immunopathogenesis of other diseases characterized by the formation of ectopic lymphoid structures and local immunoglobulin production.

Clinical implications: Effective strategies can be designed to target nasal IL-4$^+$ T_{FH} cells in eosinophilic nasal polyps to suppress local IgE production.

REFERENCES

1. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. European position paper on rhinosinusitis and nasal polyps 2012. Rhinol Suppl 2012;23:1-299.
2. Cao PP, Li HB, Wang BF, Wang SB, You XJ, Cui YH, et al. Distinct immunopathologic characteristics of various types of chronic rhinosinusitis in adult Chinese. J Allergy Clin Immunol 2009;124:478-84.
3. Bachert C, Gavaert P, Holtappels G, Johansson SG, Van Cauwenberge P. Total and specific IgE in nasal polyps is related to local eosinophilic inflammation. J Allergy Clin Immunol 2001;107:607-14.
4. Cao PP, Zhang YN, Liao B, Ma J, Wang BF, Wang H, et al. Increased local IgE production induced by common aeroallergens and phenotypic alteration of mast cells in Chinese eosinophilic, but not non-eosinophilic, chronic rhinosinusitis with nasal polyps. Clin Exp Allergy 2014;44:690-700.
5. Gevaert P, Nouri-Aria KT, Wu H, Harper CE, Takhar P, Fear DJ, et al. Local receptor revision and class switching to IgE in chronic rhinosinusitis with nasal polyps. Allergy 2013;68:55-63.
6. Mechtcheriakova D, Sobanov Y, Holtappels G, Bajna E, Svoboda M, Jaritz M, et al. Activation-induced cytidine deaminase (AID)-associated multigene signature to assess impact of AID in etiology of diseases with inflammatory component. PLoS One 2011;6:e25611.
7. Chen M, Guo Z, Ju W, Ryffel B, He X, Zheng SG. The development and function of follicular helper T cells in immune responses. Cell Mol Immunol 2012;9:375-9.
8. Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG. Follicular helper T cells: lineage and location. Immunity 2009;30:324-35.
9. Ma CS, Deenick EK. Human T follicular helper (Tfh) cells and disease. Immunol Cell Biol 2014;92:64-71.
10. Tangye SG, Ma CS, Brink R, Deenick EK. The good, the bad and the ugly—TFH cells in human health and disease. Nat Rev Immunol 2013;13:412-26.
11. Lindqvist M, van Lunzen J, Soghoian DZ, Kuhl BD, Ranasinghe S, Kranias G, et al. Expansion of HIV-specific T follicular helper cells in chronic HIV infection. J Clin Invest 2012;122:3271-80.
12. He J, Tsai LM, Leong YA, Hu X, Ma CS, Chevalier N, et al. Circulating precursor CCR7(lo)PD-1(hi)CXCR5$^+$CD4$^+$ T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. Immunity 2013;39:770-81.
13. Morita R, Schmitt N, Bentebibel SE, Ranganathan R, Bourdery L, Zurawski G, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. Immunity 2011;34:108-21.
14. Locci M, Havenar-Daughton C, Landais E, Wu J, Kroenke MA, Arlehamn CL, et al. Human circulating PD-1+CXCR3-CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. Immunity 2013;39:758-69.
15. Schmitt N, Bentebibel SE, Ueno H. Phenotype and functions of memory Tfh cells in human blood. Trends Immunol 2014;35:436-42.
16. Gu-Trantien C, Loi S, Garaud S, Equeter C, Libin M, de Wind A, et al. CD4$^+$ follicular helper T cell infiltration predicts breast cancer survival. J Clin Invest 2013;123:2873-92.
17. Chu Y, Wang F, Zhou M, Chen L, Lu Y. A preliminary study on the characterization of follicular helper T (Tfh) cells in rheumatoid arthritis synovium. Acta Histochem 2014;116:539-43.
18. Mazerolles F, Picard C, Kracker S, Fischer A, Durandy A. Blood CD4+CD45RO+CXCR5+ T cells are decreased but partially functional in signal transducer and activator of transcription 3 deficiency. J Allergy Clin Immunol 2013;131:1146-56.
19. Bentebibel SE, Schmitt N, Banchereau J, Ueno H. Human tonsil B-cell lymphoma 6 (BCL6)-expressing CD4+ T-cell subset specialized for B-cell help outside germinal centers. Proc Natl Acad Sci U S A 2011;108:E488-97.
20. Hulse KE, Norton JE, Suh L, Zhong Q, Mahdavinia M, Simon P, et al. Chronic rhinosinusitis with nasal polyps is characterized by B-cell inflammation and EBV-induced protein 2 expression. J Allergy Clin Immunol 2013;131:1075-83.
21. Dullaers M, De Bruyne R, Ramadani F, Gould HJ, Gevaert P, Lambrecht BN. The who, where, and when of IgE in allergic airway disease. J Allergy Clin Immunol 2012;129:635-45.

22. Vicario M, Blanchard C, Stringer KF, Collins MH, Mingler MK, Ahrens A, et al. Local B cells and IgE production in the oesophageal mucosa in eosinophilic oesophagitis. Gut 2010;59:12-20.
23. Crotty S. T follicular helper cell differentiation, function, and roles in disease. Immunity 2014;41:529-42.
24. Le Coz C, Joublin A, Pasquali JL, Korganow AS, Dumortier H, Monneaux F. Circulating TFH subset distribution is strongly affected in lupus patients with an active disease. PLoS One 2013;8:e75319.
25. Kato A, Peters A, Suh L, Carter R, Harris KE, Chandra R, et al. Evidence of a role for B cell-activating factor of the TNF family in the pathogenesis of chronic rhinosinusitis with nasal polyps. J Allergy Clin Immunol 2008;121:1385-92.
26. Tan BK, Li QZ, Suh L, Kato A, Conley DB, Chandra RK, et al. Evidence for intranasal antinuclear autoantibodies in patients with chronic rhinosinusitis with nasal polyps. J Allergy Clin Immunol 2011;128:1198-206.
27. Shi LL, Song J, Xiong P, Cao PP, Liao B, Ma J, et al. Disease-specific T-helper cell polarizing function of lesional dendritic cells in different types of chronic rhinosinusitis with nasal polyps. Am J Respir Crit Care Med 2014;190:628-38.
28. Wollenberg I, Agua-Doce A, Hernández A, Almeida C, Oliveira VG, Faro J, et al. Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. J Immunol 2011;187:4553-60.
29. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, et al. Foxp3+ follicular regulatory T cells control the germinal center response. Nat Med 2011;17:975-82.
30. Vinuesa CG, Fagarasan S, Dong C. New territory for T follicular helper cells. Immunity 2013;39:417-20.
31. Ballesteros-Tato A, Randall TD. Priming of T follicular helper cells by dendritic cells. Immunol Cell Biol 2014;92:22-7.