

# Stromal cells and B cells orchestrate ectopic lymphoid tissue formation in nasal polyps

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## Abstract

**Background:** Although the importance of ectopic lymphoid tissues (eLTs) in the pathophysiology of nasal polyps (NPs) is increasingly appreciated, the mechanisms underlying their formation remain unclear. **Objective:** To study the role of IL-17A, CXCL13 and lymphotoxin (LT) in eLT formation in NPs. **Methods:** The expression of CXCL13 and LT as well as their receptors, and the phenotypes of stromal cells in NPs were studied by flow cytometry, immunostaining, and RT-PCR. Purified nasal stromal cells and polyp B cells were cultured and a murine model with nasal type 17 inflammation was established for the mechanistic study. **Results:** Excessive CXCL13 production was found in NPs and correlated with enhanced IL-17A expression. Stromal cells, with an expansion of CD31-Pdpn+ fibroblastic reticular cell (FRC) type, were the major source of CXCL13 in NPs without eLTs. IL-17A induced FRC expansion and CXCL13 production in nasal stromal cells. In contrast, B cells were the main source of CXCL13 and LT $\alpha$ 1 $\beta$ 2 in NPs with eLTs. CXCL13 upregulated LT $\alpha$ 1 $\beta$ 2 expression on polyp B cells, which in turn promoted CXCL13 production from polyp B cells and nasal stromal cells. LT $\alpha$ 1 $\beta$ 2 induced expansion of FRCs and CD31+Pdpn+ lymphoid endothelial cells, corresponding to the phenotypic characteristic of stromal cells in NPs with eLTs. IL-17A gene knockout, and CXCL13 and LT $\beta$ R blockage diminished nasal eLT formation in the murine model. **Conclusion:** We identified an important role of IL-17A-induced stromal cell remodeling in the initiation, and crosstalk between B and stromal cells via CXCL13 and LT $\alpha$ 1 $\beta$ 2 in the enlargement of eLTs in NPs.

## INTRODUCTION

Nasal polyps (NPs) are benign edematous outgrowth of the sinonasal mucosa characterized by persistent and exaggerated inflammation.<sup>1</sup> Clinical management of NPs is largely unsatisfying,<sup>2, 3</sup> reflecting our limited understanding of the pathogenesis of NPs. Emerging evidence highlight an important role of local immunoglobulin (Ig) hyperproduction in the pathogenesis of NPs. Local IgE, IgG, IgA and IgD may lead to the activation of mast cells, eosinophils and classic complement pathway in NPs, and associate with poor treatment outcome.<sup>4-7</sup> Recently, it has been revealed that local immunoglobulin production is supported by ectopic lymphoid tissues (eLTs) in NPs, which are composed of T/B cell aggregation and germinal center (GC) like structure.<sup>8</sup> The eLT formation associates with refractory disease in patients with NPs as well.<sup>8, 9</sup> Therefore, disrupting the formation of eLTs, which likely function as lymphoid organs in NPs, may hold significant clinical implications for NP treatment, particularly for the refractory type. However, little is known about what drives the appearance of eLTs in NPs.

The eLTs share morphological and functional similarities with secondary lymphoid organs (SLOs).<sup>10</sup> In the development of SLOs, lymphotoxin (LT)  $\alpha_1\beta_2$ -expressing lymphoid tissue inducer (LTi) cells induce the differentiation of the LT $\beta$  receptor (LT $\beta$ R) positive stromal organizer cells and subsequent secretion of homeostatic chemokines, such as CXCL13, CCL21 and CXCL12, from those cells, which lead to the recruitment and compartmentalization of T and B cells.<sup>11, 12</sup> In contrast to the well documented formation of SLOs, the mechanisms controlling the eLT development are limited understood. Although homeostatic chemokines have also been implicated in the eLT formation in inflamed tissues in response to infection, autoimmunity, cancer and transplantation,<sup>13-17</sup> the involvement of specific homeostatic chemokines is highly tissue and disease-dependent. In addition, inflammatory cells may substitute for embryonic LTi cells to induce the production of lymphorganogenic chemokines during ectopic lymphoid neogenesis. For example, IL-17A has been implicated in driving microbial infection-induced lung eLT formation by inducing CXCL12 or CXCL13 production in murine models.<sup>18, 19</sup>

Previous studies have demonstrated elevated IL-17A levels and increased accumulation of IL-17A positive CD4<sup>+</sup> and CD8<sup>+</sup> T cells in NPs, especially in Asian patients.<sup>20, 21</sup> We discovered that the presence of eLTs associated with elevated levels of IL-17A, LT and CXCL13 in NPs.<sup>8</sup> IL-17A-producing cells have been identified inside and surrounding the eLTs in NPs.<sup>8</sup> We therefore hypothesized that IL-17A, LT and CXCL13 may have a role in the development of eLTs in NPs. In this study, we determined the cellular sources of LT and CXCL13 as well as their receptors in NPs. We also performed mechanistic studies to explore the function of IL-17A, LT and CXCL13 in eLT formation in NPs by using nasal stromal cell and B cell culture, and a murine model with local IL-17A inflammation.

## **METHODS**

### **Subjects and specimens**

This study was approved by the Ethics Committee of Tongji Hospital and written informed consents were obtained from all subjects. A total of 78 NP patients and 49 control subjects were recruited in this study. The diagnosis of NPs was made according to the current guideline.<sup>1</sup> Patients undergoing septoplasty owing to anatomic variations and without any other inflammatory sinonasal disorders were enrolled as control subjects. During surgery, polyp tissues from patients with NPs and inferior turbinate mucosal tissues from control subjects were collected. Not all samples were included in every experiment protocol because of limited quantity. More information is provided in this article's Online Supplement including Table E1.

### **Immunohistochemistry and immunofluorescence**

The histology, immunohistochemistry and immunofluorescence study were conducted as described elsewhere.<sup>8</sup> In NPs, eLTs were defined as lymphoid aggregates with GC like structure as previously reported.<sup>8</sup> More information is provided in this article's Online Supplement including Tables E2 and E3.

### **Quantitative RT-PCR**

Quantitative RT-PCR was performed with specific primers (Table E4 and E5) as previously reported.<sup>7</sup> More information is provided in this article's Online Supplement.

### **Flow cytometry**

Nasal single cell suspensions were prepared, stained, and analyzed by flow cytometry as previously described.<sup>8</sup> More information including Table E6 is provided in this article's Online Supplement.

### **Purification and culture of B cells and stromal cells**

Nasal stromal cells from control tissues and B cells from polyp tissues with eLTs were isolated by means of immunomagnetic cell sorting, and cultured as previously stated.<sup>18, 22, 23</sup> More information is provided in this article's Online Supplement.

### **Mouse experiment**

Female C57BL/6 wild type (WT) mice and *il17a*<sup>-/-</sup> mice were treated with curdian intranasally.<sup>24-26</sup> In some experiment, WT mice were given anti-CXCL13 antibody or LT $\beta$ R-Ig fusion protein intraperitoneally. Histology and RT-PCR study were performed as mentioned elsewhere.<sup>27</sup> As previously described,<sup>28</sup> lymphoid aggregates with a dense lymphocyte core and > 50 cells were defined as eLTs, and those with fewer cells and/or dispersed lymphocyte localization were defined as lymphocyte clusters. All mice were used following protocols approved by the Animal Care and Use Committee of Tongji Hospital. More information is provided in this article's Online Supplement.

## Statistics

Statistical analysis was performed with SPSS 18.0 software (SPSS, Chicago, IL, USA). Data derived from human tissue and animal studies are presented in dot plots with horizontal bars representing medians and error bars showing interquartile ranges unless specifically addressed. A Kruskal-Wallis *H* test was used to assess intergroup variability and a Mann-Whitney *U* test was used for between-group comparisons. The Spearman rank test was used for correlations. Chi-square test or Fisher's exact test was applied to analyze differences in proportions between groups. Data from cell culture experiments are expressed as means  $\pm$  SEMs and were analyzed by the paired Student's *t* test. Significance was accepted at a *P* value of less than 0.05.

## RESULTS

### Distinct CXCL13 expression pattern in NPs with and without eLTs

Although both CXCL12/CXCR4 and CXCL13/CXCR5 axis are vital for B cell attraction and aggregation, our previous study only found an elevation of CXCL13, but not CXCL12, in NPs with eLTs.<sup>8</sup> In this study, we first confirmed increase of CXCL13 production in NPs with eLTs in comparison to NPs without eLTs and control tissues at both mRNA and protein level (Fig 1, A and B). Compared to control tissues, NPs without eLTs also demonstrated a mild increase of CXCL13 production (Fig 1, A and B). We therefore explored whether there is a difference in cellular sources of CXCL13 in NPs with and without eLTs. Immunofluorescence study showed that CXCL13 was mainly expressed by CD20<sup>+</sup> B cells and vimentin<sup>+</sup> stromal cells in NPs with and without eLTs (Fig 1, C). Given to the difficulty in counting the number of CXCL13 positive cells in eLTs in immunofluorescence study and the interference of membrane-bound CXCL13 on quantification of cytoplasmic CXCL13 in B cells by flow cytometry, we detected CXCL13 mRNA levels in different portions of cells in NPs. We depleted stromal cells from total polyp cells and found that CXCL13 mRNA expression was remarkably reduced in NPs without eLTs (mean, 66% reduction), while mildly reduced in NPs with eLTs (mean, 36% reduction) (Fig 1, D). On the contrary, after B cell depletion, there was about 31% and 60% (mean) reduction of CXCL13 mRNA expression in relation to the expression of total cells from NPs without and with eLTs, respectively (Fig 1, E). These results suggest that CXCL13 is mainly produced by stromal cells in NPs without eLTs, whereas by B cells in NPs with eLTs.

### The phenotypic changes of stromal cells in NPs with or without eLTs

The production of CXCL13 by nasal stromal cells in NPs without eLTs may point a role of stromal cells in the recruitment of B cells and initiation of eLT development. We then compared the CXCL13 expression in stromal cells from different types of sinonasal tissues. We found that compared to those in control tissues, CXCL13 mRNA expression was increased in stromal cells purified from both NPs with and without eLTs, although a more prominent upregulation was noted in NPs with eLTs than those without eLTs (Fig 2, A).

Based on CD31 and podoplanin (Pdpn, also known as gp38) expression,<sup>29, 30</sup> CD45 and epithelial cell adhesion molecule double negative stromal cells are classified into four subpopulations including CD31<sup>+</sup>Pdpn<sup>-</sup> blood endothelial cell (BEC), CD31<sup>-</sup>Pdpn<sup>+</sup> fibroblastic reticular cell (FRC), CD31<sup>+</sup>Pdpn<sup>+</sup> lymphoid endothelial cell (LEC), and CD31<sup>-</sup>Pdpn<sup>-</sup> double negative cell (DN) (Fig 2, B), which have different functions. We studied whether specific stromal cell types are involved in eLT formation in NPs. We discovered that the frequencies of FRCs were increased in both NPs with and without eLTs, and the frequencies of LECs were only increased in NPs with eLTs compared with those in control tissues (Fig 2, C and D). In contrast, the frequencies of

DNs were reduced in NPs with eLTs and no significant change of BECs was found in NPs in comparison to those in control tissues (Fig 2, C and D).

Analyzing CXCL13 expression in different nasal stromal cell types, we found that CXCL13 expression was enhanced in FRCs and LECs in both types of NPs in comparison to control tissues, with NPs with eLTs having a higher upregulation than NPs without eLTs (Fig E1, A and B). CXCL13 expression in DNs was only upregulated in NPs with eLTs and no upregulation of CXCL13 was found in BECs in NPs (Fig E1, A and B). We determined the contribution of different types of stromal cells to CXCL13 expression and found that FRCs were the major source of stromal cell derived CXCL13 in NPs (Fig 2, E and F).

### IL-17A induces FRC expansion and CXCL13 production in nasal stromal cells

Several studies have demonstrated a requirement for IL-17A signaling in the initiation of eLT formation in animal models of chronic inflammation.<sup>18, 19, 31-33</sup> In this study, we confirmed increased mRNA (Fig 3, A) and protein (Fig E2) expression of IL-17A in NPs, especially in those with eLTs, as compared with control tissues. Moreover, we revealed a positive correlation between IL-17A and CXCL13 mRNA expression in both NPs with and without eLTs (Fig 3, B).

Given the finding that nasal stromal cells were the major source of CXCL13 in NPs without eLTs (Fig 1, D), we explored whether IL-17A can regulate CXCL13 production in nasal stromal cells. We first verified that there was IL-17RA expression on vimentin<sup>+</sup> stromal cells in nasal tissues by immunofluorescence staining (Fig E3, A). Additionally, we found that the mRNA expression of IL-17RA was increased in stromal cells isolated from NPs as compared with those from control tissues (Fig E3, B). We further found that among different types of stromal cells, the FRC population had the highest frequencies of IL-17RA expression in control tissues and NPs without eLTs (Fig E3, C and D).

Next, we found that IL-17A induced CXCL13 mRNA production in nasal stromal cells purified from control tissues in a dose-dependent manner (Fig 3, C). IL-17A also promoted CXCL13 protein production in nasal stromal cells (Fig 3, D), in which FRCs were the major stromal cell type producing CXCL13, accounting for 61% (mean) of CXCL13<sup>+</sup> stromal cells (Fig 3, E). In addition, we discovered that IL-17A stimulation led to a specific expansion of FRC population and reduction of DN population in a time-dependent manner (Fig 3, F). We did not find a change of CXCL12 mRNA expression in nasal stromal cells after IL-17A stimulation for 12 hours (Fig E3, E).

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In NPs with eLTs, B cells were found to be the major producer of CXCL13 (Fig 1, E), suggesting an involvement of B cells at a later stage of eLT formation in NPs. B cells are also known as the main source of the membrane-bound form of LT (LTα<sub>1</sub>β<sub>2</sub>) in SLOs.<sup>34, 35</sup> We found here that the mRNA expression levels of LTα were upregulated in both types of NPs, whereas the levels of LTβ were only upregulated in NPs with eLTs compared to those in control tissues (Fig 4, A), indicating an upregulation of membrane LTα<sub>1</sub>β<sub>2</sub> in NPs with eLTs rather than in NPs without eLTs. Consistent with the ability of B cells to express LT, our immunofluorescence staining demonstrated expression of LTα and LTβ on B cells in eLTs in NPs (Fig 4, B). Furthermore, we found that after B cell depletion, LTβ mRNA expression was markedly decreased in patients with NPs with eLTs (mean, 62% reduction), but not in patients with NPs without eLTs (mean, 32% reduction). In contrast, a mild and comparable reduction of LTα mRNA expression was noted in both NPs with and without eLTs after depleting B cells (mean, 27% and 38% reduction, respectively) (Fig 4, C). These results suggest that B cells are the main producer of LTα<sub>1</sub>β<sub>2</sub>, but not LTα<sub>3</sub>, in NPs with eLTs.

Of a particular interest, it has been shown that activation of CXCR5 induced murine splenic B cells to produce membrane LTα<sub>1</sub>β<sub>2</sub> which is required for GC formation in SLOs.<sup>36</sup> Here, the expression of CXCR5 on B cells in eLTs in NPs was verified by immunofluorescence (Fig E4, A). We further discovered that CXCL13 induced the upregulation of LTβ mRNA expression and membrane LTα expression in purified polyp B cells (Fig 4, D and E). However, there was no increase of secreted LTα<sub>3</sub> in the supernatant of B cell culture (Fig E4, B). Collectively, these findings indicate an upregulation of membrane type, but not

secretory type of LT, in polyp B cells under CXCL13 stimulation.

In SLOs, LT $\beta$ R pathway is critical for the induction of CXCL13.<sup>37, 38</sup> In this study, we found that B cells were the major source of CXCL13 in NPs with eLTs (Fig 1, E). Thereby we tested whether the production of CXCL13 in B cells can be induced via LT $\beta$ R pathway. We found that LT $\beta$ R was expressed by B cells in eLTs in NPs by immunofluorescence staining (Fig E4, C). Moreover, we found that LT $\alpha_1\beta_2$  treatment promoted mRNA (Fig 4, F) and protein production of CXCL13 in polyp B cells as reflected by CXCL13<sup>+</sup> B cell frequencies (Fig E4, D) and CXCL13 levels in culture supernatants (Fig 4, G).

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In addition to B cells, we found that LT $\beta$ R was also expressed on vimentin<sup>+</sup> stromal cells in eLTs in NPs by immunofluorescence study (Fig E5, A) and the mRNA expression levels of LT $\beta$ R was enhanced in purified stromal cells from NPs with eLTs compared to those from NPs without eLTs and control tissues (Fig E5, B). Flow cytometric analysis showed that LT $\beta$ R expression was upregulated in four subsets of stromal cells in NPs with eLTs (Fig E5, C and D).

We next found that LT $\alpha_1\beta_2$  induced CXCL13 mRNA production in nasal stromal cells in a dose-dependent manner (Fig 5, A). LT $\alpha_1\beta_2$  also induced CXCL13 protein production in nasal stromal cells (Fig 5, B). FRCs and LECs were found to be the major stromal cell types producing CXCL13 under LT $\alpha_1\beta_2$  stimulation (Fig 5, C). Different from the effect of IL-17A, LT $\alpha_1\beta_2$  induced the remodeling of nasal stromal cells towards not only FRCs but also LECs (Fig 5, D).

#### IL-17A is required for *de novo* nasal follicle formation in an IL-17A high mouse model

To further determine the role of IL-17A in eLT formation in nasal mucosa *in vivo*, we established a murine model with high local IL-17A inflammation by nasal administration of curdlan (Fig E6, A).<sup>24-26</sup> Interestingly, we revealed that both 20  $\mu$ g and 100  $\mu$ g curdlan treatment promoted lymphoid aggregate formation in nasal mucosa, however, only 100  $\mu$ g curdlan treatment induced the eLT formation characterized by the presence of CD21<sup>+</sup> follicular dendritic cell networks in B cells aggregates (Fig E6, B-D), which was supported by the elevated mRNA expression of activation induced cytidine deaminase (AID) and CD21 in those mice (Fig E7, A). In addition, CXCL13 and LT $\beta$  mRNA levels were enhanced in 100  $\mu$ g curdlan treated mice compared to other groups (Fig E7, B). The upregulation of both Pdpn and CD31 in 100  $\mu$ g curdlan group suggests a remodeling of stromal cells towards FRCs and LECs during eLT development (Fig E7, C). IL-17A and IL-17F mRNA expression in nasal mucosa were upregulated in both 20  $\mu$ g and 100  $\mu$ g curdlan group compared to control group, however, 100  $\mu$ g curdlan group demonstrated a marked greater upregulation than 20  $\mu$ g curdlan group (Fig E7, D).

In contrast to WT mice, *il17a*<sup>-/-</sup> mice treated with 100  $\mu$ g curdlan demonstrated no lymphocyte cluster or eLT formation (Fig 6, A and B). No upregulation of CXCL13 and LT $\beta$  (Fig 6, C), AID and CD21 (Fig E8, A), or Pdpn and CD31 (Fig E8, B) mRNA expression was found in *il17a*<sup>-/-</sup> mice.

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Next, we investigated the role of CXCL13 and LT $\alpha_1\beta_2$  on eLT formation in the IL-17A high mouse model induced by 100  $\mu$ g curdlan. We found that blockage of CXCL13 or LT $\alpha_1\beta_2$  completely prevented eLT formation, and partially diminished lymphocyte cluster development in nasal mucosa (Fig 7). Consistently, AID and CD21 mRNA expression in nasal mucosa were reduced in anti-CXCL13 and LT $\beta$ R-Ig treated mice (Fig E9, A and D). The mRNA expression of CXCL13 and LT $\beta$  were decreased in anti-CXCL13 treatment mice (Fig 7, C), while only CXCL13 mRNA expression was significantly inhibited in mice treated with LT $\beta$ R-Ig (Fig 7, F). The mRNA expression of CD31, but not Pdpn, was significantly inhibited by anti-CXCL13 and LT $\beta$ R-Ig treatment (Fig E9, B and E). Nevertheless, there was no change of IL-17A or IL-17F mRNA levels in mice treated with anti-CXCL13 or LT $\beta$ R-Ig (Fig E9, C and F). Together with the finding of abolished upregulation of CXCL13 and LT $\beta$  in *il17a*<sup>-/-</sup> mice, these data suggest that CXCL13 and LT $\alpha_1\beta_2$  reside downstream of IL-17A in this model.

## DISCUSSION

The eLTs can produce antibodies locally and therefore perpetuate inflammation in NPs.<sup>8</sup> However, the mechanisms underlying the formation and expansion of eLTs in NPs remain unexplored. In this study, for the first time, we identified a critical role for IL-17A-induced stromal cell remodeling in the initiation, and crosstalk between B cells and stromal cells via CXCL13 and  $LT\alpha_1\beta_2$  in the enlargement and maintenance of eLTs in NPs.

Expression of homeostatic chemokines is central to the initiating events that lead to lymphoneogenesis.<sup>13-15, 39, 40</sup> In NPs without eLTs, we found that stromal cells were the major cellular source of CXCL13, indicating that stromal cells are critical for the initiation of B cell recruitment and compartmentalization in NPs. Evidence that IL-17A may play an important role in eLT formation by inducing the production of lymphoid chemokine CXCL12 and CXCL13 emerge from recent animal studies of bronchial infection and autoimmune encephalitis.<sup>18, 32</sup> In this study, we found that IL-17A expression correlated with CXCL13 expression in NPs. We further discovered that nasal stromal cells had the expression of IL-17RA and IL-17A induced CXCL13 production in nasal stromal cells. Although IL-17A induced the production of CXCL12, another important B cell chemokine, during bacteria-induced lung lymphoid neogenesis,<sup>18</sup> we failed to find an induction of CXCL12 in nasal stromal cells by IL-17A, which is consistent with our previous finding of no association between CXCL12 expression and eLT formation in NPs.<sup>8</sup> Therefore, distinct mechanisms may underline the effect of IL-17A in promoting eLT development in different organs and pathological conditions.

Stromal cells have a complex role at local microenvironments, which induce immune cell migration, activation and survival, and support lymphoid enlargement. FRCs provide homeostatic chemokines, and secrete extracellular matrix proteins to form the structural framework for immune cell interaction in SLOs.<sup>41</sup> BECs and LECs regulate lymphocyte entry into SLOs. DNs have recently been shown to contain a novel subset of fibroblastic contractile pericytes.<sup>42</sup> Although the phenotype and function of stromal cells are well documented in SLOs, little is known of their role in eLT formation. In this study, we revealed an expansion of FRC population of stromal cells in both NPs with and without eLTs, which was likely induced by IL-17A and  $LT\alpha_1\beta_2$ . The expansion of FRCs in NPs without eLTs indicates a role of FRCs, but not other types of stromal cells, in eLT formation in its infant stage given to the findings that stromal cells were the main producer of CXCL13 in NPs without eLTs and FRCs were the major source for CXCL13 in stromal cells in NPs. LECs is also a fundamental compartment in controlling SLOs organogenesis.<sup>43</sup> Nevertheless, LECs were only expanded in NPs with eLTs, and the expansion of LECs was induced by  $LT\alpha_1\beta_2$  but not IL-17A. Since  $LT\alpha_1\beta_2$  was only upregulated in NPs with eLTs, LECs are more likely involved in the enlargement rather than the initiation of eLTs in NPs by facilitating the entry of lymphocytes into NPs.

In NPs with eLTs, we found that B cells were main producer of CXCL13. After the B cell recruitment under the control of CXCL13 derived from stromal cells, B cells themselves may provide a “second wave” of supply of CXCL13.  $LT\alpha_3$  and  $LT\alpha_1\beta_2$  are reported to be involved in eLT formation.<sup>44, 45</sup> Compared with control tissues, the mRNA expression of  $LT\beta$  was only upregulated in NPs with eLTs, suggesting an involvement of membrane form of LT in the later stage of eLT formation in NPs. Previous studies show that CXCL13 induces murine splenic B cells to upregulate membrane-bound LT via Grb2, and CXCL13 expression induction is dependent on  $LT\beta R$  pathway in SLOs.<sup>37, 38, 46</sup> In this study, we demonstrated a positive feedback loop between CXCL13 and  $LT\alpha_1\beta_2$  on B cells in eLTs in NPs, which obviously exaggerates the B cell recruitment and compartmentalization. In addition to B cells, 36% reduction of CXCL13 expression was founded in NPs with eLTs after depletion of stromal cells. Nasal stromal cells also had  $LT\beta R$  expression. We found that  $LT\alpha_1\beta_2$  reshaped stromal cells to FRC and LEC type and promoted their CXCL13 production. Thus, the crosstalk between stromal cells and B cells further perpetuate the eLT development in NPs.

Using a murine model with high nasal type 17 inflammation, we confirmed that IL-17A was able to induce eLT formation in nasal mucosa and this process was dependent on CXCL13 and  $LT\alpha_1\beta_2$  *in vivo*. In the animal study, we found that only high levels of IL-17A induced by 100  $\mu\text{g}$  curdlan led to eLT formation. In contrast, the comparatively lower IL-17A levels induced by curdlan at 20  $\mu\text{g}$  only induced lymphocyte

clusters. This is in line with the finding in humans that the IL-17A levels were more prominently elevated in NPs with eLTs than those without eLTs.

There are several limitations in this study. We established a mouse model by using curdlan, which elicits a high local IL-17A inflammation but may not mirror the pathogenesis of NPs in humans. It is considered that the type 17 response is less important for Caucasian patients with NPs than for Chinese patients. Nevertheless, eLTs have also been reported in Caucasian patients,<sup>47</sup> indicating that additional mechanisms may underlie eLT formation in Caucasian patients.

These comments notwithstanding, for the first time, we have established a paradigm of how eLTs are formed in NPs in Chinese patients. We suspect that targeting IL-17A, CXCL13 and  $LT\alpha_1\beta_2$  may provide opportunities for the design of therapies to manipulate eLT formation and alleviate inflammation in patients with NPs.

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## FIGURE LEGENDS

**Fig 1. CXCL13 expression in NPs with and without eLT formation.** **A**, The mRNA expression levels of CXCL13 in different study groups as detected by quantitative RT-PCR. **B**, The protein levels of CXCL13 in different study groups as detected by ELISA. **C**, Representative photomicrographs showing immunofluorescence staining of CXCL13 on vimentin<sup>+</sup> stromal cells and CD20<sup>+</sup> B cells in polyp tissues with

and without eLTs. Original magnification  $\times 200$ . Insets show a higher magnification of the outlined area, and arrows denote representative positive cells. **D**, Reduction of CXCL13 mRNA expression after depletion of stromal cells in relation to total polyp cells from NPs with and without eLTs. Data are expressed as means  $\pm$  SEMs. **E**, Reduction of CXCL13 mRNA expression after depletion of B cells in relation to total polyp cells from NPs with and without eLTs. Data are expressed as means  $\pm$  SEMs. NPs, nasal polyps; eLTs<sup>+</sup>, with ectopic lymphoid tissues; eLTs<sup>-</sup>, without ectopic lymphoid tissues; DAPI, 4, 6-Diamidino-2-phenylindole dihydrochloride. \* $P < 0.05$ ; \*\*\* $P < 0.001$ .

**Fig 2. Phenotypic changes of stromal cells in NPs** **A**, The mRNA expression levels of CXCL13 in nasal stromal cells purified from different types of tissues as detected by quantitative RT-PCR. **B**, The gating strategy and representative flow plots showing nasal stromal cells in tissues. **C**, The frequencies of different stromal cell subpopulations in different study groups as detected by flow cytometry. **D**, The composition of stromal cell subsets in different study groups. Mean percentages are shown. **E**, The gating strategy of CXCL13<sup>+</sup> stromal cells. **F**, The composition of different types of stromal cells for CXCL13<sup>+</sup> stromal cells in different types of tissues as detected by flow cytometry. Mean percentages of different stromal cell types are shown. NPs, nasal polyps; eLTs<sup>+</sup>, with ectopic lymphoid tissues; eLTs<sup>-</sup>, without ectopic lymphoid tissues; EpCAM, epithelial cell adhesion molecule; Pdpn, podoplanin; FRCs, fibroblastic reticular cells; LECs, lymphoid endothelial cells; BECs, blood endothelial cells; DNs, double negative cells. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

**Fig 3. IL-17A induces CXCL13 production and FRC expansion in nasal stromal cells.** **A**, The mRNA expression levels of IL-17A in different study groups as detected by quantitative RT-PCR. **B**, The correlation between IL-17A and CXCL13 mRNA expression levels in NPs with and without eLTs. **C**, Purified nasal stromal cells from control tissues were stimulated with various doses of IL-17A for 12 hours and mRNA expression of CXCL13 were measured by quantitative RT-PCR (n = 6). **D**, Purified nasal stromal cells from control tissues were stimulated with IL-17A at 100 ng/mL for 36 hours and CXCL13 positive cells were quantified by flow cytometry (n = 6). Representative flow plots are shown. **E**, Purified nasal stromal cells from control tissues were stimulated with IL-17A at 100 ng/mL for 36 hours, and CXCL13 positive cells were analyzed for the composition of different types of stromal cells by flow cytometry (n = 6). Mean percentages of different stromal cell types are shown. **F**, After treatment with IL-17A at 100 ng/mL for different time points, the frequencies of different types of stromal cells were detected by flow cytometry (n = 6). NPs, nasal polyps; eLTs<sup>+</sup>, with ectopic lymphoid tissues; eLTs<sup>-</sup>, without ectopic lymphoid tissues; Pdpn, podoplanin; FRCs, fibroblastic reticular cells; LECs, lymphoid endothelial cells; BECs, blood endothelial cells; DNs, double negative cells. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

**Fig 4. Α ποσITIVE φεεδβασκ λOOπ βετωεεV "Ξ"Λ13 ανδ ΑΤα1β2 οV Β σελλεε. A**, The mRNA expression levels of LT $\alpha$  and LT $\beta$  in different study groups as detected by quantitative RT-PCR. **B**, Representative photomicrographs showing immunofluorescence staining of LT $\alpha$  and LT $\beta$  on CD20<sup>+</sup> B cells in eLTs in NPs. Original magnification  $\times 200$ . Insets show a higher magnification of the outlined area, and arrows denote representative positive cells. **C**, Reduction of LT $\alpha$  and LT $\beta$  mRNA expression after depletion of B cells in relation to total polyp cells from NP with and without eLTs. Data are expressed as means  $\pm$  SEMs. **D**, B cells purified from polyp tissues with eLTs were stimulated with CXCL13 at various doses for 12 hours, and the mRNA expression of LT $\beta$  in B cells was detected by RT-PCR (n = 6). **E**, B cells purified from poly tissues with eLTs were stimulated with CXCL13 at 1000 ng/mL for 36 hours, the membrane expression of LT $\alpha$  on B cells was detected by flow cytometry (n = 6). The representative flow plots are shown. **F**, B cells purified from polyp tissues with eLTs were stimulated with LT $\alpha_1\beta_2$  at various doses for 12 hours, and the mRNA expression of CXCL13 in B cells was detected by RT-PCR (n = 6). **G**, B cells purified from polyp tissues with eLTs were stimulated with LT $\alpha_1\beta_2$  at 1000 ng/mL for 48 hours, the protein levels of CXCL13 in culture supernatants were detected by ELISA (n = 6). NPs, nasal polyps; eLTs<sup>+</sup>, with ectopic lymphoid tissues; eLTs<sup>-</sup>, without ectopic lymphoid tissues; LT, lymphotoxin. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

**Fig 5. ΑΤα1β2 Vνδυσεεε ΦΡ" ανδ ΑΕ" εεπανσIον ανδ "Ξ"Λ13 προδυετIον Iν νασαλ ετρομαλ σελλεε. A**, Nasal stromal cells purified from control tissues were treated with LT $\alpha_1\beta_2$  at various doses for

12 hours, and the mRNA expression of CXCL13 in stromal cells were detected by RT-PCR (n = 6). **B**, After stimulation with  $LT\alpha_1\beta_2$  at 100 ng/mL for 36 hours, CXCL13 positive nasal stromal cells were analyzed by flow cytometry (n = 6). Representative flow plots are shown. **C**, After stimulation with  $LT\alpha_1\beta_2$  at 100 ng/mL for 36 hours, CXCL13 positive stromal cells were analyzed for the composition of different stromal cell types by flow cytometry (n = 6). Mean percentages of different types of stromal cells are shown. **D**, After stimulation with  $LT\alpha_1\beta_2$  at 100 ng/mL for different time points, the frequencies of different stromal cell populations were detected by flow cytometry (n = 6). NPs, nasal polyps; eLTs<sup>+</sup>, with ectopic lymphoid tissues; eLTs<sup>-</sup>, without ectopic lymphoid tissues; LT, lymphotoxin; Pdpn, podoplanin; FRCs, fibroblastic reticular cells; LECs, lymphoid endothelial cells; BECs, blood endothelial cells; DNs, double negative cells. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

**Fig 6. IL-17A is required for *de novo* nasal follicle formation in an IL-17A high mouse model.**

**A**, Histologic studies showed lymphoid aggregates in WT and *il17a*<sup>-/-</sup> mice challenged by curdlan at 100  $\mu$ g or PBS (control). **B**, The number of eLTs and lymphocyte clusters in different study groups. **C**, The mRNA levels of CXCL13 and  $LT\beta$  in nasal mucosa in different study groups. WT, wild type; eLTs, ectopic lymphoid tissues; LT, lymphotoxin. \* $P < 0.05$ ; \*\* $P < 0.01$ .

**Φιγ 7. Νασαλ εΛΤ φορματιον ις διμινισηδ βψ “Ξ”Λ13 ανδ ΛΤβΡ βλοσκαγε ιν τηε μουσε μοδελ.**

**A**, Histologic studies showed lymphoid aggregates in WT mice challenged by curdlan at 100  $\mu$ g and treated with anti-CXCL13 or rat control IgG1. **B**, The number of eLTs and lymphocyte clusters in WT mice with and without anti-CXCL13 treatment. **C**, The mRNA levels of CXCL13 and  $LT\beta$  in nasal mucosa in WT mice with and without anti-CXCL13 treatment. **D**, Histologic studies showed lymphoid aggregates in WT mice challenged by curdlan at 100  $\mu$ g and treated with  $LT\beta$ R-Ig or mouse control IgG1. **E**, The number of eLTs and lymphocyte clusters in WT mice with and without  $LT\beta$ R-Ig treatment. **F**, The mRNA levels of CXCL13 and  $LT\beta$  in WT mice with and without  $LT\beta$ R-Ig treatment. WT, wild type; eLTs, ectopic lymphoid tissues; LT, lymphotoxin. \* $P < 0.05$ ; \*\* $P < 0.01$ .

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