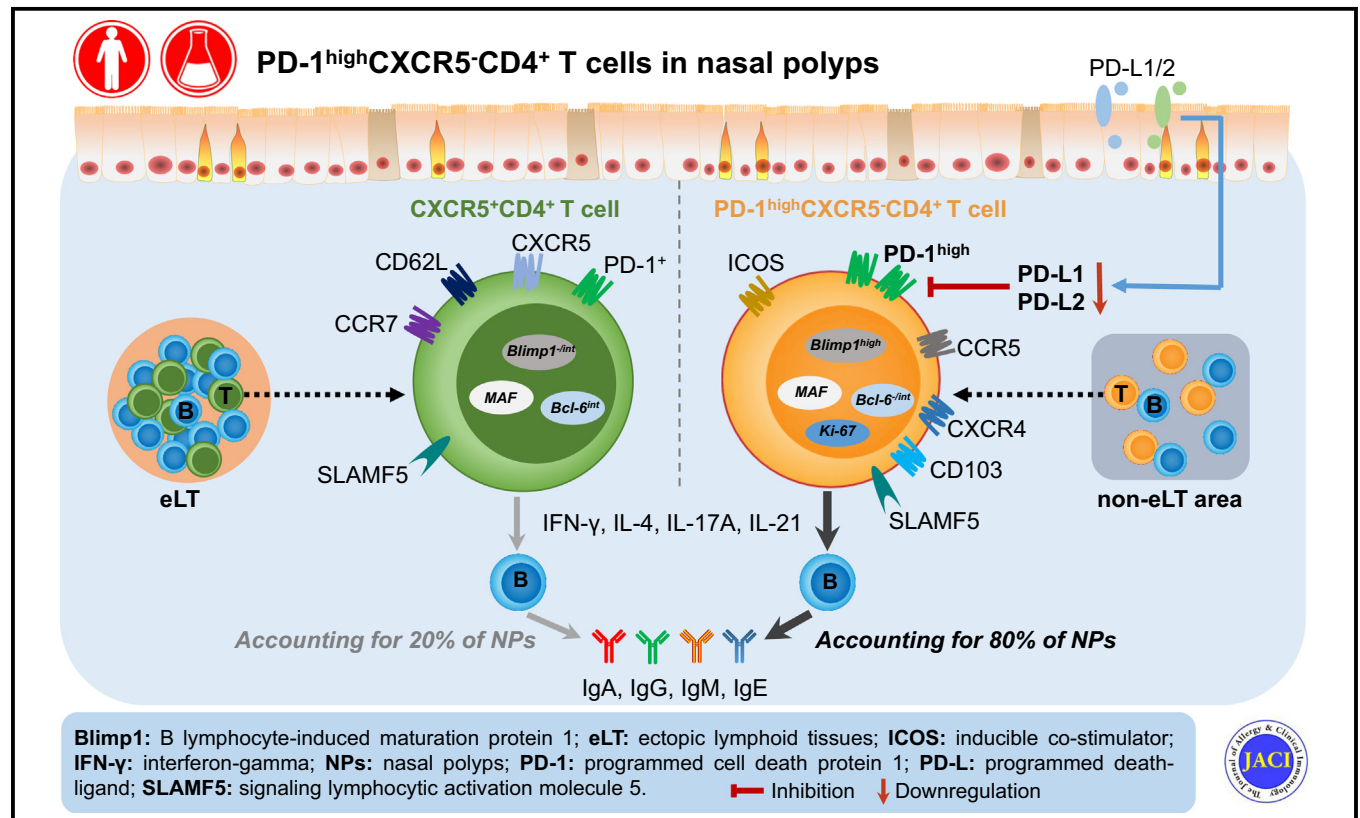


Extrafollicular PD-1^{high}CXCR5⁺CD4⁺ T cells participate in local immunoglobulin production in nasal polyps

Zhi-Chao Wang, MD, PhD,^{a*} Yin Yao, MD, PhD,^{a*} Cai-Ling Chen, MD,^a Cui-Lian Guo, MD, PhD,^a Hong-Xia Ding, MD,^a Jia Song, MD, PhD,^a Zhe-Zheng Wang, MD,^a Nan Wang, MD, PhD,^a Xue-Li Li, MD,^a Bo Liao, MD, PhD,^a Yang Yang, PhD,^b Di Yu, PhD,^a and Zheng Liu, MD, PhD^a *Wuhan, China, and Brisbane, Australia*

GRAPHICAL ABSTRACT



Background: Local immunoglobulin hyperproduction is observed in nasal polyps (NPs) with and without ectopic lymphoid tissues (eLTs).

Objective: Our aim was to identify the T-cell subsets involved in local immunoglobulin production independent of eLTs in NPs.

Methods: The localization, abundance, and phenotype of CD4⁺ T-cell subsets were studied by immunofluorescence, flow cytometry, and single-cell RNA sequencing. Purified nasal T-cell

subsets were cultured with autologous peripheral naive B cells to explore their function. Programmed death ligand 1 and programmed death ligand 2 expression in NPs was investigated by immunofluorescence staining and flow cytometry. **Results:** Accumulation of PD-1^{high}CXCR5⁺CD4⁺ T cells outside lymphoid aggregates was found in NPs. Nasal PD-1^{high}CXCR5⁺CD4⁺ T cells were characterized by a unique phenotype that was related to B-cell help and tissue residency

From ^athe Department of Otolaryngology-Head and Neck Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, and ^bThe University of Queensland Diamantina Institute, Faculty of Medicine, The University of Queensland, Brisbane.

*These authors contributed equally to this work

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Corresponding author: Zheng Liu, MD, PhD, Department of Otolaryngology-Head and Neck Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, No. 1095 Jiefang Ave, Wuhan 430030, China. E-mail: zhenglizheng@hotmail.com.

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and distinct from PD-1^{int}CXCR5⁺ and CXCR5⁺ CD4⁺ T cells in NPs as well as PD-1^{high}CXCR5^{high}CD4⁺ follicular helper T cells in tonsils. Compared with the frequencies of PD-1^{high}CXCR5⁺CD4⁺ T cells and their IFN- γ ⁺, IL-17A⁺, and IL-21⁺ subsets in the control inferior turbinate tissues, the frequencies of these cells and their subsets were increased in both eosinophilic and noneosinophilic NPs, whereas the frequencies of the IL-4⁺ and IL-4⁺IL-21⁺ subsets were increased only in eosinophilic NPs. Nasal PD-1^{high}CXCR5⁺CD4⁺ T cells induced immunoglobulin production from B cells in a potency comparable to that induced by tonsillar follicular helper T cells. PD-1^{high}CXCR5⁺CD4⁺ T-cell frequencies were correlated with IgE levels in eosinophilic NPs. PD-L1 and PD-L2 suppressed the function of PD-1^{high}CXCR5⁺CD4⁺ T cells, and their levels were reduced in NPs. PD-1^{high}CXCR5⁺CD4⁺ T-cell abundance was associated with the postsurgical relapse of NPs.

Conclusion: PD-1^{high}CXCR5⁺CD4⁺ T cells participate in local immunoglobulin production independent of eLTs in NPs. (J Allergy Clin Immunol 2021;■■■:■■■-■■■.)

Key words: Nasal polyps, immunoglobulin, T cell, programmed cell death protein 1, programmed death ligand

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a common upper airway disorder characterized by persistent inflammation in the sinonasal mucosa and polyp formation.^{1,2} Up to 20% to 40% of patients with CRSwNP respond poorly to current medical and surgical treatments, reflecting our limited understanding of the disease pathogenesis and a paucity of effective therapeutic interventions.²⁻⁶ Recently, emerging evidence has implicated local immunoglobulin overproduction as having a central role in the pathogenesis of CRSwNP.⁷⁻¹³ Enhanced levels of IgA, IgG, IgE, and IgD have been reported in nasal polyps (NPs).⁷⁻¹³ IgE and IgD activate mast cells and exaggerate eosinophilic inflammation,^{10,13} and IgG potentially activates the classical pathway of complements in NPs.⁹ Importantly, increased local IgE and IgG levels have been found to be associated with refractory NPs, requiring repeated surgery.^{9,11,12} In addition, recent phase 3 trials demonstrated that omalizumab (an anti-IgE mAb) was able to shrink NPs and significantly improve endoscopic, clinical, and patient-reported outcomes in patients with CRSwNP with inadequate response to intranasal corticosteroids.¹⁴ Thus, a better understanding of the mechanisms underlying the local immunoglobulin hyperproduction will benefit the development of novel therapeutic and prevention strategies for patients with CRSwNP.

T-cell-dependent immunoglobulin response requires a series of signals provided by T cells.¹⁵ We have recently identified the presence in NPs of ectopic lymphoid tissues (eLTs) and chemokine (C-X-C motif) receptor 5 (CXCR5)⁺CD4⁺ follicular helper T (T_{FH}) cells, which support local immunoglobulin production in an interleukin (IL)-21 dependent manner.^{7,16} The eLT formation is demonstrated in approximately 20% of NPs.¹⁶⁻¹⁸ However, an upregulation of local immunoglobulin production has also been noted in NPs without eLTs, albeit to a lower degree than that in NPs with eLTs,¹⁶ suggesting the presence of additional mechanisms beyond T_{FH} cells and eLTs in orchestrating local immunoglobulin production in NPs. IL-21 is a potent driver of B-cell terminal differentiation and immunoglobulin production, and it is considered a signature cytokine of T_{FH} cells.^{19,20}

Abbreviations used

AID:	Activation-induced cytidine deaminase
Bcl-6:	Transcription factor B-cell lymphoma 6
Blimp1:	B lymphocyte-induced maturation protein 1
CCR:	CC-chemokine receptor
CTLA-4:	Cytotoxic T-lymphocyte-associated protein 4
CXCL13:	Chemokine (C-X-C motif) ligand 13
CXCR:	Chemokine (C-X-C motif) receptor
CRSwNP:	Chronic rhinosinusitis with nasal polyps
eLT:	Ectopic lymphoid tissue
GC:	Germinal center
HLA-DR:	HLA-DR isotype
ICOS:	Inducible costimulator
int:	Intermediate
IT:	Inferior turbinate
NP:	Nasal polyp
PD-1:	Programmed cell death protein 1
PD-L1:	Programmed death ligand 1
PD-L2:	Programmed death ligand 2
SAP:	Signaling lymphocytic activation molecule-associated protein
SLAMF5:	Signaling lymphocytic activation molecule 5
SEB:	Staphylococcal enterotoxin B
T _{reg} :	Regulatory T
T _{FH} :	Follicular helper T
Tim-3:	T-cell immunoglobulin and mucin-domain containing-3

Nevertheless, we and other researchers have found that IL-21 is also produced by CXCR5⁺ T cells in NPs,^{7,21} raising the possibility that CXCR5⁺ T cells may contribute to immunoglobulin production in an eLT-independent mechanism.

In this study, we investigated the tissue localization, phenotype, and function of CD4⁺ T-cell subsets with different expression levels of IL-21, CXCR5, and programmed cell death 1 (PD-1) in NPs. We aimed to identify CXCR5⁺CD4⁺ T-cell subsets with the capacity to induce local immunoglobulin production in NPs.

METHODS

Study participants

This study was approved by the Ethics Committee of Tongji Hospital, and written informed consent was obtained from each patient. The diagnosis of CRSwNP was made on the basis of European and American guidelines.^{2,22} CRSwNP was defined as eosinophilic when the percentage of tissue eosinophils exceeded 10% of the total infiltrating cells, as previously reported.²³ Subjects undergoing septoplasty because of anatomic variations and without other sinonasal diseases were enrolled as control subjects. NP tissues from patients with CRSwNP and inferior turbinate (IT) mucosal tissues from control subjects, as well as nasal epithelial cell and peripheral blood samples from those subjects, were obtained. Disease severity before surgery and NP recurrence at 12 months after surgery were assessed as previously described.^{16,24} Tonsil samples were collected from patients who had tonsil hypertrophy and were undergoing tonsillectomy. Not all subjects were included in every experiment protocol because of the limited quantity of samples. The numbers of samples for each experiment are indicated in the figures or figure legends. More information on the subjects is provided in Table E1 and the Methods section of this article's Online Repository (both of which are available at www.jacionline.org).

Histology, immunohistochemistry, and immunofluorescence staining

Detailed information is provided in the Methods section of the Online Repository, including in Table E2 (in the Online Repository at www.jacionline.org).

org). Isotype control staining, and picro-Mallory trichrome staining are shown in Fig E1 (in the Online Repository at www.jacionline.org).

Mononuclear cell isolation

Dispersed nasal and tonsil tissue cells were prepared by dissociating tissues mechanically.¹⁶ Blood and tissue mononuclear cells were isolated as previously mentioned.²⁵ Additional information is provided in the [Methods](#) section of the Online Repository.

Flow cytometry

PBMCs and tissue mononuclear cells were stained with specific primary antibodies (see [Table E3](#) in the Online Repository at www.jacionline.org) and analyzed by flow cytometry.²⁶ Additional information is provided in the [Methods](#) section of the Online Repository.

Cell sorting

Immune cells were sorted on a BD FACSARIA II cell sorter (BD Biosciences, San Jose, Calif). The purity of the sorted cells was higher than 95%. Detailed information is provided in the [Methods](#) section of the Online Repository.

T-cell-B-cell coculture

The sorted tissue T-cell subsets were cultured with autologous naive B cells purified from PBMCs. Additional information is provided in the [Methods](#) section of the Online Repository.

Single-cell RNA sequencing

CD45⁺ live cells isolated from 2 eosinophilic and 2 noneosinophilic NP samples were subjected to single-cell RNA sequencing by using 10× Genomics (Pleasanton, Calif) as previously described.²⁷ A total of 35,854 cells from 4 samples were analyzed. Quality control measurements are provided in [Table E4](#) (in the Online Repository at www.jacionline.org). More information is provided in the [Methods](#) section of the Online Repository.

Cytokine and immunoglobulin measurement

Immunoglobulin levels in tissue homogenates and culture supernatants were detected by using the Bio-Plex suspension chip method (Bio-Rad, Hercules, Calif).³ IL-21, IL-4, and chemokine (C-X-C motif) ligand 13 (CXCL13) levels in culture supernatants were detected by using commercial ELISA kits (Thermo Fisher Scientific, Waltham, Mass).¹⁶ Detection limits are listed in [Table E5](#) (in the Online Repository at www.jacionline.org). More information is provided in the [Methods](#) section of the Online Repository.

Quantitative real-time PCR

Quantitative real-time PCR was conducted with specific primers (see [Table E6](#) in the Online Repository at www.jacionline.org) and detailed information is provided in the [Methods](#) section of the Online Repository.

Statistical analysis

All statistical analyses were carried out with GraphPad Prism Software 8.0 (GraphPad Software Inc, San Diego, Calif). Expression results are presented in dot plots unless specifically stated. Symbols represent individual samples, horizontal bars represent medians, and error bars show interquartile ranges. The Kruskal-Wallis *H* test was used to assess significant intergroup variability. The Mann-Whitney *U* test was used for between-group comparisons. For paired samples, data were analyzed with the paired-sample *t* test. Differences in proportions between groups were compared by using the Fisher exact or chi-square test. Cell culture data are expressed as means plus or minus SEMs and were analyzed by using the Mann-Whitney *U* test. The Spearman rank test was used for correlation analysis. Significance was accepted at *P* values less than .05. For multiple comparisons among 3 study groups, Bonferroni correction

was used to adjust the significance level by using a *P* value of .017 for each comparison.

RESULTS

PD-1^{high}CXCR5⁺CD4⁺ T cells are located adjacent to B cells in NPs

IL-21 is a potent driver of B-cell terminal differentiation and immunoglobulin production.^{19,20} We first analyzed IL-21 expression in gated lymphocytes in mononuclear cells in NPs by flow cytometry. We found that CXCR5⁺CD4⁺ T cells were a more abundant cellular source of IL-21, accounting for 46.2% (mean) of the total IL-21⁺ lymphocytes, in comparison with CXCR5⁺CD4⁺ T_{FH} cells (11.4%) and CXCR5⁺CD4⁺ cells (42.4%) in NPs ([Fig 1, A](#)). A similar result was observed by gating IL-21^{dim} cells (see [Fig E2, A](#) in this article's Online Repository at www.jacionline.org). In NP lymphocytes, we found that the frequencies of CXCR5⁺CD4⁺ cells were 6 times those of CXCR5⁺CD4⁺ cells (see [Fig E2, B](#)), although the percentages of IL-21⁺ cells in CXCR5⁺CD4⁺ cells were twice those in CXCR5⁺CD4⁺ cells (see [Fig E2, C](#)). By gating IL-21⁺ lymphocytes, we found that CXCR5⁺CD4⁺ and CXCR5⁺CD4⁺ T cells demonstrated an expression intensity comparable to that of IL-21, which was higher than that of CXCR5⁺CD4⁺ T cells ([Fig 1, B](#)). Taken together, these results suggest that CXCR5⁺CD4⁺ T cells are a main source of IL-21 in NPs.

In addition to IL-21, antigen-specific T-cell-B-cell interaction is essential for efficient antibody production;¹⁹ we therefore explored the potential T-cell-B-cell interaction in NPs by immunofluorescence staining. Of interest, we found that the majority of CD4⁺ T cells adjacent to diffused B cells in NPs without eLTs were negative for CXCR5, and only a few of them were CXCR5⁺CD4⁺ T_{FH} cells ([Fig 1, C and D](#)). Antigen presentation or T-cell-B-cell interactions lead to the induction of activated markers of T cells.¹⁹ PD-1 is a classical marker of *bona fide* T_{FH} cells in second lymphoid organs, which indicates the activation of CD4⁺ T cells.¹⁹ Consistent with our previous study,⁷ we found that NP CXCR5⁺CD4⁺ T cells had no significant PD-1 expression; however, most of NP CXCR5⁺CD4⁺ T cells had bright PD-1 staining ([Fig 1, E](#)), which was comparable to the PD-1 staining intensity on *bona fide* T_{FH} cells in tonsils (see [Fig E3, A](#) in the Online Repository at www.jacionline.org). We thus defined this population of cells in NPs as PD-1^{high}CXCR5⁺CD4⁺ T cells. For PD-1^{high}CD4⁺ cells, the numbers of CXCR5⁺ cells were significantly higher than those of CXCR5⁺ cells in NPs without eLTs ([Fig 1, F](#)). We additionally explored IL-21 expression in CD4⁺ T cells in NPs by immunostaining. In contrast to the low IL-21 expression in PD-1⁺CD4⁺ T cells, a strong IL-21 expression in PD-1^{high}CD4⁺ T cells was observed ([Fig 1, G](#)).

We further investigated the distribution of PD-1^{high}CXCR5⁺CD4⁺ T cells in NPs with eLTs. We found that PD-1^{high}CD4⁺ T cells with bright PD-1 staining were located mainly in areas outside eLTs ([Fig 2, A](#) and see [Fig E3, B](#)). On the contrary, CD4⁺ T cells within the eLTs, presumably CXCR5⁺ T_{FH} cells in NPs, had only intermediate (int) or weak PD-1 staining ([Fig 2, A](#) and see [Fig E3, B](#)). Moreover, we found that the numbers of PD-1^{high}CD4⁺ T cells close to B cells were significantly higher than those of PD-1^{int}CD4⁺ T cells adjacent to B cells in areas outside or without eLTs ([Fig 2, B and C](#)). Collectively, these results indicate that PD-1^{high}CXCR5⁺CD4⁺ T-cell and B-cell cooperation takes place in NPs irrelevant to eLTs.

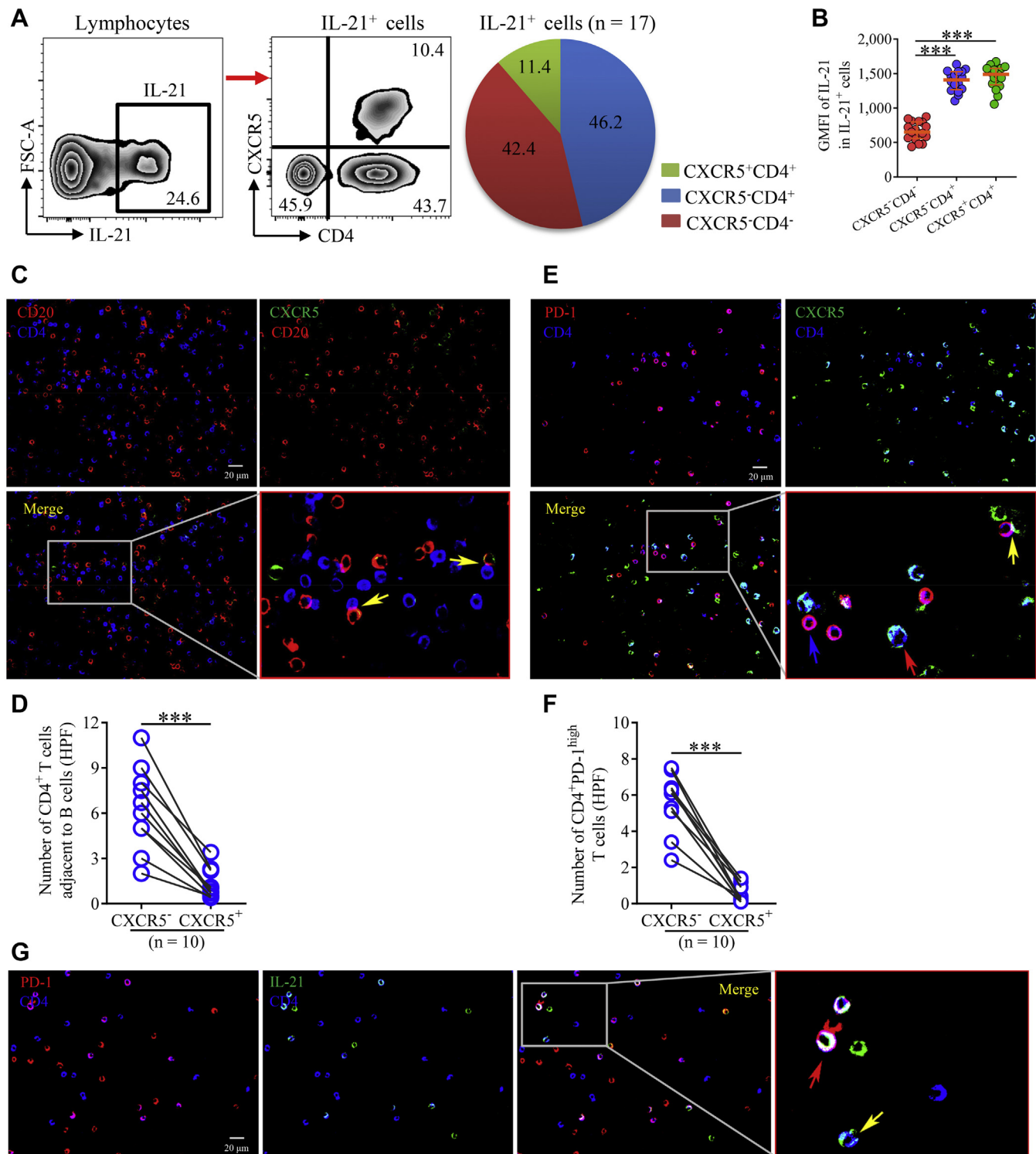


FIG 1. IL-21, CXCR5, and PD-1 expression by T cells in NPs. **A**, Flow cytometric analysis of IL-21⁺ cells in gated lymphocytes in mononuclear cells in NPs. Mean percentages of CXCR5⁺CD4⁺, CXCR5⁺CD4⁺, and CXCR5⁺CD4⁺ cells accounting for the IL-21⁺ cells. **B**, Geometric mean fluorescence intensity (GMFI) of IL-21 in IL-21⁺ cells in indicated cell subsets. In **(A)** and **(B)**, for eosinophilic (Eos) NPs, n = 8, and for noneosinophilic (non-Eos) NPs, n = 9. **C**, Representative photomicrographs showing immunofluorescence staining for CD4, CXCR5, and CD20 in a non-Eos NP sample. The lower right image shows greater magnification of the outlined area. Yellow arrows indicate the CXCR5⁺CD4⁺ T cells in contact with B cells. **D**, Numbers of CXCR5⁺ and CXCR5⁺CD4⁺ T cells adjacent to diffused CD20⁺ B cells in the same NP samples without eLTs (for Eos NPs, n = 7; for non-Eos NPs, n = 3). **E**, Representative photomicrographs showing immunofluorescence staining for PD-1, CXCR5, and CD4 in an Eos NP sample. The lower right image shows greater magnification of the outlined areas. Blue arrow indicates PD-1^{high}CXCR5⁺CD4⁺ T cells, red arrow indicates

Expansion of PD-1^{high}CXCR5⁺CD4⁺ T cells in NPs

We next studied the abundance and phenotype of PD-1^{high}CXCR5⁺CD4⁺ T cells by flow cytometry. Consistent with the histology findings, flow cytometric analysis revealed a clear population of PD-1^{high}CXCR5⁺CD45RA⁺CD4⁺ T cells in NPs (Fig 3, A). We found that the frequencies of PD-1^{high}CXCR5⁺CD45RA⁺CD4⁺ T cells within total CD4⁺ T cells were significantly increased, whereas the frequencies of PD-1⁺CXCR5⁺CD45RA⁺CD4⁺ T cells were reduced in NPs versus in control IT tissues (Fig 3, B). In line with the findings of previous reports,^{7,21} a significant increase in CXCR5⁺CD4⁺ T_{FH} cells was observed in NPs versus in control IT tissues (Fig 3, B).

The phenotypic characteristics of PD-1^{high}CXCR5⁺CD4⁺ T cells in NPs

We used flow cytometry to investigate the phenotypic characteristics of PD-1^{high}CXCR5⁺CD4⁺ T cells by comprehensively comparing the expression of an array of cytokines, surface markers, chemokine receptors, and transcription factors in PD-1^{high}, PD-1^{int}, and PD-1⁺CXCR5⁺CD4⁺ and CXCR5⁺CD4⁺ T cells in NPs, as well as in PD-1^{high}CXCR5^{high}CD4⁺ T_{FH} cells in tonsils (Fig 4, A). The gating strategy for tonsillar T_{FH} cells is shown in Fig E4 (see the Online Repository at www.jacionline.org). We first revealed higher percentages of IL-21⁺ cells in PD-1^{high}CXCR5⁺CD4⁺ T cells than in other types of CD4⁺ T cells in NPs (Fig 4, A and B). In addition, PD-1^{high}CXCR5⁺CD4⁺ T cells displayed stronger capacity to produce CXCL13, IFN- γ , IL-4, and IL-17A than PD-1^{int}CXCR5⁺CD4⁺ T-cell subsets in NPs, with some of them having a capacity comparable even to that of T_{FH} cells in tonsils (Fig 4, A and B and see Fig E5, A in the Online Repository at www.jacionline.org).

We found that NP PD-1^{high}CXCR5⁺CD4⁺ T cells had higher percentages of expression of inducible T-cell costimulator (ICOS), CD69, and HLA-DR isotype (the activation markers) than did PD-1^{int}CXCR5⁺CD4⁺ T-cell subsets in NPs (Fig 4, A and B and see Fig E5, B). Compared with other T-cell subsets in NPs, PD-1^{high}CXCR5⁺CD4⁺ T cells showed increased percentages of expression of CC-chemokine receptor 2 (CCR2) and CCR5 (the chemokine receptors mediating cell migration to nonlymphoid tissues) but reduced percentages of CD62L and CCR7 expression (the molecule required by lymphocytes to recirculate to secondary lymphoid) (Fig 4, A and B and Fig E5, B). CD103 is the canonic resident memory T-cell marker. Notably, the frequencies of CD103⁺ cells were markedly higher in NP PD-1^{high}CXCR5⁺CD4⁺ T cells than in other types of CD4⁺ T cells (Fig 4, A and B), and more than 65% (mean) of PD-1^{high}CXCR5⁺CD4⁺ T cells coexpressed CD103 and CD69 (see Fig E5, C). Consistent with the expression patterns of chemokine receptors and tissue-resident markers, substantially lower frequencies of PD-1^{high}CXCR5⁺CD4⁺ T cells were noted in PBMCs and tonsils than in NPs (see Fig E6, A and B in the Online Repository at www.jacionline.org). In addition, no difference in the frequencies of circulating PD-1^{high}CXCR5⁺CD4⁺ T cells

was discovered between patients with CRSwNP and control subjects (see Fig E6, C).

We revealed that PD-1^{high}CXCR5⁺CD4⁺ T cells showed higher percentages of expression of the transcription factor MAF, signaling lymphocytic activation molecule-associated protein (SAP), and signaling lymphocytic activation molecule 5 (SLAMF5), the factors necessary for T-cell–B-cell interaction, than did the PD-1^{int}CXCR5⁺CD4⁺ T-cell subsets and CXCR5⁺CD4⁺ T_{FH} cells in NPs (Fig 4, A and see Fig E7, A in the Online Repository at www.jacionline.org). Notably, the percentages of expression of MAF and SLAMF5 in PD-1^{high}CXCR5⁺CD4⁺ T cells were comparable to those of *bona fide* T_{FH} cells in tonsils (Fig 4, A and see Fig E7, A).

The extremely low expression percentages of B-cell lymphoma 6 (Bcl-6), which is the master transcription factor controlling T_{FH} cell differentiation, and the high expression percentages of B-lymphocyte-induced maturation protein-1 (Blimp1), which is an antagonist of Bcl-6, distinguished NP PD-1^{high}CXCR5⁺CD4⁺ T cells from *bona fide* T_{FH} cells in tonsils (Fig 4, A and B). Instead, the high percentages of CXCR4 expression suggest a role of PD-1^{high}CXCR5⁺CD4⁺ T cells in extrafollicular B-cell responses (see Fig E5, B). PD-1^{high}CXCR5⁺CD4⁺ T cells displayed significantly higher expression percentages of Ki-67, a nuclear protein associated cellular proliferation, than did other T-cell subsets in NPs and tonsil T_{FH} cells, supporting a local expansion of PD-1^{high}CXCR5⁺CD4⁺ T cells in NPs (Fig 4, A and B). In contrast, the exhaustion markers (T-cell immunoglobulin and mucin-domain containing-3 [Tim-3] and cytotoxic T-lymphocyte-associated protein 4 [CTLA-4]) and the regulatory T (T_{reg}) cell-related marker Foxp3 did not appear to be enriched in PD-1^{high}CXCR5⁺CD4⁺ T cells in NPs (Fig 4, A and see Fig E7, B).

To further investigate T-cell subsets in NPs, we did single-cell RNA sequencing of CD45⁺ mononuclear cells isolated from 2 eosinophilic and 2 noneosinophilic NP samples. By analysis of cells expressing CD4, we observed an aggregation of cells with high *PDCD1* expression and low or absent *CXCR5* expression, which had high expression of *ICOS*, *CD69*, *ITGAE*, and *CCR5* and low expression of *SELL*, *CCR7*, *IL2RA*, and *FOXP3* (see Fig E8 in the Online Repository at www.jacionline.org). These findings further indicate an activated, tissue-resident, extrafollicular, and B-cell–helping phenotype of PD-1^{high}CXCR5⁺CD4⁺ T cells in NPs.

Distinct IL-4–producing capacity of PD-1^{high}CXCR5⁺CD4⁺ T cells in eosinophilic and noneosinophilic NPs

We next compared the difference in the PD-1^{high}CXCR5⁺CD4⁺ T-cell phenotype between eosinophilic and noneosinophilic NPs. Our preliminary analysis based on a limited sample size revealed no significant difference in expression of the aforementioned markers by PD-1^{high}CXCR5⁺CD4⁺ T cells between eosinophilic and noneosinophilic NPs, except for an increased trend of IL-4 production by

PD-1^{int}CXCR5⁺CD4⁺ T cells, and *yellow arrow* indicates PD-1^{high}CXCR5⁺CD4⁺ T cells adjacent to CXCR5⁺CD4⁺ B cells. **F**, Numbers of PD-1^{high}CD4⁺ T cells with positive or negative CXCR5 expression in the same NP samples without eLTs (for Eos NPs, n = 4; for non-Eos NPs, n = 6). **G**, Representative photomicrographs showing immunofluorescence staining for PD-1, IL-21, and CD4 in an Eos NP sample. The right image shows greater magnification of the outlined areas. *Red arrow* indicates PD-1^{high}IL-21⁺CD4⁺ T cells, and *yellow arrow* indicates PD-1^{int}IL-21^{low}CD4⁺ T cells. ***P < .001. FSC, Forward scatter.

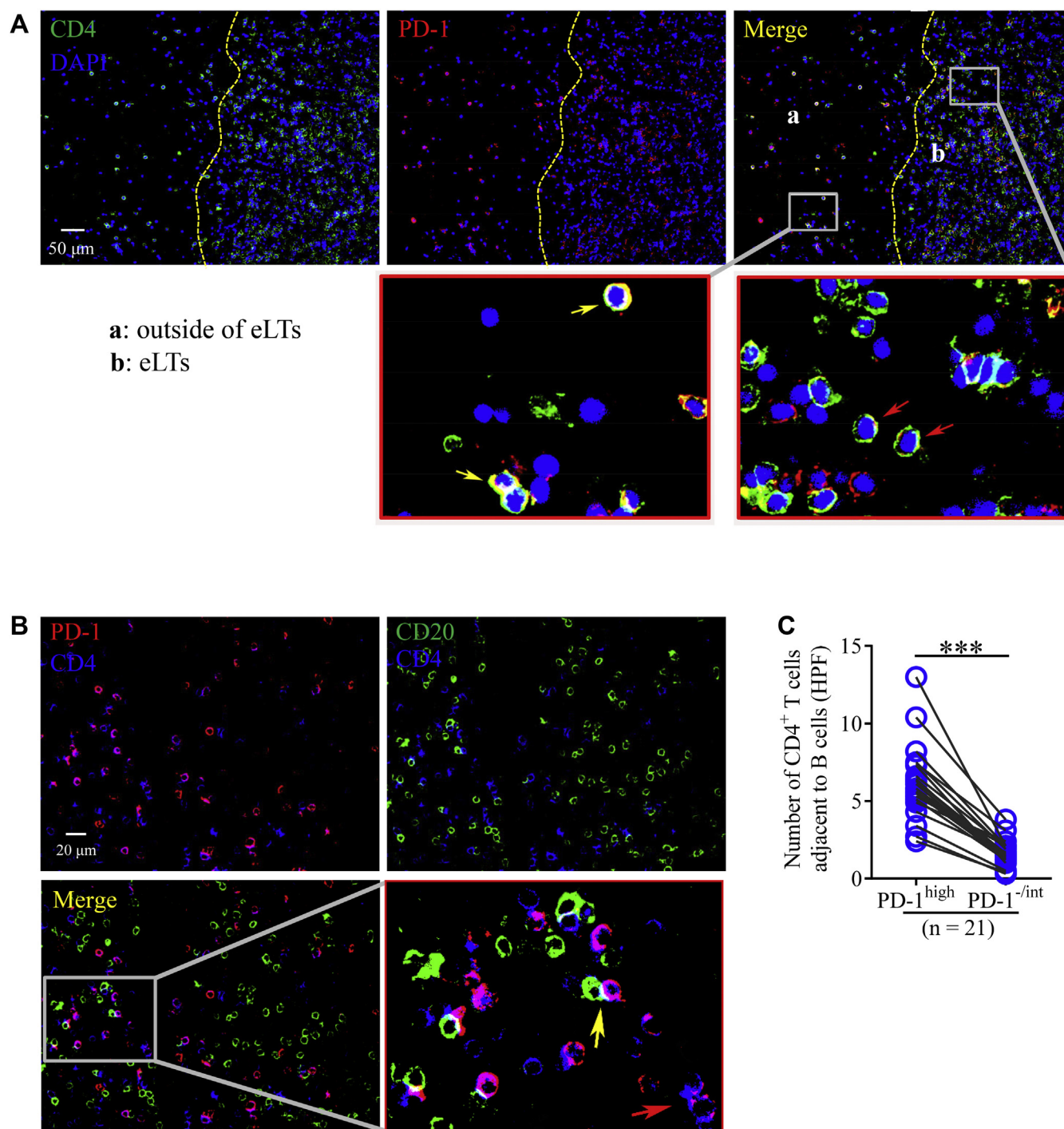


FIG 2. Tissue distribution of PD-1^{high}CXCR5⁻CD4⁺ T cells in NPs. **A**, Representative photomicrographs showing immunofluorescence staining for PD-1 and CD4 in an eosinophilic NP sample with eLTs. Lower images show greater magnification of the outlined areas. **Red arrows** indicate PD-1^{int}CD4⁺ T cells within eLTs (*lower right image*), and **yellow arrows** indicate PD-1^{high}CD4⁺ T cells in areas outside eLTs (*lower middle image*). **B**, Representative photomicrographs showing immunofluorescence staining for PD-1, CD4, and CD20 in the non-eLT area in NPs. Lower right image shows greater magnification of the outlined areas. **Yellow arrow** indicates PD-1^{high}CD4⁺ T cells adjacent to CD20⁺ B cells, and **red arrow** indicates PD-1^{int}CXCR5⁻CD4⁺ T cells. **C**, Numbers of PD-1^{high} and PD-1^{-int} CD4⁺ T cells adjacent to diffused CD20⁺ B cells in the non-eLT area in the same NP samples (for Eos NPs, n = 11; for noneosinophilic NPs, n = 10). ***P < .001.

PD-1^{high}CXCR5⁻CD4⁺ T cells in eosinophilic NPs (see Fig E9 in the Online Repository at www.jacionline.org). Given the limited resources of big NP samples, we then focused on the expression of

activation and tissue-resident markers and type 1, 2, and 17 cytokines and compared the difference between eosinophilic and non-eosinophilic NPs after increasing the sample size.^{7,28} There was

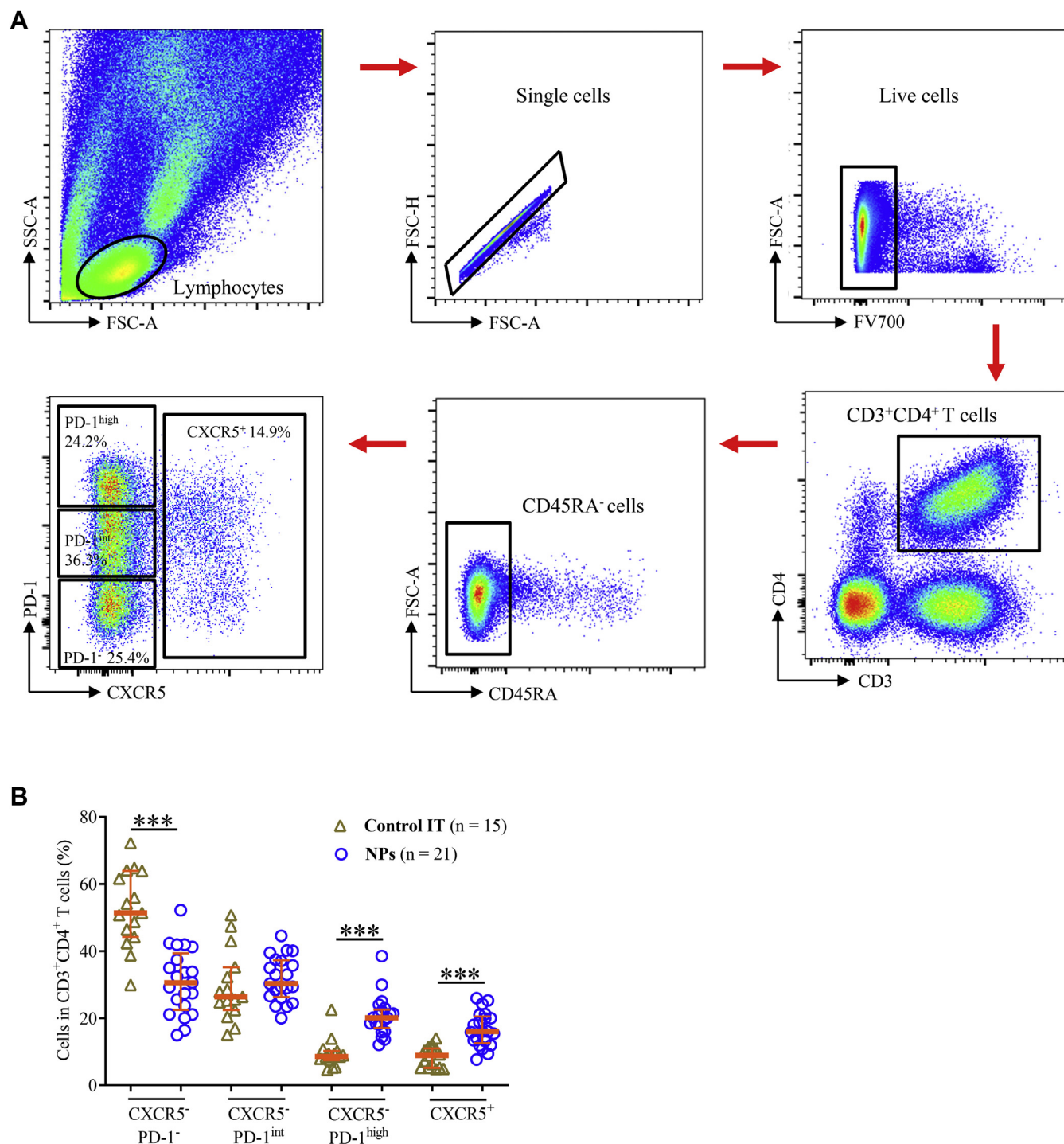
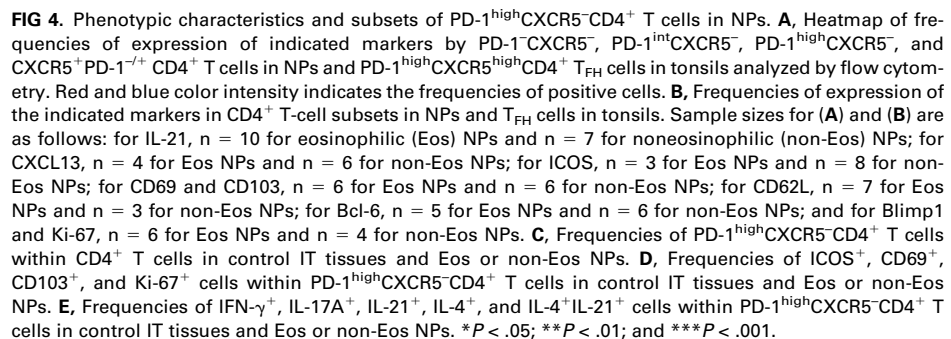


FIG 3. Expanded PD-1^{high}CXCR5⁻CD4⁺ T cells in NPs. **A**, Gating strategy used to define different T-cell subsets in NPs. **B**, Frequencies of PD-1⁻CXCR5⁻, PD-1^{int}CXCR5⁻, PD-1^{high}CXCR5⁻, and CXCR5⁺ cells within CD4⁺ T cells in control IT tissues and NPs (for Eos NPs, n = 13; for noneosinophilic NPs, n = 8). ****P* < .001. FSC, Forward scatter; SSC, side scatter.

no difference in the frequencies of total PD-1^{high}CXCR5⁻CD4⁺ T cells within CD4⁺ T cells (Fig 4, C) and ICOS⁺, CD69⁺, CD103⁺, and Ki-67⁺ cells within PD-1^{high}CXCR5⁻CD4⁺ T cells between eosinophilic and noneosinophilic NPs (Fig 4, D). Although we found that the frequencies of IFN- γ ⁺, IL-17A⁺, and IL-21⁺ cells within PD-1^{high}CXCR5⁻CD4⁺ T cells

were comparably increased in eosinophilic and noneosinophilic NPs when compared with their frequencies in control IT tissues, the frequencies of IL-4⁺ and IL-4⁺IL-21⁺ cells within PD-1^{high}CXCR5⁻CD4⁺ T cells were significantly increased in eosinophilic NPs versus in noneosinophilic NPs and control IT tissues (Fig 4, E).



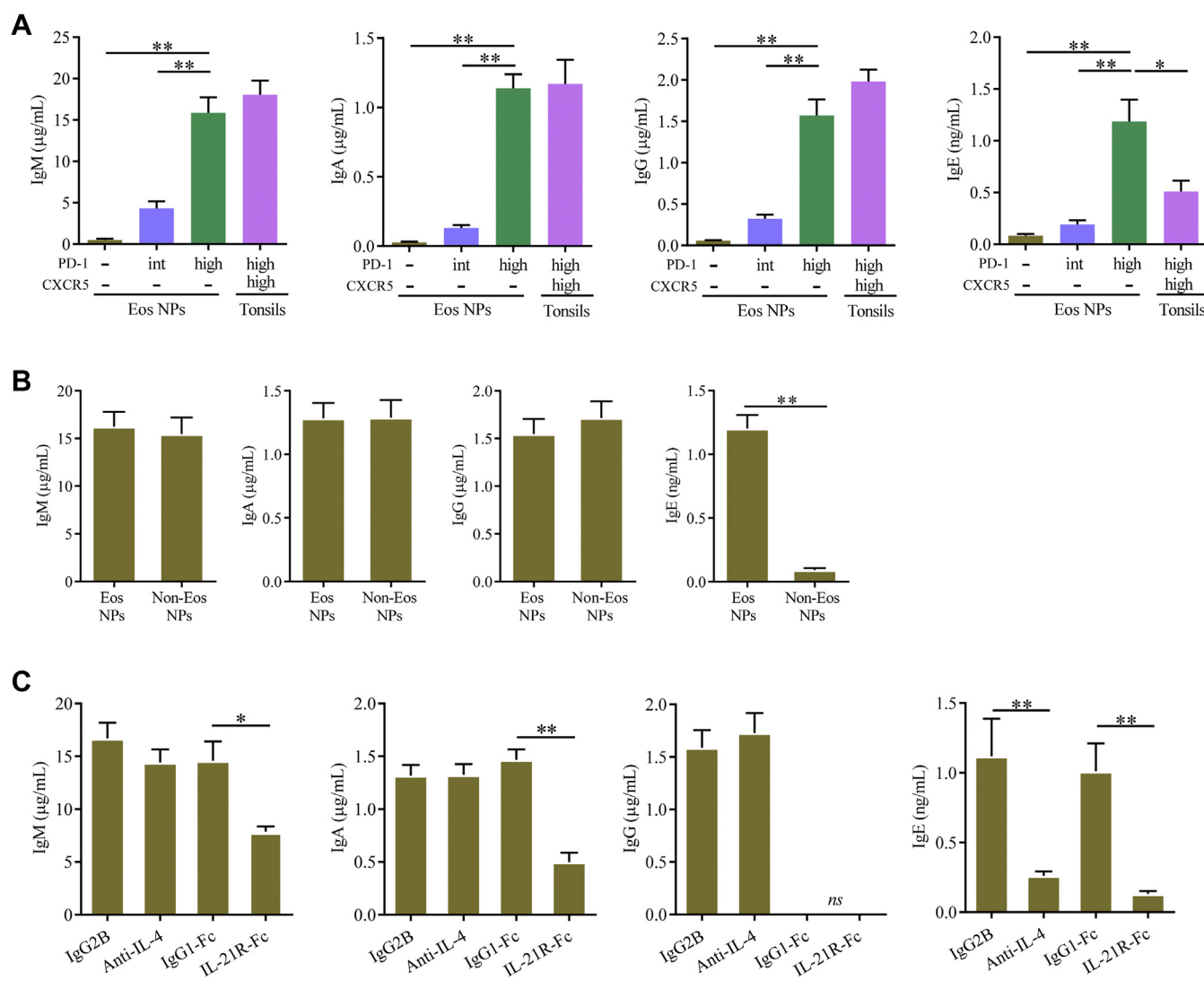


FIG 5. PD-1^{high}CXCR5⁻CD4⁺ T cells in NPs drive immunoglobulin production **A**, T-cell subsets isolated from eosinophilic (Eos) NPs and T_H cells from tonsils were cocultured with autologous naive B cells, and immunoglobulin levels were determined at day 12 (n = 6). **B**, PD-1^{high}CXCR5⁻CD4⁺ T cells purified from Eos or noneosinophilic (non-Eos) NPs were cocultured with autologous naive B cells, and immunoglobulin levels were determined at day 12 (n = 6). **C**, PD-1^{high}CXCR5⁻CD4⁺ T cells purified from Eos NPs were cocultured with autologous naive B cells in the presence of blocking antibody or protein, and immunoglobulin levels were determined at day 12 (n = 6). *P < .05 and **P < .01. NS, Not shown.

PD-1^{high}CXCR5⁻CD4⁺ T cells promote immunoglobulin production by B cells

Using a T-cell–B-cell coculture assay,^{7,29} we found that PD-1^{high}CXCR5⁻CD4⁺ T cells, but not their PD-1⁻ and PD-1^{int} counterparts isolated from eosinophilic NPs, had potent capacities to induce IgM, IgA, IgG, and IgE production from B cells, which were comparable to or even higher than those of tonsil T_H cells (Fig 5, A). As expected, the IL-21, CXCL13, and IL-4 levels in culture supernatants were significantly higher in the coculture of B cells with NP PD-1^{high}CXCR5⁻CD4⁺ T cells than with NP PD-1⁻ and PD-1^{int} CXCR5⁻CD4⁺ T cells (see Fig E10, A in the Online Repository at www.jacionline.org). The mRNA expression of activation-induced cytidine deaminase (AID) in B cells and the number of recovered B cells after culture were significantly increased after coculture with NP PD-1^{high}CXCR5⁻CD4⁺

T cells versus after coculture with other NP T-cell subsets (see Fig E10, B and C).

We discovered that compared with PD-1^{high}CXCR5⁻CD4⁺ T cells from eosinophilic NPs, those from noneosinophilic NPs had a significantly reduced capacity to induce IgE production, although PD-1^{high}CXCR5⁻CD4⁺ T cells from both types of NPs had a comparable ability to induce production of IgM, IgA, and IgG (Fig 5, B). We further found that IL-21 blockade dramatically reduced the production of IgA, IgM, and IgE, whereas blocking IL-4 suppressed only IgE production from B cells cocultured with PD-1^{high}CXCR5⁻CD4⁺ T cells from eosinophilic NPs (Fig 5, C). In the blocking experiment with IL-21R-Fc, IgG is not shown because of the potential cross-reactivity to the Fc portion of IL-21R-Fc.

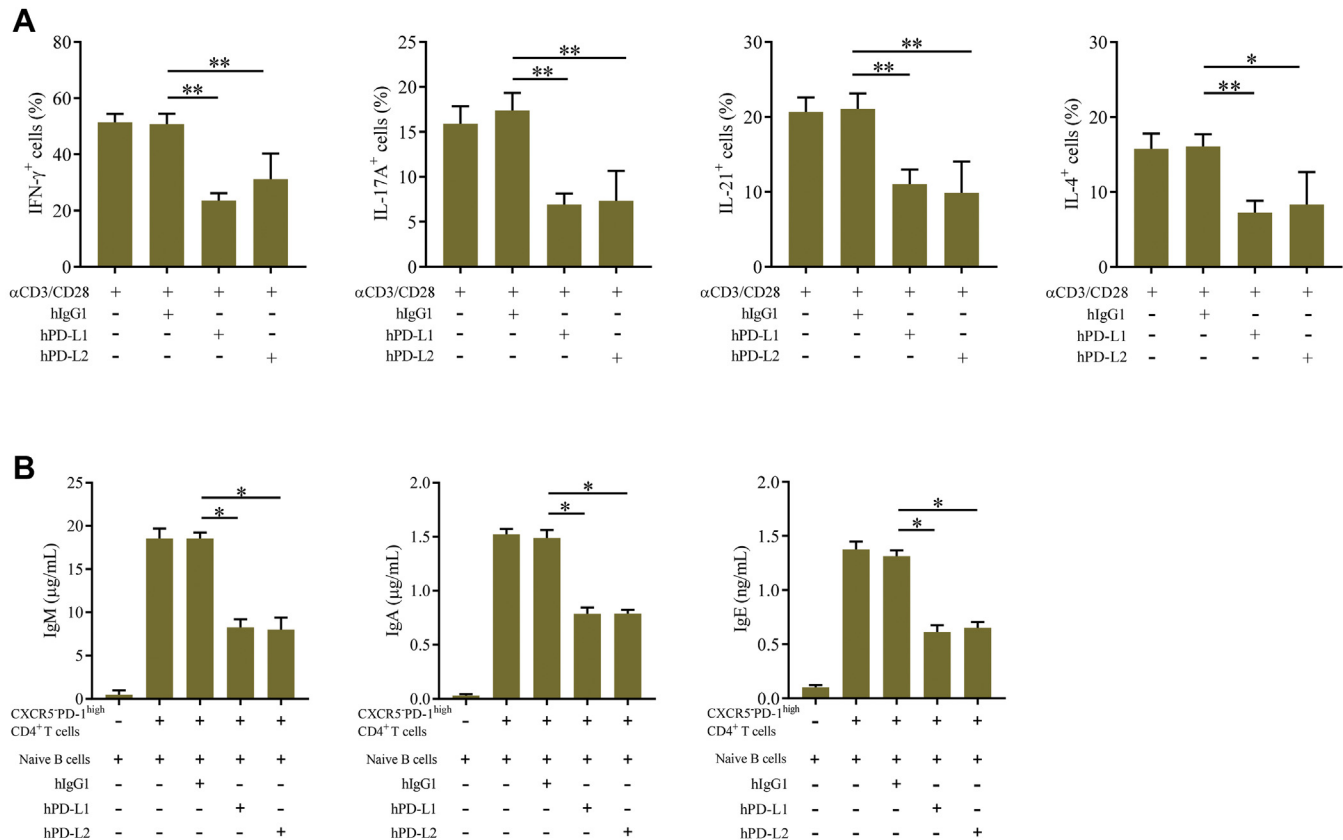


FIG 6. PD-1 ligands suppress PD-1^{high}CXCR5⁻CD4⁺ T-cell function. PD-1^{high}CXCR5⁻CD4⁺ T cells purified from eosinophilic NPs were activated by anti-CD3/CD28 in the presence or absence of recombinant human PD-L1 and PD-L2. **A**, Frequencies of IFN- γ^+ , IL-17A $^+$, IL-21 $^+$, and IL-4 $^+$ cells within PD-1^{high}CXCR5⁻CD4⁺ T cells were determined after a 48-hour stimulation by flow cytometry (n = 6). **B**, Immunoglobulin levels were determined at day 12 after stimulation (n = 6). *P < .05 and **P < .01.

Activation of PD-1 signaling suppresses the function of PD-1^{high}CXCR5⁻CD4⁺ T cells

PD-1 provides negative feedback to suppress the effector functions of T cells.³⁰ We thus asked whether PD-1 signaling is functional in NP PD-1^{high}CXCR5⁻CD4⁺ T cells. We found that the production of IFN- γ , IL-17A, IL-21, and IL-4 was dramatically inhibited when PD-1^{high}CXCR5⁻CD4⁺ T cells from eosinophilic NPs were activated by anti-CD3 and anti-CD28 antibody in the presence of the PD-1 agonist PD-L1 or PD-L2 (Fig 6, A). Accordingly, the addition of PD-L1 or PD-L2 to the coculture system of eosinophilic NP PD-1^{high}CXCR5⁻CD4⁺ T cells and autologous peripheral naive B cells significantly suppressed IgM, IgA, and IgE production (Fig 6, B). IgG is not shown because of the potential cross-reactivity to the PD-L1 and PD-L2.

Reduced PD-L1 and PD-L2 expression in NPs

The aforementioned results raise the question of how T cells with high PD-1 expression maintain their activation status in NPs. To answer this question, we explored PD-L1 and PD-L2 expression in NPs and observed a downregulation of both mRNA and protein expression of PD-L1 and PD-L2 in both eosinophilic and noneosinophilic NPs versus in control IT tissues (Fig 7, A and B). Immunofluorescence staining revealed that immunoreactivity of PD-L1 and PD-L2 was predominant in epithelial cells and to a much lesser degree in infiltrating cells

in the lamina propria in control IT tissues (Fig 7, C). The expression of PD-L1 and PD-L2 was significantly reduced in nasal epithelial cells in NPs. Again, only scattered cells with PD-L1 and PD-L2 immunoreactivity were found in the lamina propria in NPs (Fig 7, C). No difference in the numbers of PD-L1⁺ and PD-L2⁺ cells in the lamina propria was found for control IT tissues and eosinophilic and noneosinophilic NPs (see Fig E11, A in the Online Repository at www.jacionline.org). Flow cytometric analysis of homogenized NP tissues confirmed that the majority of PD-L1⁺ and PD-L2⁺ cells in NPs were CD45⁺CD326⁺ epithelial cells (see Fig E11, B and C). We further found that PD-L1 and PD-L2 mRNA expression in scraped nasal epithelial cells was significantly reduced in both eosinophilic and noneosinophilic NPs as compared with in control IT tissues (Fig 7, D).

The associations between PD-1^{high}CXCR5⁻CD4⁺ T cells and immunoglobulin levels and clinical outcomes

Our previous study demonstrated upregulated levels of IgG in both eosinophilic and noneosinophilic NPs but increased IgE levels only in eosinophilic NPs.⁷ Here we analyzed the relationship between PD-1^{high}CXCR5⁻CD4⁺ T cells and local immunoglobulin levels in 29 prospectively recruited patients with CRSwNP. We discovered that the frequencies of PD-1^{high}CXCR5⁻CD4⁺ T cells were positively correlated

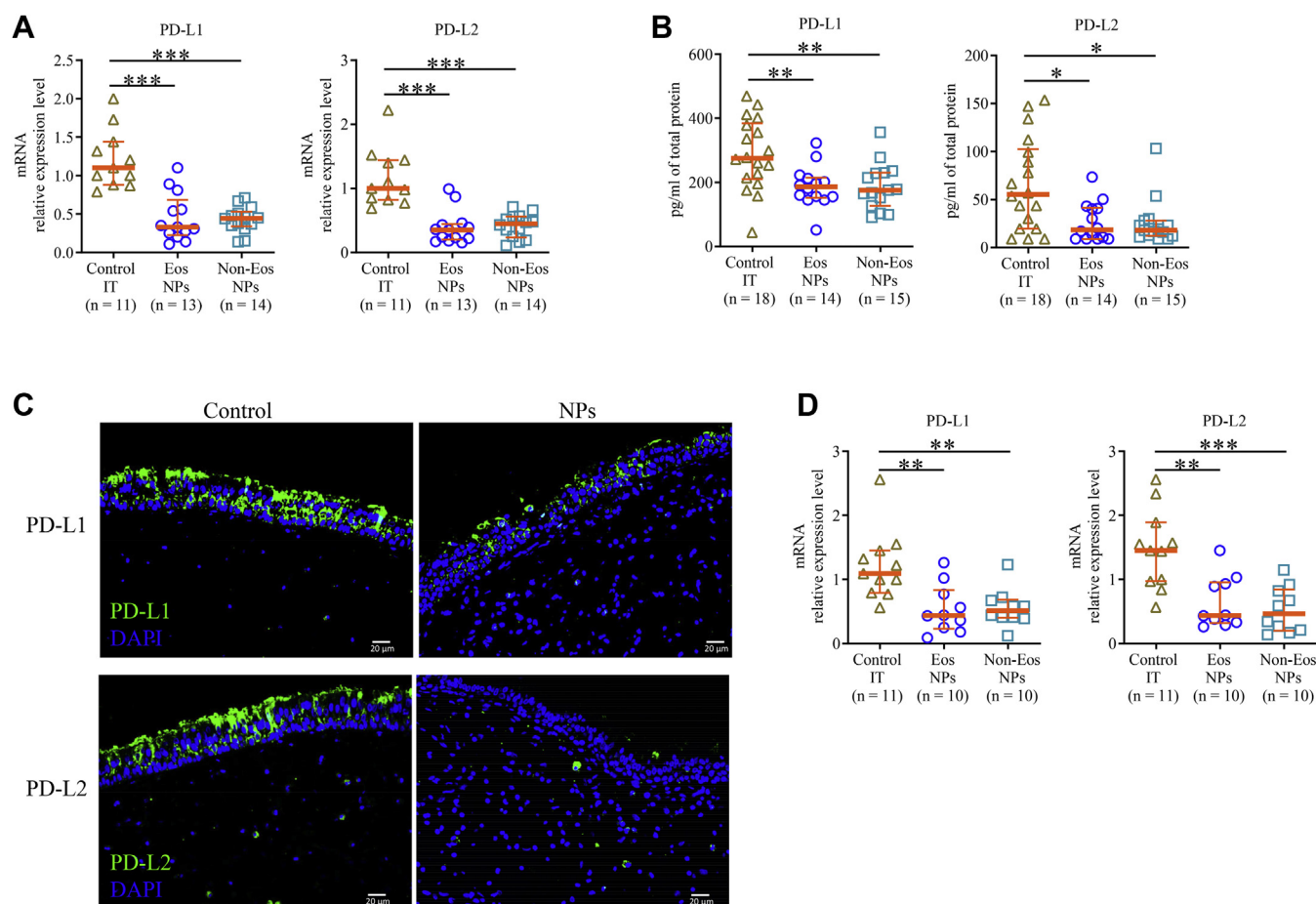


FIG 7. Reduced PD-L1 and PD-L2 expression in NPs. **A**, The mRNA levels of PD-L1 and PD-L2 in tissues as detected by real-time PCR. **B**, The protein levels of PD-L1 and PD-L2 in tissue homogenates as detected by ELISA. **C**, Representative immunostaining for PD-L1 and PD-L2 in control IT tissues and NPs. **D**, The mRNA levels of PD-L1 and PD-L2 in nasal epithelial cells as detected by real-time PCR. * $P < .017$; ** $P < .01$; and *** $P < .001$. Eos, Eosinophilic; non-Eos, noneosinophilic.

with IgG and IgM levels in NPs (Fig 8, A) but correlated with IgE levels only in eosinophilic NPs (Fig 8, B). We further examined the association between PD-1^{high}CXCR5⁺CD4⁺ T cells and disease severity in this set of patients with CRSwNP (see Table E7 in the Online Repository at www.jacionline.org). We found that PD-1^{high}CXCR5⁺CD4⁺ T-cell frequencies were associated with disease duration and total endoscopic scores (Fig 8, C). Moreover, we found that patients with prior sinus surgery displayed higher frequencies of PD-1^{high}CXCR5⁺CD4⁺ T cells than did those having no surgical history (Fig 8, D). The frequencies of PD-1^{high}CXCR5⁺CD4⁺ T cells in NP samples obtained during surgery were higher in patients with NP relapse (n = 7; 24.1%) than in the samples obtained from patients without relapse 12 months after the surgery (Fig 8, E).

DISCUSSION

Although our previous study revealed that nasal CXCR5⁺CD4⁺ T_{FH} cells and eLTs promote local immunoglobulin production in NPs, this cannot explain the increased local immunoglobulin production in NPs without eLTs,^{7,16} which accounts for 80% of NPs. Here, for the first time, we identified an

expanded PD-1^{high}CXCR5⁺CD4⁺ T-cell population in NPs that are in close contact with B cells independent of eLTs. This subset of NP CD4⁺ T cells have a peripheral tissue-resident feature and comparable capacity to induce immunoglobulin production by B cells as *bona fide* tonsil T_{FH} cells. The PD-1^{high}CXCR5⁺CD4⁺ T-cell abundance is associated with disease severity and relapse in patients with CRSwNP. Our study highlights a broader role of PD-1^{high}CXCR5⁺CD4⁺ T cells than that of T_{FH} cells in the pathogenesis of NPs.

IL-21 is recognized as the most potent driver of B-cell terminal differentiation and controller of antibody production, which is also the signature cytokine of T_{FH} cells.^{7,29,31} Our flow cytometric analysis revealed that IL-21 was produced primarily by CXCR5⁺ cells and not by CXCR5⁺ T cells in NPs. By using immunofluorescence staining, we identified a subset of CXCR5⁺CD4⁺ T cells with high PD-1 expression comparable to that in *bona fide* T_{FH} cells, which were in close contact with B cells in areas outside or without eLTs in NPs. PD-1^{high}CXCR5⁺CD4⁺ T cells had high expression of IL-21 and factors necessary for T-cell–B-cell interaction, making them among the important candidates driving local immunoglobulin production in NPs, particularly in those without eLTs. Recently, Rao et al found a similar T-cell population in both the synovium and peripheral blood of patients with

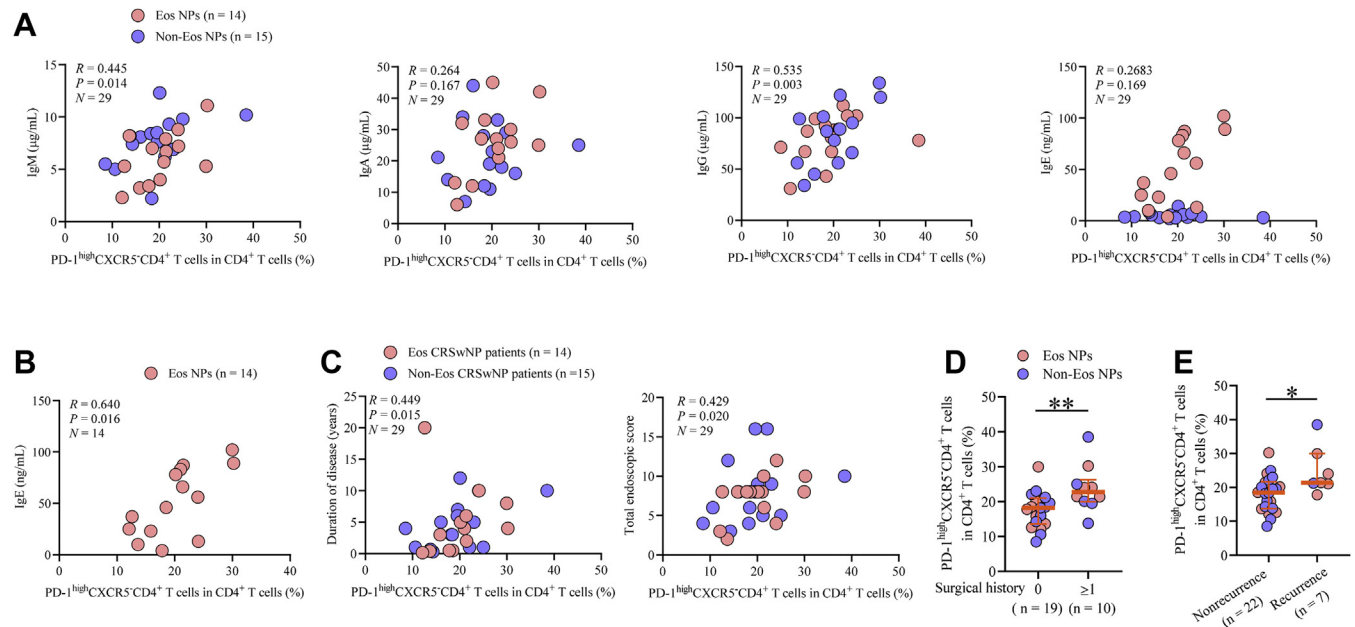


FIG 8. PD-1^{high}CXCR5⁻CD4⁺ T cells associated with polyp recurrence. **A**, Correlations between immunoglobulin levels and frequencies of PD-1^{high}CXCR5⁻CD4⁺ T cells in NPs. **B**, Correlation between IgE and PD-1^{high}CXCR5⁻CD4⁺ T cells in eosinophilic (Eos) NPs. **C**, Correlations between duration of disease and total endoscopic scores and frequencies of PD-1^{high}CXCR5⁻CD4⁺ T cells in patients with CRSwNP. **D**, Frequencies of NP PD-1^{high}CXCR5⁻CD4⁺ T cells in patients with CRSwNP with and without previous sinus surgery. Sample size for the group with no history of surgery: for Eos NPs, n = 9; for noneosinophilic (non-Eos) NPs, n = 10. Sample size for the group with a history of 1 or more operations: Eos NPs, n = 5; for non-Eos NPs, n = 5. **E**, Frequencies of PD-1^{high}CXCR5⁻CD4⁺ T cells in NPs in patients with or without recurrence of NPs 12 months after the surgery. Sample size for the nonrecurrence group: for Eos NPs, n = 9; for non-Eos NPs, n = 13. Sample size for the recurrence group: for Eos NPs, n = 5; for non-Eos NPs, n = 2. **P* < .05 and ***P* < .01.

rheumatoid arthritis.³² In contrast, we found low frequencies of PD-1^{high}CXCR5⁻CD4⁺ T cells in the peripheral blood in patients with CRSwNP, and no difference in circulating PD-1^{high}CXCR5⁻CD4⁺ T cells was found between control subjects and patients with CRSwNP. These data are consistent with the high expression of CD103, CCR2, and CCR5, and low expression of CD62L and CCR7 in PD-1^{high}CXCR5⁻CD4⁺ T cells in NPs, indicating peripheral residency of this cell type in NPs. The low abundance of PD-1^{high}CXCR5⁻CD4⁺ T cells in tonsils and peripheral blood also suggests a potentially localized differentiation and expansion of PD-1^{high}CXCR5⁻CD4⁺ T cells in NPs, which is supported by the high expression of Ki-67 in these cells. PD-1^{high}CXCR5⁻CD4⁺ T cells displayed lower Bcl-6 and higher Blimp1 expression than did T_{FH} cells, suggesting that this cell type may have a unique differentiation pathway distinct from that of T_{FH} cells. Recently, TGF- β has been reported to induce the differentiation of human CXCL13-producing CXCR5^{low}Bcl-6^{low} CD4⁺ T cells from naive CD4⁺ T cells *in vitro*.³³ Oxidized mitochondrial DNA-activated plasmacytoid dendritic cells induce the generation of PD-1^{high}CXCR5⁻CD4⁺ T cells *in vitro*.³⁴ The mechanisms underlying the generation of PD-1^{high}CXCR5⁻CD4⁺ T cells in NPs obviously demand further studies.

PD-1 has been considered an exhaustion marker of CD8⁺ T cells in cancer and chronic infectious diseases.³⁵ However, we found that NP PD-1^{high}CXCR5⁻CD4⁺ T cells represented a more activated population in CD4⁺ T cells in NPs than their PD-1⁻ and PD-1^{int} counterparts, as indicated by their high expression of HLA-DR, CD69, and ICOS and profound production of

IFN- γ , IL-17A, IL-21, and IL-4. Recently, a subset of T_{reg} cells with high PD-1 expression has been noted in PBMCs.^{36,37} However, we did not find significant expression of T_{reg} cell markers, including Foxp3 and CD25, by NP PD-1^{high}CXCR5⁻CD4⁺ T cells. These findings further support the notion that PD-1^{high}CXCR5⁻CD4⁺ T cells are activated rather than regulatory or exhausted T cells in NPs. Although Rao et al found that PD-1^{high}CXCR5⁻CD4⁺ T cells from synovial tissue of patients with rheumatoid arthritis induced IgG production by B cells,³² the role of these cells in IgE-mediated diseases remains unknown. Cytokines secreted by T cells direct the isotype class switching.³⁸ We have characterized PD-1^{high}CXCR5⁻CD4⁺ T cells on the basis of expression of IFN- γ , IL-17A, and IL-4 for the first time. We found that although the numbers of IFN- γ ⁺, IL-17A⁺, and IL-21⁺ subsets were increased in both eosinophilic and noneosinophilic NPs, IL-4⁺ and IL-4⁺IL-21⁺ subsets were increased only in eosinophilic NPs compared with in control IT tissues. We discovered that NP PD-1^{high}CXCR5⁻CD4⁺ cells, but not their PD-1⁻ and PD-1^{int} counterparts, efficiently promoted IgG, IgA, and IgM production from B cells. Nevertheless, only PD-1^{high}CXCR5⁻CD4⁺ T cells from eosinophilic NPs, and not those from noneosinophilic NPs, had a potent capacity to induce IgE production via IL-4. These results highlight a critical role of the IL-4⁺ subset of PD-1^{high}CXCR5⁻CD4⁺ T cells in inducing class switch toward IgE. This finding is consistent with our previous finding that local IgE levels were increased only in eosinophilic NPs.⁷ In this study, we found a positive correlation between PD-1^{high}CXCR5⁻CD4⁺ T-cell frequencies and IgE levels only in eosinophilic NPs.

PD-1 ligation inhibits T-cell receptor-initiated cell proliferation and survival and cytokine production.³⁰ PD-L1 and PD-L2 treatment resulted in a significant but modest decrease in IgM, IgA, and IgE production in the PD-1^{high}CXCR5⁺CD4⁺ T-cell and B-cell coculture system, suggesting the involvement of other regulatory mechanisms beyond the PD-1/PD-L pathway. In addition, it would be interesting to explore in future studies whether combination of PD-L1 or PD-L2 with anti-IL-21 or anti-IL-4 would cause a more profound inhibition of immunoglobulin production. Why are the PD-1^{high}CXCR5⁺CD4⁺ T cells not kept in check by their high PD-1 expression but, in contrast, highly activated in NPs? In line with the findings of a previous report,³⁹ we observed significantly reduced PD-L1 and PD-L2 expression in NPs, particularly in nasal epithelial cells. Thus, it is possible that low levels of PD-L1 and PD-L2 expression in a local inflammatory site may release the suppression and lead to the sustained activation of PD-1^{high}CXCR5⁺CD4⁺ T cells. However, why there is a downregulation of PD-L1 and PD-L2 expression in NPs remains an open question. PD-L1 has also been reported to be expressed by B cells, which may regulate T-cell function during T-cell-B-cell interaction.⁴⁰ We examined PD-L1 expression in B-cell subsets in NPs but failed to find changes in PD-L1 expression when compared with that in B cells in control IT tissues (data not shown). In addition to the lack of inhibitory signaling in NPs, the factors that primarily activate PD-1^{high}CXCR5⁺CD4⁺ T cells in NPs remain to be elucidated. T-cell activation is a complex process that is mainly regulated by antigen-presenting cells. In addition, staphylococcal enterotoxins and an imbalanced microbiome, which are known to activate T cells, have been implicated in the pathogenesis of NPs. Whether these factors are involved in the activation of PD-1^{high}CXCR5⁺CD4⁺ T cells in NPs awaits further studies.

There are several limitations associated with the present study. First, only 4 NP samples were used for single-cell RNA sequencing analysis. The sequencing results need to be validated with additional NP samples. Second, both PD-L1 and PD-L2 have a soluble and a membrane-bound form. The level and function of different forms of PD-L1 and PD-L2 in NPs remain to be elucidated. Third, our preliminary analysis showed that there was no difference in the phenotype of PD-1^{high}CXCR5⁺CD4⁺ T cells between NPs with and without eLTs (see Fig E12 in the Online Repository at www.jacionline.org), suggesting that eLTs might not have significant influence on PD-1^{high}CXCR5⁺CD4⁺ T cells. Obviously, this should be validated in further investigations with larger sample sizes. Fourth, eosinophils, mast cells, neutrophils, eLTs, an imbalanced microenvironment, and dysfunction of coagulation and fibrinolysis system have all been reported to play a role in the pathology of NPs. NPs are more likely caused by the interaction and cooperation of various cells and molecules. Fifth, although our study identified potential associations between PD-1^{high}CXCR5⁺CD4⁺ T-cell abundance and disease severity and relapse in patients with CRSwNP, the clinical relevance of PD-1^{high}CXCR5⁺CD4⁺ T cells in predicting disease relapse and their efficacy versus that of other potential predictors, such as eosinophils, deserve further study with a large and well-controlled cohort. Sixth, as we have already mentioned, further exploration of the mechanisms underlying nasal PD-1^{high}CXCR5⁺CD4⁺ T-cell differentiation would deepen our understanding of the pathogenesis of NPs.

In conclusion, we have identified an expansion of PD-1^{high}CXCR5⁺CD4⁺ T cells in NPs, which are distinct from

other T-cell populations in NPs and T_{FH} cells in tonsils. PD-1^{high}CXCR5⁺CD4⁺ T cells may participate in immunoglobulin production in NPs independent of eLTs. The downregulation of PD-L1 and PD-L2 in NPs potentially leads to persistent activation of PD-1^{high}CXCR5⁺CD4⁺ T cells. Our study provides novel insights into the production of immunoglobulin in NPs. Reestablishing the inhibition of PD-1^{high}CXCR5⁺CD4⁺ T cells by PD-1 ligands might be a potential therapeutic strategy to alleviate local immunoglobulin production in patients with CRSwNP.

Key messages

- An expanded population of PD-1^{high}CXCR5⁺CD4⁺ T cells is observed in nasal polyps in areas outside or without ectopic lymphoid tissues.
- PD-1^{high}CXCR5⁺CD4⁺ T cells in nasal polyps demonstrate an activated and peripheral resident memory phenotype, and they robustly induce immunoglobulin production by naive B cells.
- Downregulation of PD-L1 and PD-L2 may contribute to persistent activation of PD-1^{high}CXCR5⁺CD4⁺ T cells in nasal polyps.

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Online Repository

**Extrafollicular PD-1^{high}CXCR5⁺CD4⁺ T cells participate in local immunoglobulin
production in nasal polyps**

Zhi-Chao Wang, M.D., Ph.D.^{1*}, Yin Yao, M.D., Ph.D.^{1*}, Cai-Ling Chen, M.D.¹, Cui-
Lian Guo, M.D., Ph.D.¹, Hong-Xia Ding, M.D.¹, Jia Song, M.D., Ph.D.¹, Zheng-Zhe
Wang, M.D.¹, Nan Wang, M.D., Ph.D.¹, Xue-Li Li, M.D.¹, Bo Liao, M.D., Ph.D.¹, Yang
Yang, Ph.D.², Di Yu, Ph.D.², Zheng Liu, M.D., Ph.D.¹

* These authors contributed equally to this work.

METHODS

Subjects

This study was approved by the Ethics Committee of Tongji Hospital of Huazhong University of Science and Technology and was conducted with written informed consent from each patient. A total of 347 adult subjects including 141 patients with eosinophilic chronic rhinosinusitis with nasal polyps (CRSwNP), 130 patients with noneosinophilic CRSwNP, and 76 control subjects undergoing septoplasty because of anatomic variations and without other sinonasal diseases were enrolled in this study. All experiments were performed in accordance with the approved guidelines and regulations. The diagnosis of CRSwNP was made according to European and American guidelines.^{E1,2} CRSwNP was defined as eosinophilic when the percent of tissue eosinophils exceeded 10% of total infiltrating cells as we previously reported.^{E3} This cut-off value was calculated as twice the standard deviation of the mean of eosinophil percentage in controls.^{E3} Atopic status was evaluated by skin prick test to a standard panel of 16 aeroallergens in our region that was performed in accordance with WHO guideline.^{E4-6} The diagnosis of asthma was based on disease history and airway responsiveness test according to Global Initiative for Asthma guideline.^{E7} Patients with antrochoanal polyps, cystic fibrosis, pregnancy, primary ciliary dyskinesia, systemic vasculitis, fungal sinusitis, acute upper respiratory tract infection, acute asthma episode within 4 weeks of entering the study, and under immunotherapy were excluded from the study. Owing to the low prevalence of aspirin-exacerbated respiratory disease (AERD) in our cohort (3 out of 274), we excluded the patients with AERD in this study.

The low prevalence of AERD is consistent with previous reports in Chinese CRS patients.^{E8} Oral and intranasal steroids were discontinued at least 3 months and 1 month before the surgery, respectively. Antileukotrienes were discontinued at least 1 month before inclusion.

Nasal polyp (NP) tissues and inferior turbinate (IT) mucosal tissues were obtained during surgery from CRSwNP and control subjects, respectively. Tissue samples were used for histology, real-time RT-PCR, Bio-Plex chip assay, ELISA, flow cytometry, single-cell RNA sequencing, and T cells isolation and culture study. For some subjects, peripheral blood samples were collected 1-3 days before surgery for flow cytometric study. Nasal epithelial cells were scraped from inferior turbinate of control subjects and from NPs of CRSwNP patients using a sterile Rhino-Pro curette (Arlington Scientific Inc, Springville, UT) under endoscope.^{E9} Nasal epithelial cells were used for real-time RT-PCR study. Demographic and clinical characteristics of CRSwNP patients and control subjects undergoing septoplasty were summarized in Table E1 in the Online Repository.

Among CRSwNP patients, 29 patients were prospectively recruited for the clinical characteristic and post-surgical NP recurrence study. At the baseline, pre-operative computed tomography (CT) scans in the axial and coronal plane were evaluated by using the Lund-Mackay CT scoring system.^{E10} Pre-operative subjective symptoms focused on nasal obstruction, rhinorrhea, loss of sense of smell, and facial pain were scored on a visual analogue scale (VAS) of 0 to 10 as previously described.^{E10} The total VAS symptom scores were calculated based on the sum of these four VAS symptom

domains. The overall disease burden was also scored on a VAS by asking patients how troublesome their disease was in affecting their daily activity and sleep.^{E10} Endoscopic scoring was performed before surgery according to Lund-Kennedy score system as previously described.^{E10} The patients' baseline clinical characteristics are list on Table E7. After surgery, each patient was routinely given fluticasone propionate nasal spray, 200 µg, and saline irrigation, twice daily. VAS scoring and endoscopic examination were performed at each follow-up. Subjects with uncontrolled symptoms were given 1-2 weeks of broad-spectrum antibiotics and /or 20 days of oral prednisone tablets (30 mg daily on days 1-5, 20 mg daily on days 6-10, 10 mg daily on days 11-15, and 5mg daily on days 16-20) as rescue treatment. The recurrence of NPs was defined as the presence of NPs by nasal endoscopy 12 months after surgery.^{E11}

In addition, 37 subjects [24 males and 13 females; age, 37 (23, 44) (median and interquartile range) for males and 32 (25, 40) for females], who were healthy other than tonsil hypertrophy (without chronic tonsillitis) were enrolled. Tonsil samples were collected during tonsillectomy.^{E12}

Not all subjects were included in every experiment protocol because of limited quantity of samples. The numbers of samples for every experiment were indicated in figures or figure legends.

Histology

Tissue samples were fixed in formaldehyde solution and embedded in paraffin. Paraffin sections (4 µm) were prepared from tissue blocks. After deparaffinization and

rehydration, sections were stained with hematoxylin and eosin routinely to screen the eosinophilic inflammation, inflammatory cell infiltration, and presence of ectopic lymphoid tissues as previously described.^{E10} The ectopic lymphoid tissues in NPs were identified as lymphoid aggregates with GC-like structures as previously stated.^{E10} The picro-Mallory trichrome (PMT) staining was conducted as previously addressed.^{E13}

Immunofluorescence staining was conducted as previously stated.^{E10} The tissue sections were first stained with specific primary antibodies (Table E2) overnight at 4 °C. After washing, the tissue sections were incubated with fluorescence conjugated secondary antibodies (Table E2) at room temperature for 2 hours. T cell subsets and B cells were studied.

The numbers of eosinophils and positive cells in the lamina propria were counted at high power magnification (× 400). Three discrete tissue sections from each sample and 10 high power fields (HPFs) of every tissue section were randomly selected and analyzed by 2 independent physicians who were blinded to the clinical data as we previously described.^{E10}

Tissue and blood mononuclear cell isolation

NP and tonsil tissue samples were dissociated mechanically with the GentleMACS Dissociator (Miltenyi Biotec, San Diego, CA, USA).^{E10,12,14} The resulting cell suspensions were filtered 2 times through a 40 µm cell strainer (BD Biosciences, San Jose, CA, USA), and washed twice with a culture medium RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA).^{E12} The mononuclear cells were

isolated by density-gradient centrifugation on Lymphoprep (AXIS-SHIELD PoC AS, Oslo, Norway) as previously described.^{E10} The peripheral blood mononuclear cells (PBMCs) were purified using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) as previously mentioned.^{E12}

Flow cytometry

Purified tissue mononuclear cells or PBMCs were incubated with 5% fetal bovine serum (FBS, Thermo Fisher Scientific) for 10 min on ice to block non-specific staining. Then, cells were stained with fixable viability stain 700 (BD Bioscience) to exclude dead cells. For cell surface staining, primary antibodies were incubated with cells for 30 min at room temperature. For intracellular cytokine staining, mononuclear cells were treated with cell stimulation cocktail containing protein transport inhibitors (BD Bioscience) in RPMI-1640 medium at 37°C for 4 hours. Then cells were washed 2 times after surface staining and permeabilized using Fixation/Permeabilization Solution Kit (BD Bioscience) for 30 min on ice. Antibodies specific for cytokines were diluted in Perm/Wash Buffer (BD Bioscience) and incubated with cells for 40 min at 4 °C. For intranuclear staining, cells were washed 2 times after surface staining and permeabilized using Transcription Factor Fix/Perm Buffer (BD Bioscience) for 30 min on ice. Antibodies specific for intranuclear proteins were diluted in Perm/Wash Buffer (BD Bioscience) and incubated with cells for 40 min at 4 °C. The stained cells were analyzed by flow cytometry using a BD LSRFortessa™ X-20 (BD Bioscience) instrument and FlowJo software (TreeStar, Ashland, OR, USA). At least 50 events for

each subpopulation were recorded. The information of antibodies used in flow cytometry are provided in Table E3 in the Online Repository.

Cell sorting

Cells were sorted on a BD FACS Aria II cell sorter (BD Biosciences). PD-1⁻, PD-1^{int}, and PD-1^{high} CD3⁺CD4⁺CD45RA⁻CXCR5⁻ cells in NPs and CD3⁺CD4⁺CD45RA⁻CXCR5^{high}PD-1^{high} T_{FH} cells in tonsils were sorted. Peripheral blood naive B cells were sorted as CD3⁻CD19⁺IgD⁺CD27⁻ cells.^{E12}

T cell culture

Sorted polyp PD-1^{high}CXCR5⁻CD4⁺ cells were cultured in RPMI-1640 supplemented with 10% FBS (Thermo Fisher Scientific) and 1% penicillin/streptomycin (Sigma Aldrich, St Louis, Mo, USA) in 96-well plates. T cells were activated in the presence of anti-CD3/CD28 (100 ng/mL; Miltenyi Biotec). To activate the PD-1 signaling, recombinant human programmed death-ligand (PD-L)1 (100 ng/mL; R&D Systems, Minneapolis, MN, USA) or recombinant human PD-L2 (50 ng/mL; R&D Systems) was added. Human recombinant IgG1 (R&D Systems) was served as a control for PD-L1 and PD-L2. Two days after culture, T cells were stimulated with leukocyte activation cocktail (BD Bioscience) for 4-hours and then subjected to flow cytometric analysis of cytokine expression.

T-B cell co-culture

Sorted polyp PD-1⁻, PD-1^{int}, and PD-1^{high} CD3⁺CD4⁺CD45RA⁻CXCR5⁻ T cells and tonsil *bona fide* CD3⁺CD4⁺CD45RA⁻CXCR5^{high}PD-1^{high} T_{FH} cells (3×10^4 /well) were cultured with autologous CD3⁻CD19⁺IgD⁺CD27⁻ naive B cells (2.5×10^4 /well) in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin in the presence of *Staphylococcal enterotoxin* B (SEB, 1 μ g/mL; Toxin Technology, Inc., Sarasota, FL, USA) and interleukin (IL)-2 (10 U/mL, Thermo Fisher Scientific) in U-bottomed 96-well plates as previously described for 2, 7, or 12 days.^{E12,14} In the blocking experiments, anti-IL-4 mouse monoclonal antibody (1 μ g/mL, R&D Systems), or IL-21R-Fc (20 μ g/mL, R&D Systems) was added.^{E14-16} Mouse IgG2B and human IgG1-Fc (R&D Systems) were served as a control for anti-IL-4 and IL-21R-Fc, respectively. For PD-1 signaling activation experiments, recombinant human PD-L1 (100 ng/mL) or PD-L2 (50 ng/mL) was added. Human recombinant IgG1 was served as a control for PD-L1 and PD-L2. The levels of cytokines and immunoglobulins in the culture supernatants were measured at day 2 and 12, respectively. AID mRNA expression in cultured B cells were detected at day 7. Recovered B cells were counted at day 12.

cDNA library preparation for single cell RNA sequencing

The mononuclear cells from NPs were isolated as described above. Cells were stained with fixable viability stain 700 and anti-human CD45 antibody for 20 min at 4 °C, and then CD45⁺ live cells were sorted on a BD FACS Aria II cell sorter. Cell viability was determined by propidium iodide (Biolegend, CA, USA) staining again.

Single cells were barcoded using the 10× Chromium Single Cell platform, and cDNA libraries were prepared according to the manufacturer’s protocol (Single Cell 5’ Reagent Kits v2, 10x Genomics, USA). Briefly, sorted single-cell suspension, 10× 5’ barcoded gel beads, and oil were loaded into Chromium Single Cell K Chip to capture single cells in nanoliter-scale oil droplets by Chromium Controller and to generate Gel Bead-In-Emulsions (GEMs). Full-length cDNA libraries were prepared by incubation of GEMs in a thermocycler machine. GEMs containing cDNAs were broken and all single-cell cDNA libraries were pooled together, cleaned using Dynabeads MyOne Silane (PN37002D; Fisher), and preamplified by PCR to generate sufficient mass for sequencing library construction. Sequencing libraries were constructed by following the steps, cDNA fragmentation, end repair and A-tailing, size selection by SPRI select beads (PNB23318; Beckman Coulter), adaptor ligation, sample index PCR amplification, and repeat SPRI select beads size selection. Next, library construction quantification of cDNA was determined by Qubit (Thermo Fisher Scientific, Waltham, MA, USA) and Agilent TapeStation HS D1000 (Santa Clara, Calif, USA).

Single-cell RNA sequencing computational pipeline and analysis

The final constructed single-cell libraries were sequenced by Illumina Novaseq 6000 (Illumina, San Diego, CA, USA) machine with total reads per cell targeted, for a minimum of 50,000. We demultiplexed raw base call (BCL) files generated by Illumina sequencers into FASTQ files, upon which alignment was carried out to the GRCh38 human transcriptome; filtering, barcode counting, and UMI counting were performed

using Cell Ranger software version 3.1 and default parameters (10X Genomics). Quality control measurements are provided in Table E4. A further downstream analysis was performed using Seurat v4.0.2 R package (<https://github.com/satijalab/seurat>). Cells were filtered based the following parameters: (1) genes expressed in less than three cells and cells expressing less than 200 and more than 4000 genes were excluded; (2) cells with more than 20% mitochondrial gene expression were removed; and (3) cells expressing *CD4* (gene > 0) were selected for subsequent analysis. After this process, the remaining data was log-normalized with scaling factor 10,000. To reduce the dimensionality of the data, we determined the 2000 most highly variable genes with the Find Variable Features-function with “vst” method. A t-distributed stochastic neighbor embedding (tSNE) plot showed the cells distribution (Fig E8). Co-expression of *PDCDI* and *CXCR5* and the expression of indicated genes in *CD4* expressing cells in NPs were analyzed and shown with “Seurat”. Similar procedures were used in previous studies and improved the downstream dimensionality reduction and clustering.^{E17,18}

Measurement of immunoglobulin and cytokine levels

Tissue samples were weighted and homogenized, and the supernatants were harvested.^{E10,19} Supernatants of cell culture were collected. The protein levels of IgG, IgM, IgA, and IgE in supernatants were detected by using Bio-Plex suspension chip method according to the manufacturer’s instructions (Bio-Rad, Hercules, Calif, USA). The protein levels of chemokine (C-X-C motif) ligand (CXCL)13, IL-4, and IL-21 in

supernatants were measured by using commercial ELISA kits (Thermo Fisher Scientific). The protein levels of PD-L1 and PD-L2 in tissue homogenates were measured with commercial ELISA kits (Multi sciences Biotech, Hangzhou, China), and normalized to total tissue protein levels quantified by using a BCA protein detection kit (Guge Biotechnology, Wuhan, China). The lower detection limits are shown in Table E5.

Quantitative RT-PCR

Total RNA was extracted from human nasal epithelial cells, nasal tissues, and cultured B cells using Trizol reagent (Thermo Fisher Scientific). Quantitative RT-PCR assay was performed as previously described.^{E10,12} Total RNA (1 µg) from each sample was reverse-transcribed to cDNA by using a PrimeScript RT reagent kit (TaKaRa Biotechnology, Dalian, China). RT-PCR was carried out by using the SYBR Premix Ex Taq kit (TaKaRa Biotechnology) with specific primers (Table E6). cDNA equivalent to 50 ng of total RNA was used and real time PCR was performed on StepOnePlus (Applied Biosystems, Foster City, Calif, USA). The reaction of PCR was performed according to the following protocol: 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, a specific annealing temperature for 10 sec, and 72 °C for 15 sec.^{E9,12} After PCR, a melting curve was constructed by increasing the temperature from 65 to 95 °C at a rate of 0.1 °C/s. Relative mRNA levels of target genes were analyzed with the 2(-Delta Delta CT) method as previously report.^{E9} A normal inferior turbinate mucosal sample and a control cell culture sample were used as the calibrator for tissue samples

232 and cell culture samples, respectively. Glucuronidase beta (GUSB) was used as a
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Figure Legends

Fig E1. Isotype control staining for immunofluorescence study and PMT staining of control IT and eosinophilic and noneosinophilic NP samples.

Fig E2. Flow cytometric analysis of IL-21 expression in gated lymphocytes in NPs.

(A) Mean percentages of CXCR5⁺CD4⁺, CXCR5⁺CD4⁺, CXCR5⁺CD4⁺, and CXCR5⁺CD4⁺ cells accounting for the IL-21^{dim} cells. (B) Mean percentages of CXCR5⁺CD4⁺, CXCR5⁺CD4⁺, CXCR5⁺CD4⁺, and CXCR5⁺CD4⁺ cells in lymphocytes. (C) Percentages of IL-21⁺ cells in indicated cell subsets in lymphocytes. Eos NP, n = 8; Non-Eos NP, n = 9. ****P* < 0.001.

Fig E3. Representative photomicrographs showing immunofluorescence staining for CD4 and PD-1 in tonsil tissues and nasal polyps.

(A) The right image at bottom shows greater magnification of the outlined area. Yellow arrow indicates the PD-1^{high}CD4⁺ T cells in the germinal center. (B) Representative photomicrographs showing immunofluorescence staining for PD-1 in NPs with eLTs. Right and bottom images show greater magnification of the outlined areas. Red arrows indicate PD-1^{int} T cells within eLTs (right image) and yellow arrows indicate PD-1^{high} T cells in areas outside of eLTs (bottom image). This figure shows the same tissue filed as Figure 2, A.

Fig E4. Gating strategies for PD-1^{high}CXCR5⁺CD4⁺ T cells and PD-1^{high}CXCR5^{high}CD4⁺ T_{FH} cells in tonsils.

Fig E5. The expression of cytokines, chemokine receptors, and surface markers in PD-1^{high}CXCR5⁻CD4⁺ T cells in NPs. (A) Frequencies of IFN- γ ⁺, IL-17A⁺, IL-4⁺, IL-2⁺, and IL-10⁺ cells in PD-1⁻CXCR5⁻CD4⁺, PD-1^{int}CXCR5⁻CD4⁺, PD-1^{high}CXCR5⁻CD4⁺, and CXCR5⁺CD4⁺ T cells in NPs and PD-1^{high}CXCR5^{high}CD4⁺ T_{FH} cells in tonsils. Sample size for IFN- γ and IL-4: Eos NP, n = 10; Non-Eos NP, n = 7; for IL-17A: Eos NP, n = 5; Non-Eos NP, n = 6; for IL-2 and IL-10: Eos NP, n = 4; Non-Eos NP, n = 2. (B) Frequencies of HLA-DR⁺, CCR2⁺, CCR3⁺, CCR4⁺, CCR5⁺, CCR6⁺, CCR7⁺, CXCR3⁺, and CXCR4⁺ cells in PD-1⁻CXCR5⁻CD4⁺, PD-1^{int}CXCR5⁻CD4⁺, PD-1^{high}CXCR5⁻CD4⁺, and CXCR5⁺CD4⁺ T cells in NPs and PD-1^{high}CXCR5^{high}CD4⁺ T_{FH} cells in tonsils. Sample size for HLA-DR: Eos NP, n = 4; Non-Eos NP, n = 7; for CCR2, CCR3 and CCR4: Eos NP, n = 4; Non-Eos NP, n = 6; for CCR5, CCR7 and CXCR4: Eos NP, n = 3; Non-Eos NP, n = 7; for CCR6: Eos NP, n = 3; Non-Eos NP, n = 3; for CXCR3: Eos NP, n = 4; Non-Eos NP, n = 2. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. (C) Left: representative flow plot and the gating strategy used to define CD69⁺CD103⁺ cells in PD-1^{high}CXCR5⁻CD4⁺ T cells in NPs. Right: mean percentages of CD69⁺CD103⁺ cells in PD-1^{high}CXCR5⁻CD4⁺ T cells in NPs (Eos NP, n = 6; Non-Eos NP, n = 6).

Fig E6. PD-1^{high}CXCR5⁻CD4⁺ T cells in peripheral bloods and tonsils. (A) Gating strategy used to define PD-1^{high}CXCR5⁻CD4⁺ T cells in peripheral bloods. (B) Frequencies of PD-1^{high}CXCR5⁻CD4⁺ T cells in bloods (n = 22), NPs (Eos NP, n = 13;

Non-Eos NP, n = 8), and tonsils (n = 19). (C) Frequencies of circulating PD-1^{high}CXCR5⁻CD4⁺ T cells in patients with CRSwNP (Eos NP, n = 13; Non-Eos NP, n = 8) and control subjects (n = 15). ***P* < 0.01 and ****P* < 0.001.

Fig E7. T-B cell interaction marker, transcription factor, and T_{REG} cell related marker expression in PD-1^{high}CXCR5⁻CD4⁺ T cells in NPs. (A) Frequencies of MAF⁺, SAP⁺, and SLAMF5⁺ cells in PD-1⁻CXCR5⁻CD4⁺, PD-1^{int}CXCR5⁻CD4⁺, PD-1^{high}CXCR5⁻CD4⁺, and CXCR5⁺CD4⁺ T cells in NPs and PD-1^{high}CXCR5^{high}CD4⁺ T_{FH} cells in tonsils. Eos NP, n = 5; Non-Eos NP, n = 5. (B) Frequencies of CTLA-4⁺, Tim-3⁺, Foxp3⁺, and CD25⁺ cells in PD-1⁻CXCR5⁻CD4⁺, PD-1^{int}CXCR5⁻CD4⁺, PD-1^{high}CXCR5⁻CD4⁺, and CXCR5⁺CD4⁺ T cells in NPs and PD-1^{high}CXCR5^{high}CD4⁺ T_{FH} cells in tonsils. Sample size for Tim-3 and CTLA-4: Eos NP, n = 4; Non-Eos NP, n = 4; for Foxp3: Eos NP, n = 5; Non-Eos NP, n = 4; for CD25: Eos NP, n = 4; Non-Eos NP, n = 3. (C) Frequencies of T-bet⁺ cells in PD-1⁻CXCR5⁻CD4⁺, PD-1^{int}CXCR5⁻CD4⁺, PD-1^{high}CXCR5⁻CD4⁺, and CXCR5⁺CD4⁺ T cells in NPs and PD-1^{high}CXCR5^{high}CD4⁺ T_{FH} cells in tonsils. Eos NP, n = 4; Non-Eos NP, n = 6. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

Fig E8. Analysis of indicated genes expression in CD4 expressing cells in NPs by single-cell RNA sequencing. (A) The t-distributed stochastic neighbor embedding (tSNE) plots showing the expression of CXCR5 and PDCD1 gene in CD4 expressing cells in NPs. Red color indicates PDCD1 relative expression intensity and green color

indicates *CXCR5* relative expression intensity. **(B)** tSNE plots showing the expression of indicated genes in *CD4* expressing cells in NPs. Red color indicates the relative expression intensity of specific gene. The circles indicate aggregation of cells with low or absent *CXCR5* expression and high *PDCDI* expression. The notes after the gene name indicate the protein name encoded by the gene. Eos NP, n = 2; Non-Eos NP, n = 2.

Figure E9. Heat map of frequencies of expression of indicated markers by PD-1^{high}CXCR5⁺CD4⁺ T cells in eosinophilic and noneosinophilic NPs analyzed by flow cytometry. Sample size for CXCL13, CCR2, CCR3, CCR4 and T-bet: Eos NP, n = 4; Non-Eos NP, n = 6; for IL-21, IFN- γ and IL-4: Eos NP, n = 10; Non-Eos NP, n = 7; for IL-17A and Bcl-6: Eos NP, n = 5; Non-Eos NP, n = 6; for IL-2, IL-10 and CCR6: Eos NP, n = 3; Non-Eos NP, n = 3; HLA-DR: Eos NP, n = 4; Non-Eos NP, n = 7; for CCR5, CCR7 and CXCR4: Eos NP, n = 3; Non-Eos NP, n = 7; for ICOS: Eos NP, n = 3; Non-Eos NP, n = 8; for CD69 and CD103: Eos NP, n = 6; Non-Eos NP, n = 6; for CD62L: Eos NP, n = 7; Non-Eos NP, n = 3; for Blimp1 and Ki-67: Eos NP, n = 6, Non-Eos NP, n = 4; for MAF, SAP and SLAMF5: Eos NP, n = 5; Non-Eos NP, n = 5; for Tim-3 and CTLA-4: Eos NP, n = 4; Non-Eos NP, n = 4; for Foxp3: Eos NP, n = 4; Non-Eos NP, n = 5; for CD25: Eos NP, n = 4; Non-Eos NP, n = 3.

Fig E10. Functional characteristics of PD-1^{high}CXCR5⁺CD4⁺ T cells in NPs. PD-1^{high}CXCR5⁺CD4⁺ T cells purified from eosinophilic NPs were cultured with

autologous naive B cells. (A) Concentrations of CXCL13, IL-21, and IL-4 in culture superannuants were determined by ELISA at day 2 after T-B cell coculture (n = 6). (B) AID mRNA expression was measured by RT-PCR at day 7 after T-B cell coculture (n = 6). (C) Number of viable B cells was counted at day 12 after coculture (n = 6). **P* < 0.05 and ***P* < 0.01. *nd*, no detected.

Fig E11. PD-L1 and PD-L2 expression in NPs. (A) Numbers of PD-L1 and PD-L2 positive cells in the lamina propria (LP) in nasal tissues. (B) and (C) Mean percentages of CD45⁺CD326⁺ and other cells accounting for the PD-L1⁺ (B) and PD-L2⁺ (C) cells. Majority of PD-L1 and PD-L2 positive cells were CD45⁺CD326⁺ epithelial cells in NPs (Eos NP, n = 3; Non-Eos NP, n = 5). Representative flow plots are shown.

Fig E12. Frequencies of expression of indicated markers by PD-1^{high}CXCR5⁺CD4⁺ T cells in NPs with and without eLTs. Sample size for NPs with eLTs: ICOS and CD69: Eos NP, n = 3; Non-Eos NP, n = 2; CD103: Eos NP, n = 4; Non-Eos NP, n = 1; Ki-67: Eos NP, n = 2; Non-Eos NP, n = 1; IFN- γ , IL-17A, IL-21, and IL-4: Eos NP, n = 4; Non-Eos NP, n = 2. Sample size for NPs without eLTs: ICOS, CD69, and CD103: Eos NP, n = 10; Non-Eos NP, n = 10; Ki-67: Eos NP, n = 9; Non-Eos NP, n = 11; IFN- γ , IL-17A, IL-21, and IL-4: Eos NP, n = 10; Non-Eos NP, n = 12.

Table E1. Demographic and clinical characteristics of enrolled subjects

	Control subjects	Patients with Eos NPs	Patients with Non-Eos NPs	<i>P</i> value
Total Subjects enrolled	76	141	130	
Methodology used				
<i>Histology</i>				
Subject number	76	141	130	
Gender, male	45 (59.2%)	90 (63.8%)	77 (59.2%)	0.437
Age (years)	33 (25, 43)	37 (27, 45)	39 (24, 45)	0.162
Patient with atopy	3 (3.9%)	45 (32.3%)	26 (20.3%)	< 0.001
Patient with allergic rhinitis	0	28 (20.1%)	6 (4.6%)	< 0.001
Patients with asthma	0	10 (7.2%)	3 (1.9%)	0.017
<i>RT-PCR</i>				
Subject number	18	19	20	
Gender, male	8 (44.4%)	11 (57.9%)	12 (60.0%)	0.587
Age (years)	31 (19, 41)	34 (24, 46)	40 (24, 46)	0.456
Patient with atopy	3 (16.7%)	5 (26.3%)	3 (15.0%)	0.632
Patient with allergic rhinitis	0	3 (15.7%)	1 (5.0%)	0.155
Patients with asthma	0	2 (10.5%)	0	0.126
<i>Flow cytometry</i>				

PD-1^{high}CXCR5⁻CD4⁺ T cells in nasal polyps

Subject number	45	74	75	
Gender, male	26 (55.6%)	52 (70.2%)	47 (62.6%)	0.355
Age (years)	33 (25, 45)	36 (27, 45)	40 (24, 47)	0.547
Patient with atopy	2 (4.4%)	18 (24.3%)	12 (16.0%)	0.018
Patient with allergic				
rhinitis	0	14 (18.9%)	5 (6.6%)	0.002
Patients with asthma	0	4 (5.4%)	2 (2.7%)	0.246
<i>Cell culture study</i>				
Subject number	-	15	12	
Gender, male	-	9 (60.0%)	8 (66.7%)	1.000
Age (years)	-	31 (27, 40)	30 (24, 44)	0.764
Patient with atopy	-	4 (26.7%)	2 (16.6%)	0.651
Patient with allergic				
rhinitis	-	2 (13%)	1 (8%)	1.000
Patients with asthma	-	0	0	-
<i>ELISA of tissue</i>				
<i>homogenates</i>				
Subject number	18	21	19	
Gender, male	10 (55.6%)	12 (57.1%)	12 (63.1%)	0.883
Age (years)	29 (25, 38)	36 (25, 45)	35 (24, 45)	0.522
Patient with atopy	1 (5.6%)	5 (23.8%)	2 (10.5%)	0.227

Patient with allergic				
rhinitis	0	3 (14.2%)	1 (5.2%)	0.202
Patients with asthma	0	0	0	-
<i>Clinical characteristics</i>				
<i>and post-surgical NP</i>				
<i>recurrence study</i>				
Subject number	-	14	15	
Gender, male	-	8 (57.1%)	11 (73.3%)	0.450
Age (years)	-	35 (28, 48)	40 (24, 45)	0.739
Patient with atopy	-	5 (28.5%)	2 (13.3%)	0.215
Patient with allergic				
rhinitis	-	2 (14.2%)	0	0.224
Patients with asthma	-	1 (7.1%)	0	0.483
<i>Single-cell RNA</i>				
<i>sequencing</i>				
Subject number	-	2	2	
Gender, male	-	1 (50%)	1 (50%)	
Age (years)	-	34, 39	28, 42	
Patient with atopy	-	0	0	
Patient with allergic				
rhinitis	-	0	0	
Patients with asthma	-	0	0	

400 For continuous variables, results are expressed as medians and interquartile ranges.
401 Categorical variables are summarized using percentage. Numbers in bold indicate the
402 *P* value less than .05. Eos, eosinophilic; Non-Eos, noneosinophilic; NPs, nasal polyps.

403 **Table E2. Antibodies used in immunofluorescence study**

Specificity	Species	Concentration	Clone ID	Manufacturer
CD4	Mouse	1:100	UMAB64	Zhongshan Golden Bridge Biotechnology (Beijing, China)
CD4	Goat	1:100	polyclonal	R&D systems (Minneapolis, USA)
CD4	Rabbit	1:100	EP204	Zhongshan Golden Bridge Biotechnology
CD20	Rabbit	1:100	EP7	Zhongshan Golden Bridge Biotechnology
CD20	Mouse	1:100	L26	Zhongshan Golden Bridge Biotechnology
CXCR5	Mouse	1:100	51505	R&D systems
PD-1	Goat	1:100	polyclonal	R&D systems
PD-L1	Rabbit	1:100	RM320	Boster Biotechnology (Wuhan, China)
PD-L2	Rabbit	1:100	polyclonal	Boster Biotechnology
IL-21	Rabbit	1:100	polyclonal	Abcam (Cambridge, MA, UK)
IFKine™ Green donkey anti-mouse IgG	Donkey	1:100	polyclonal	Abbkine Scientific Company (Wuhan, China)
IFKine™ Blue donkey anti-rabbit IgG	Donkey	1:100	polyclonal	Abbkine Scientific Company
IFKine™ Green donkey anti-rabbit IgG	Donkey	1:100	polyclonal	Abbkine Scientific Company
IFKine™ Red donkey anti-rabbit IgG	Donkey	1:100	polyclonal	Abbkine Scientific Company
IFKine™ Red donkey	Donkey	1:100	polyclonal	Abbkine Scientific

PD-1^{high}CXCR5⁺CD4⁺ T cells in nasal polyps

anti-goat IgG				Company
IFKine™ Blue donkey	Donkey	1:100	polyclonal	Abbkine Scientific
anti-mouse IgG				Company
404	CXCR5, C-X-C chemokine receptor type 5; Ig, immunoglobulin; IL, interleukin; PD-			
405	L, programmed death-ligand.			

406 **Table E3. Antibodies used for flow cytometry and cell sorting**

Antibody	Parameter	Clone ID	Source	Isotype	Manufacturer	Dilution
					BD	
CD3	BV510	HIT3a	Mouse	IgG2a, κ	Biosciences, (CA, USA)	1:200
CD4	APC-Cy7	RPA-T4	Mouse	IgG1, κ	BD Biosciences	1:200
CD8	APC-Cy7	RPA-T8	Mouse	IgG1, κ	BD Biosciences	1:200
CD25	PE-Cy7	M-A251	Mouse	IgG1, κ	BD Biosciences	1:100
CD127	BV421	HIL-7R-M21	Mouse	IgG1, κ	BD Biosciences	1:100
CD45RA	BV605	HI100	Mouse	IgG2b, κ	BD Biosciences	1:100
CXCR3	APC	IC6/CXCR3	Mouse	IgG1, κ	BD Biosciences	1:50
CXCR4	PerCP-Cy5.5	12G5	Mouse	IgG2a, κ	Biolegend (CA, USA)	1:100
CXCR5	BB515 (FITC)	RF8B2	Rat	IgG2b, κ	BD Biosciences	1:100
CCR2	APC	SA203G11	Rat	IgG2b, κ	Biolegend	1:100
CCR3	PerCP-Cy5.5	5E8	Mouse	IgG2b, κ	Biolegend	1:100
CCR4	PE	L291H4	Mouse	IgG1, κ	Biolegend	1:100

PD-1^{high}CXCR5⁺CD4⁺ T cells in nasal polyps

CCR5	PE	J418F1	Rat	IgG2a, κ	Biolegend	1:100
CCR6	PerCP-Cy5.5	11A9	Mouse	IgG1, κ	BD Biosciences	1:100
CCR7	Alexa Fluor 647	3D12	Rat	IgG2a, κ	BD Biosciences	1:100
CXCL13	APC	53610	Mouse	IgG1	R&D Systems, Minnesota (CA, USA)	1:50
CD62L	PE	DREG-56	Mouse	IgG1, κ	BD Biosciences	1:200
CD3	FITC	UCHT1	Mouse	IgG1, κ	BD Biosciences	1:200
CD25	PE	BC96	Mouse	IgG1, κ	Biolegend	1:100
CD27	PE-Cy7	M-T271	Mouse	IgG1, κ	BD Biosciences	1:100
CD45	APC	HI30	Mouse	IgG1, κ	BD Biosciences	1:100
CD45	FITC	HI30	Mouse	IgG1, κ	BD Biosciences	1:100
CD69	PE-Cy7	FN50	Mouse	IgG1, κ	BD Biosciences	1:100
CD103	BV421	Ber-ACT8	Mouse	IgG1, κ	BD Biosciences	1:100
CD326	PerCP-Cy5.5	9C4	Mouse	IgG2b, κ	BD Biosciences	1:100
HLA-DR	BV421	G46-6	Mouse	IgG2a, κ	BD Biosciences	1:100

PD-1^{high}CXCR5⁺CD4⁺ T cells in nasal polyps

HLA-DR	APC	G46-6	Mouse	IgG2a, κ	BD Biosciences	1:100
PD-1	PerCP-Cy5.5	EH12.1	Mouse	IgG1, κ	BD Biosciences	1:100
PD-1	BV421	29F.1A12	Rat	IgG2a, κ	BD Biosciences	1:100
PD-L1	BV605	29E.2A3	Mouse	IgG2b, κ	Biolegend	1:50
PD-L2	BV421	MIH18	Mouse	IgG1, κ	BD Biosciences	1:50
ICOS	APC	DX29	Mouse	IgG1, κ	BD Biosciences	1:100
CTLA-4	APC	BNI3	Mouse	IgG2a, κ	BD Biosciences	1:100
IFN-γ	PerCP-Cy5.5	B27	Mouse	IgG1, κ	BD Biosciences	1:100
IL-2	PE	MQ1-17H12	Rat	IgG2a, κ	Biolegend	1:50
IL-4	APC	JES3-19F1	Rat	IgG2a, κ	BD Biosciences	1:100
IL-4	PE	8D4-8	Mouse	IgG1, κ	BD Biosciences	1:100
IL-10	APC	JES3-19F1	Rat	IgG2a, κ	BD Biosciences	1:50
IL-17A	APC	BL168	Mouse	IgG1, κ	Biolegend	1:100
IL-17A	BV605	BL168	Mouse	IgG1, κ	Biolegend	1:100
IL-21	Alexa Fluor 647	3A3-N2.1	Mouse	IgG1, κ	Biolegend	1:100
IL-21	PE	3A3-N2.1	Mouse	IgG1, κ	BD Biosciences	1:100

PD-1^{high}CXCR5⁺CD4⁺ T cells in nasal polyps

Foxp3	APC	259D/C	Mouse	IgG1	BD Biosciences	1:100
Bcl-6	APC	K112-91	Mouse	IgG1, κ	BD Biosciences	1:100
Blimp1	PE	6D3	Rat	IgG2a, κ	BD Biosciences	1:100
Ki-67	PerCP-Cy5.5	B56	Mouse	IgG1, κ	BD Biosciences	1:100
T-bet	PerCP-Cy5.5	O4-46	Mouse	IgG1, κ	BD Biosciences	1:100
Tim-3	APC	F38-2E2	Rat	IgG1, κ	Biolegend	1:100
SAP	PE	XLP-1D12	Rat	IgG2a, κ	eBioscience	1:100
MAF	PE	T54-853	Mouse	IgG2a, κ	BD Biosciences	1:100
SLAMF5 (CD84)	APC	CD84.1.21	Mouse	IgG2a, κ	Biolegend	1:100
CD19	APC-Cy7	SJ25C1	mouse	IgG1, κ	BD Biosciences	1:100
CD20	BV510	2H7	mouse	IgG2b, κ	BD Biosciences	1:100
CD27	PE-Cy7	M-T271	mouse	IgG1, κ	BD Biosciences	1:100
IgD	PerCP-Cy5.5	IA6-2	mouse	IgG2a, κ	BD Biosciences	1:100

407 APC, allophycocyanin; Bcl-6: transcription factor B cell lymphoma 6; Blimp1: B
408 lymphocyte-induced maturation protein 1; BV, brilliant violet; CCR: CC-chemokine
409 receptor; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; CXCL13: chemokine
410 (C-X-C motif) ligand 13; CXCR: chemokine (C-X-C motif) receptor; FITC, fluorescein

411 isothiocyanate; Foxp3: forkhead box P3; HLA-DR: human leukocyte antigen-DR
412 isotype; ICOS: inducible co-stimulator; IFN- γ : interferon-gamma; Ig, immunoglobulin;
413 IL, interleukin; PD-1, programmed cell death protein 1; PD-L: programmed death-
414 ligand; PE, phycoerythrin; PerCP, peridinin chlorophyll protein complex; SAP:
415 signaling lymphocytic activation molecule-associated protein; SLAMF5: signaling
416 lymphocytic activation molecule 5; T-bet: T-box transcription factor expressed in T-
417 cells; TIM-3: T-cell immunoglobulin and mucin-domain containing-3.

418 **Table E4. Quality control measurements for single-cell RNA sequencing**

Numbers	Total	Fractions	Estimated	Mean	Reads mapped	Median		Median
						genes	Sequencing	UMI
								counts
	number of	of reads	number	reads	confidently to	per cell	saturation	per cell
	reads	in cells	of cells	per cell	transcriptome			
Eos NP #1	427,416,910	96.1%	9,982	42,819	59.8%	1,766	74.60%	4,919
Eos NP #2	500,931,373	97.6%	9,433	53,104	62.1%	1,833	82.7%	4,759
Non-Eos NP								
#1	402,823,654	96.4%	8,007	50,309	38.5%	1,704	73.2%	4,259
Non-Eos NP								
#2	439,368,442	94.9%	8,432	52,107	62.5%	1,679	81.0%	4,516

419

420 **Table E5. The lower detection limits for Bio-Plex chip assay and ELISA**

Target	Lower detection limit
IgG	2.74 ng/mL
IgA	0.13 ng/mL
IgM	0.78 ng/mL
IgE	0.02 ng/mL
IL-21	1.03 pg/mL
CXCL13	4.86 pg/mL
PD-L1	5.58 pg/mL
PD-L2	5.58 pg/mL
IL-4	2.2 pg/mL

421 CXCL, chemokine (C-X-C motif) ligand; Ig, immunoglobulin; IL, interleukin; PD-L,
 422 programmed death-ligand.

Table E6. Primers used for quantitative RT-PCR analysis

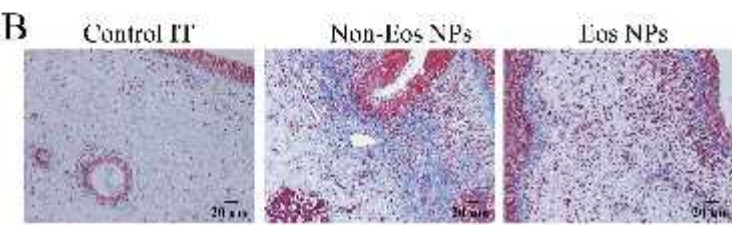
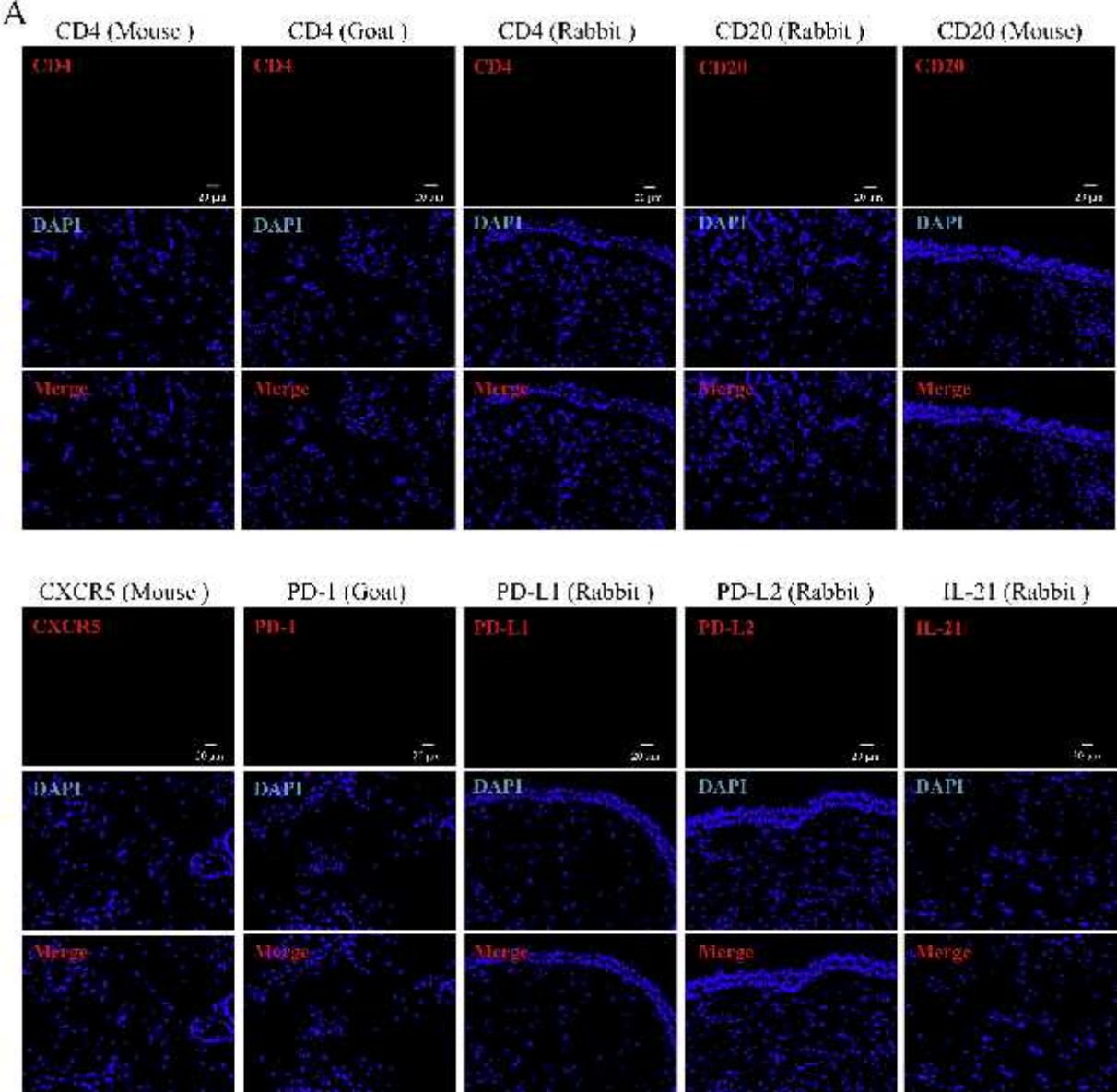
	Primer sequences	Annealing temperature (°C)
PD-L1	(F) 5'-TGGCATTGCTGAACGCATTT-3' (R) 5'-TGCAGCCAGGTCTAATTGTTTT-3'	60
PD-L2	(F) 5'-ACCGTGAAAGAGCCACTTTG-3' (R) 5'-GCGACCCCATAGATGATTATGC-3'	60
AID	(F) 5'-AAAATGTCCGCTGGGCTAAG-3' (R) 5'-TCGTGGTTTTCTTTGAAGGTCAT-3'	60
GUSB	(F) 5'-CACCAGGGACCATCCAATACC-3' (R) 5'-GCAGTCCAGCGTAGTTGAAAAA-3'	60

AID, activation induced cytidine deaminase; GUSB, glucuronidase beta; PD-L, programmed death-ligand; RT-PCR, reverse transcriptase polymerase chain reaction.

Table E7. The associations between the frequencies of PD-1^{high}CXCR5⁺CD4⁺ T cells and clinical characteristics in patients with chronic rhinosinusitis with nasal polyps

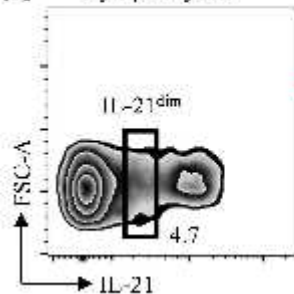
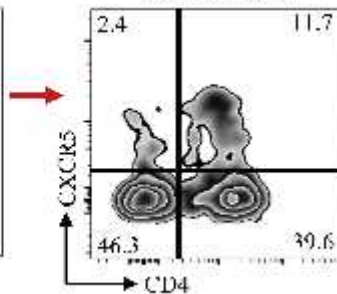
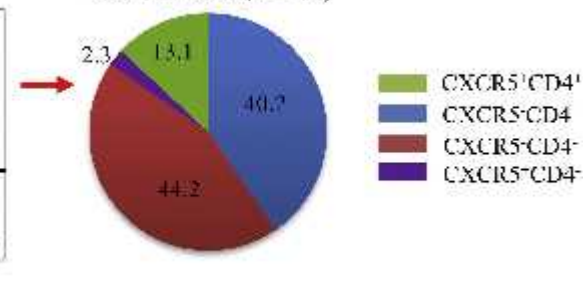
Characteristics	Values	Correlations with PD-1 ^{high} CXCR5 ⁺ CD4 ⁺ T cells (n = 29)	
		R	P value
Duration of disease (years)	4 (1, 6.25)	0.449	0.015
Symptom VAS score			
Nasal obstruction	6 (3.75, 8)	0.302	0.110
Rhinorrhea	5 (2, 7)	0.068	0.727
Facial pain	0 (0, 4)	0.268	0.160
Loss of smell	8 (3.75, 10)	0.075	0.700
Total score	19.5 (12.75, 24.25)	0.315	0.096
Overall burden	6 (5, 7)	0.138	0.475
Total endoscopic score	8 (5, 9.25)	0.429	0.020
Total CT score	14 (8, 21)	0.303	0.101

Results are expressed as medians and interquartile ranges. Numbers in bold indicate the P value less than .05. CT, computed tomography; VAS, visual analogue scale.



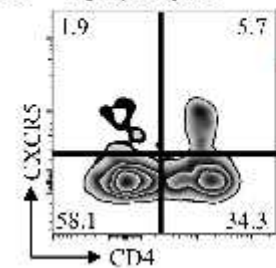
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Lymphocytes

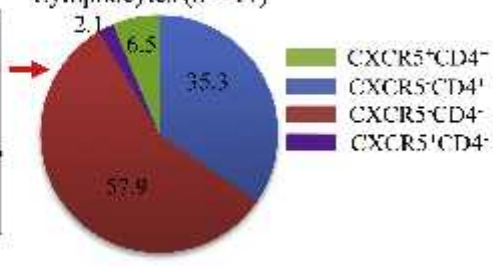
IL-21^{dim} cellsIL-21^{dim} cells (n = 17)

B

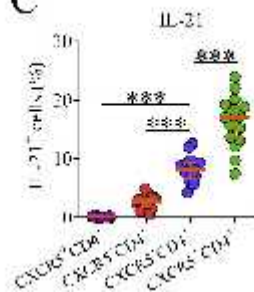
Lymphocytes



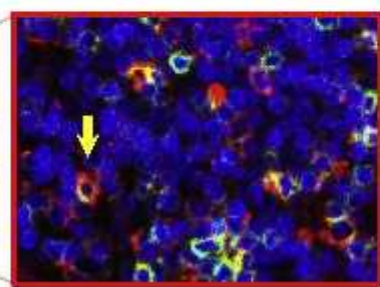
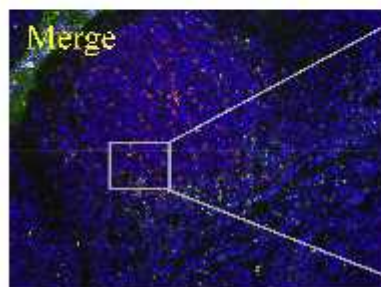
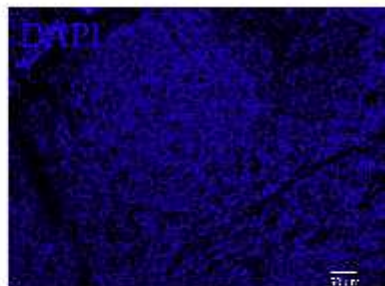
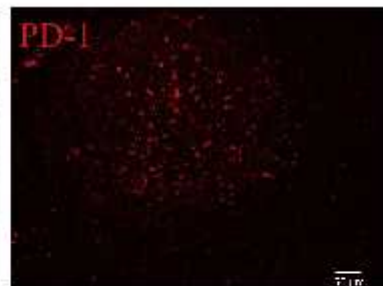
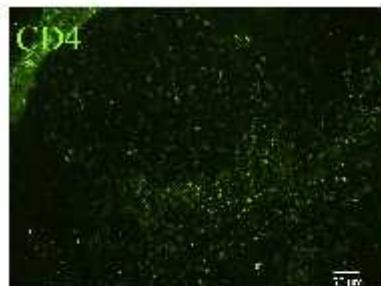
Lymphocytes (n = 17)



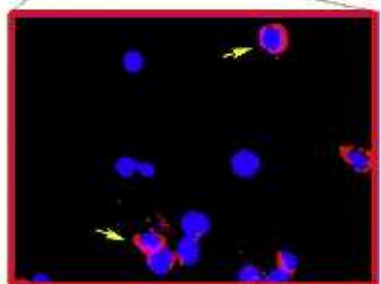
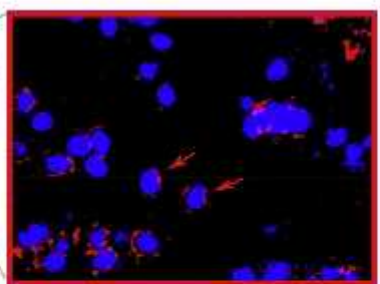
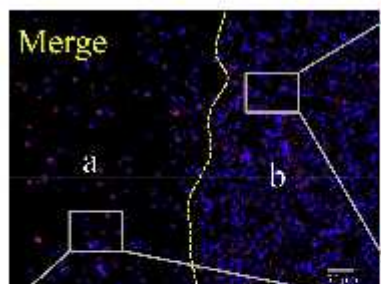
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A

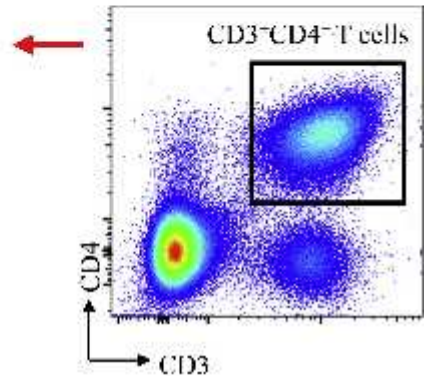
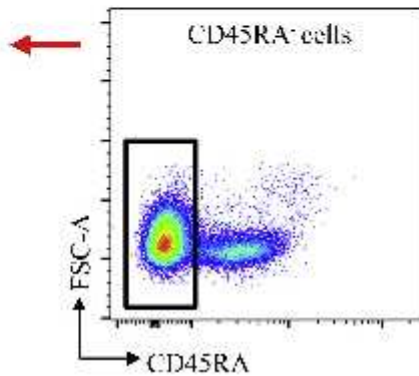
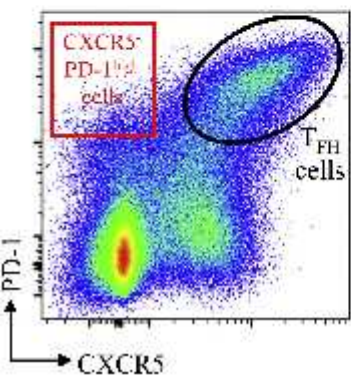
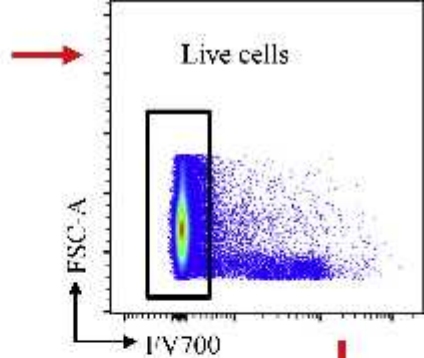
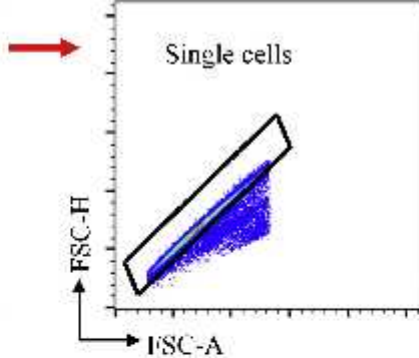
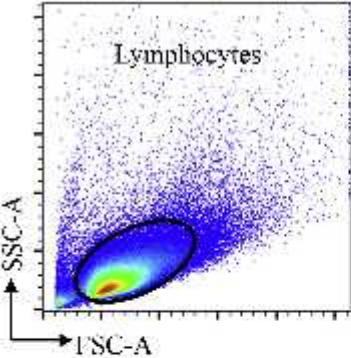


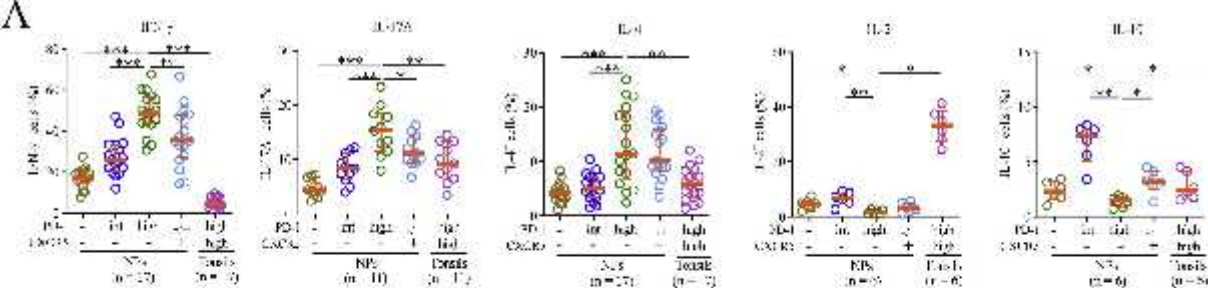
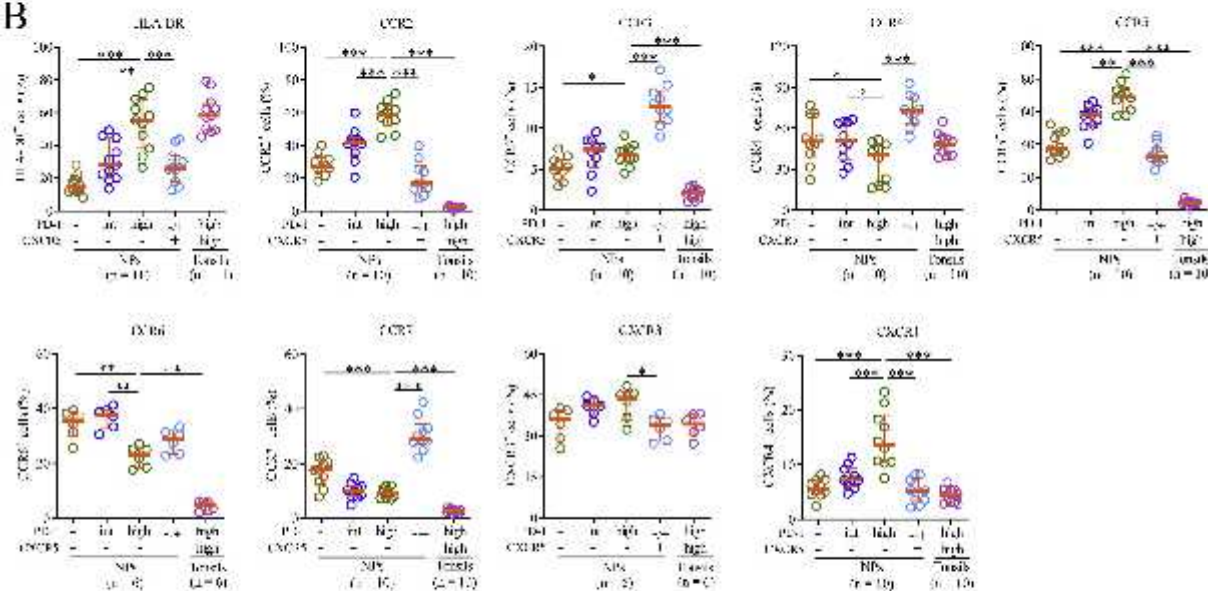
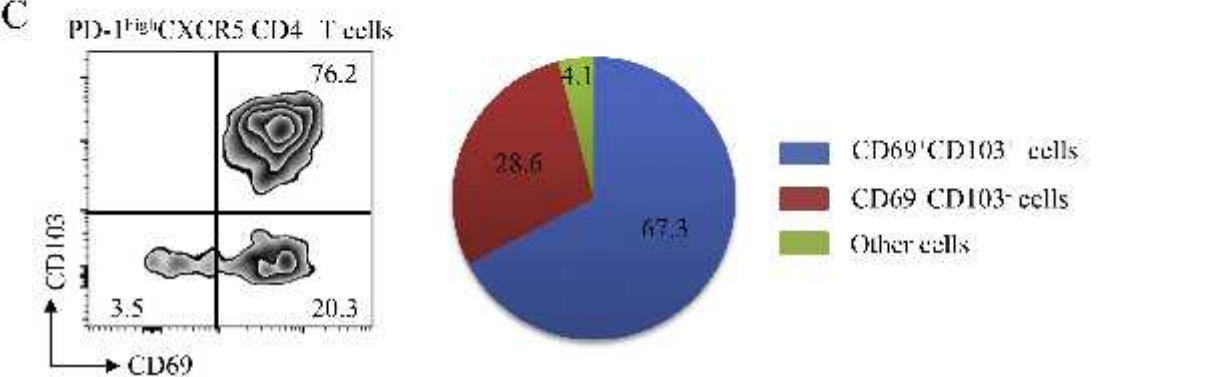
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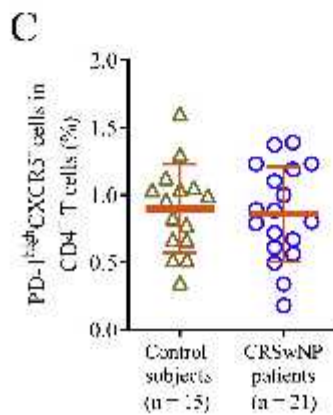
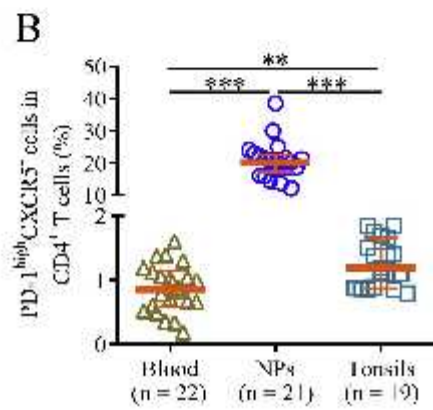
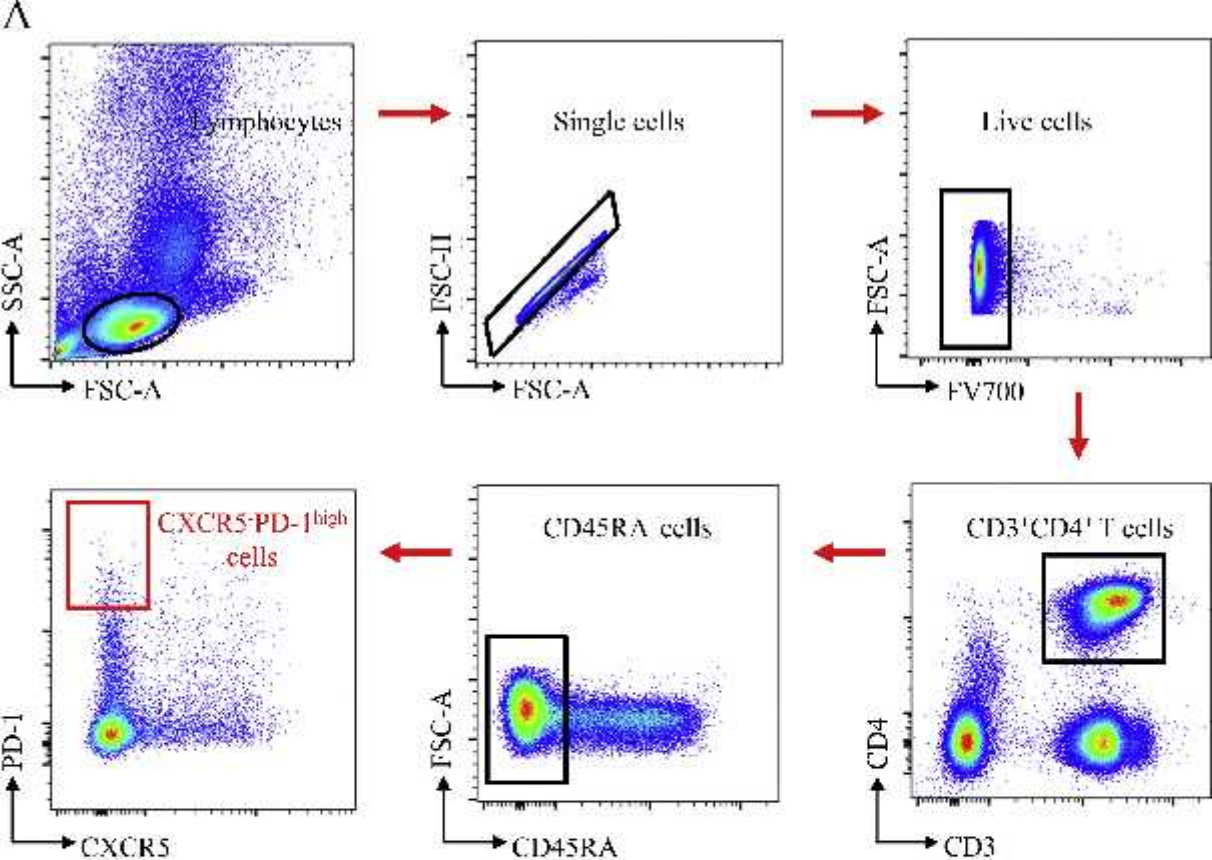


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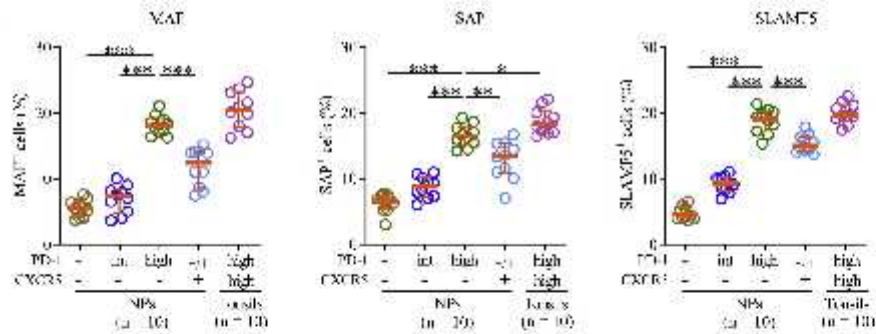
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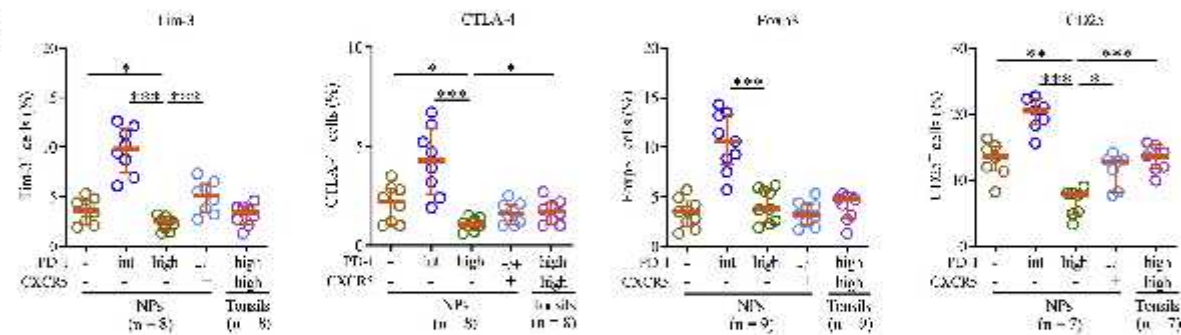
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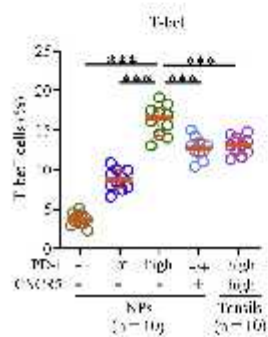
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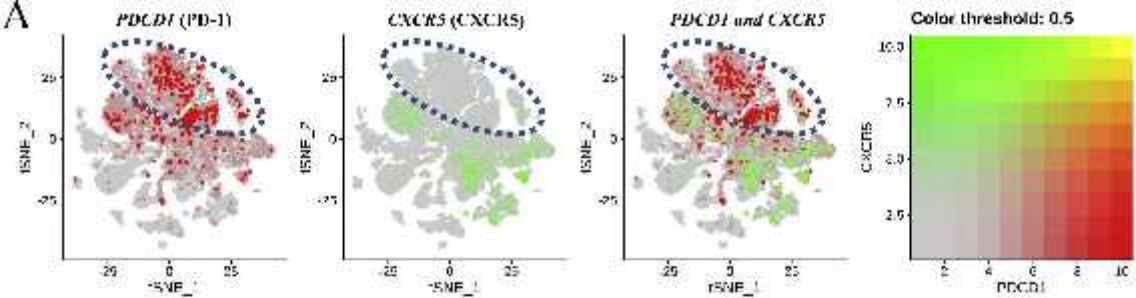
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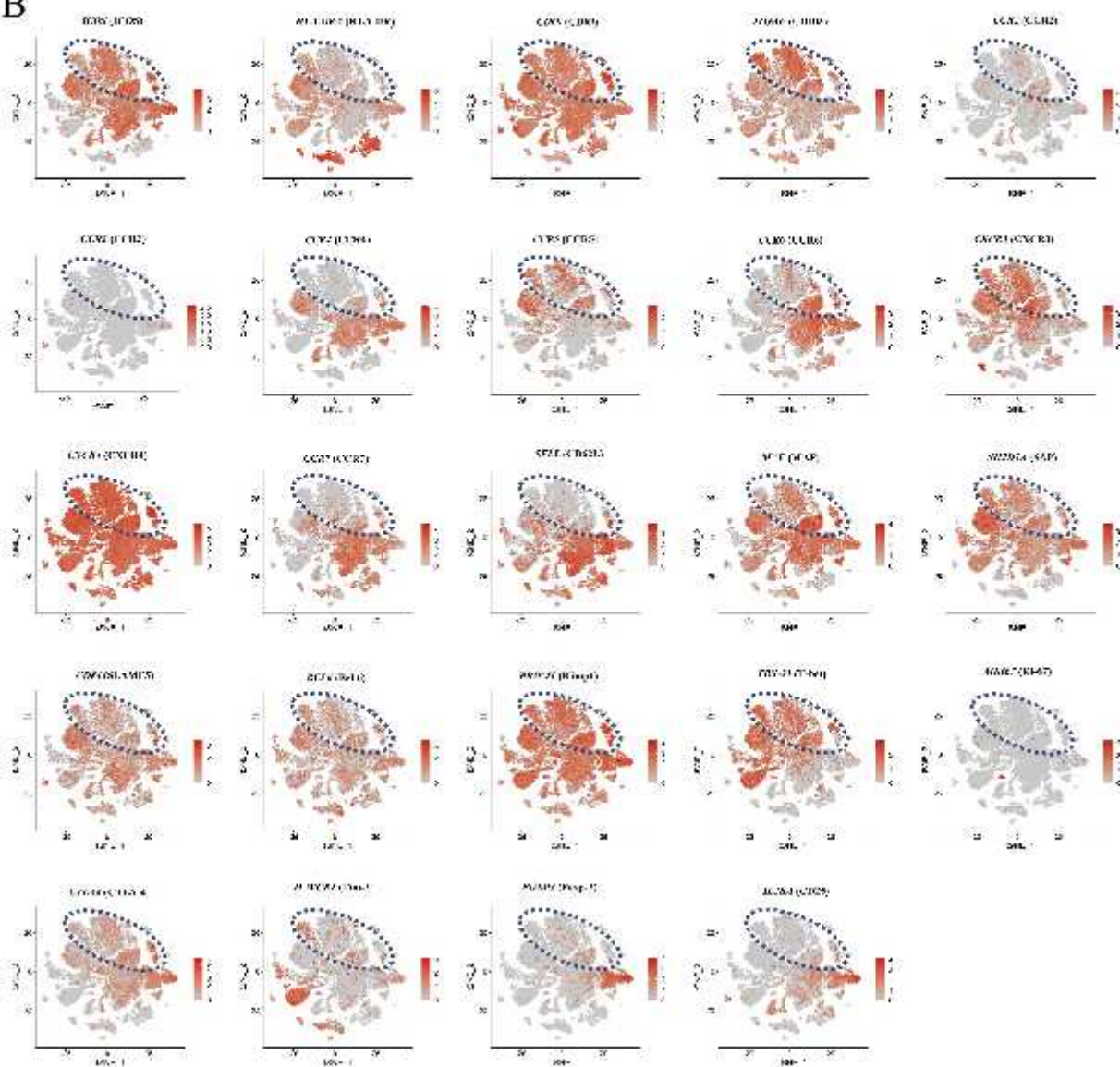
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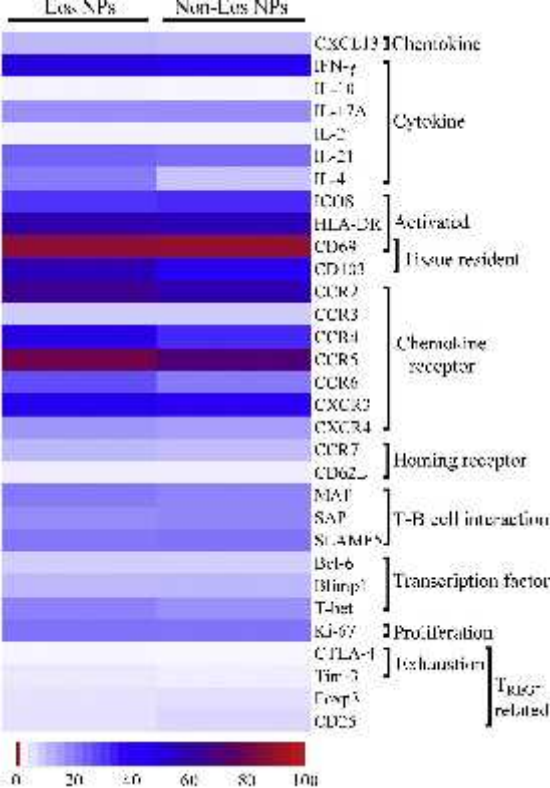


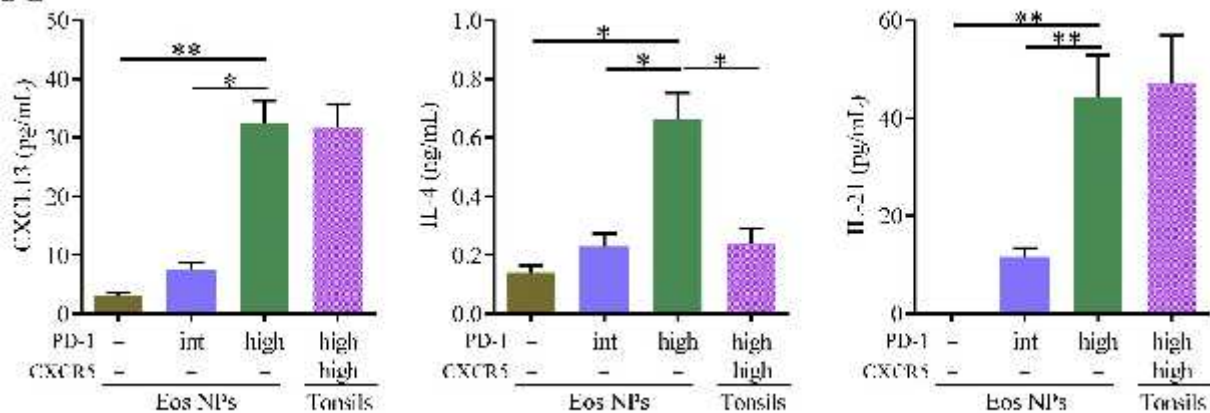
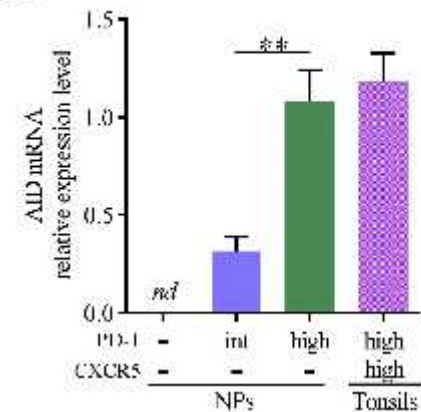
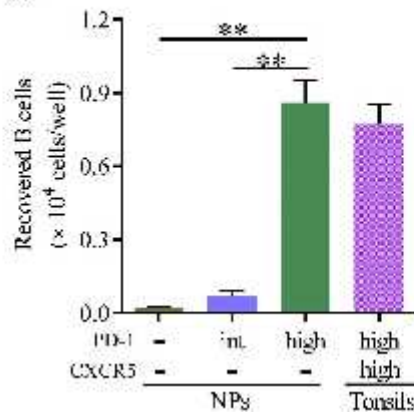
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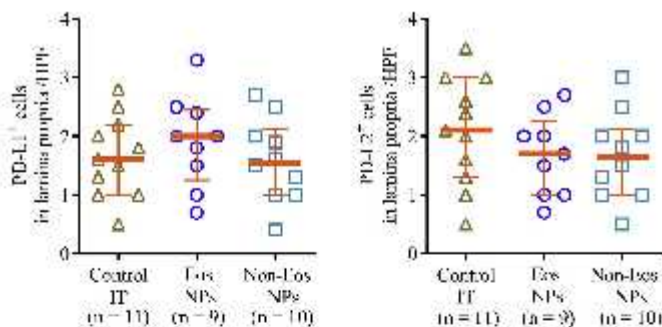
B



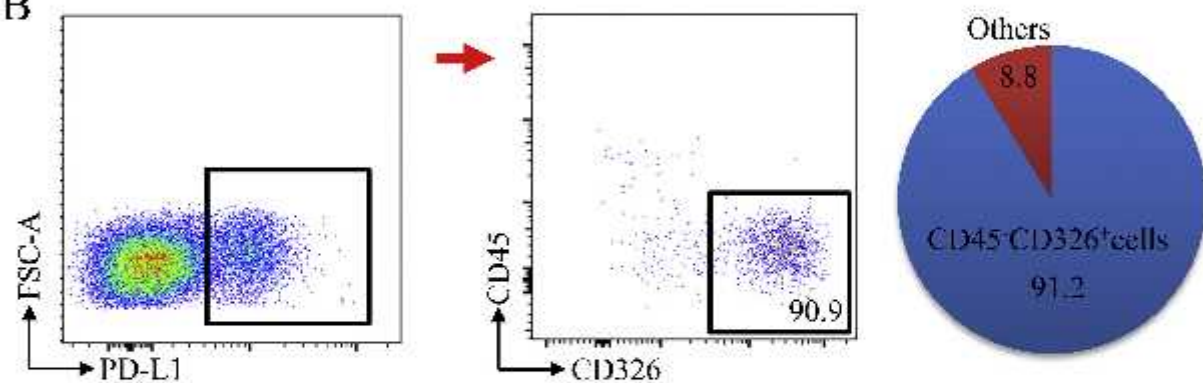


A**B****C**

A



B



C

