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**CD8⁺ T cells with distinct cytokine producing features and low
cytotoxic activity in eosinophilic and non-eosinophilic chronic
rhinosinusitis with nasal polyps**

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Authors' contributions

Z.L contributed the study idea and design; H.W, X.B.L and Y.L collected the clinical samples; J.M, L.L.S, Y.K.D and P.P.C performed the experiments; J.M and L.L.S performed data analysis; X.H.Z and M.Z participated in data discussion and manuscript preparation; J.M, L.L.S and Z.L prepared manuscript; and all authors were involved in final approval.

ABSTRACT

Background: CD8⁺ T cells are important effectors of cell-mediated immunity; however, their contribution to the pathogenesis of CRS is unclear.

Objective: This study aimed to characterize the cytokine producing features and cytotoxic activity of CD8⁺ T cells, and their correlation with inflammation patterns in CRS with nasal polyps.

Methods: The expression of IFN- γ , IL-4, IL-5, IL-17A, forkhead box P3 (FOXP3), perforin, and granzyme B in CD8⁺ T cells were studied by means of flow cytometry, immunohistochemistry, and immunofluorescence. The expression of CD8⁺ T cell subset relevant chemokines and chemokine receptors was detected by means of real-time RT-PCR or ELISA. The cytotoxic activity of sorted CD8⁺ T cells was defined by anti-CD3-redirected killing assay.

Results: Compared with controls, elevated percentages of total CD8⁺ T cells and cytotoxic T lymphocyte (Tc) 1 (IFN- γ ⁺), Tc2 (IL-4⁺) and Tc17 (IL-17A⁺) cell subset, and decreased percentages of FOXP3⁺CD8⁺ regulatory T cells, were found in both eosinophilic and non-eosinophilic polyps with a Tc2-skewed and Tc1/Tc17-dominated response in eosinophilic and non-eosinophilic polyps, respectively. Nasal CD8⁺ T cells were found to produce similar or even higher levels of IFN- γ and IL-4 compared with CD4⁺ T cells. Tc1 and Tc17, and Tc2 (IL-4⁺ and IL-5⁺) cell subset percentages positively correlated with neutrophil and eosinophil counts in sinonasal mucosa, respectively. Strikingly, the expression of perforin and granzyme B and cytotoxic activity were significantly reduced in nasal CD8⁺ T cells compared with their counterparts in peripheral blood. The expression of CXCL16, CCL17 and CCL20 positively correlated with Tc1, Tc2 and Tc17 cell subset number in sinonasal mucosa, respectively.

Conclusion and Clinical Relevance: CD8⁺ T cells have low cytotoxic activity; nevertheless, they are a significant and previously underappreciated source of inflammatory cytokine production in polyps. Different Tc cell subset domination may contribute to distinctly biased granulocyte inflammation in eosinophilic and non-eosinophilic polyps.

(300 words)

Key words: chronic rhinosinusitis, cytokine, cytotoxic T lymphocyte, cytotoxicity, eosinophil, neutrophil, nasal polyps.

INTRODUCTION

Chronic rhinosinusitis (CRS) is a multifactorial and heterogeneous disorder [1]. Based on the presence or absence of nasal polyps (NPs), CRS is currently classified into two major clinical phenotypes: CRS without nasal polyps and CRS with nasal polyps (CRSwNP) [2, 3]. Although CRS represents a significant health problem worldwide, its etiology and pathogenesis remain poorly understood [2, 3]. A variety of factors have been suggested to be involved in the pathogenesis of CRS, such as inflammatory and immune cell activation, local IgE production, epithelial barrier impairment, and tissue remodeling [1]. Eosinophilic inflammation has been considered to be a cardinal feature of CRSwNP in Caucasians [2, 3], whereas Asian CRSwNP demonstrates considerable non-eosinophilic inflammation with lower asthma comorbidity [3, 4]. Despite increased recognition that eosinophilic and non-eosinophilic CRSwNP have distinct patterns of inflammation and tissue remodeling [4-6], the underlying mechanisms remain largely unknown.

T lymphocytes play a crucial role in the regulation of immune responses and inflammation at mucosal site [4, 7, 8]. The participation of CD4⁺ T cells in the pathogenesis of CRS is well established, which orchestrate different phenotypic CRS by producing various cytokines [9]. In addition to CD4⁺ T cells, CD8⁺ T cells are

another subpopulation of adaptive immune lymphocytes. The canonical function of CD8⁺ T cells is to destruct and eliminate intracellular pathogen-invaded and tumor-transformed cells through their cytotoxic activity [10]. The cytolytic pathway of CD8⁺ T cells mainly involves the perforin and granzyme B mediated induction of target cell apoptosis or lysis [10-12]. Moreover, it is suggested that perforin and granzyme B expression and cytotoxic activity of lesional CD8⁺ T cells can be significantly modulated by local immunological environment [13]. Recently, increasing evidence have shown that CD8⁺ T cells may also regulate pathologic processes such as autoimmune and allergic disorders via functions beyond their classic cytotoxic activity [14]. Similar to CD4⁺ T helper cell (Th), CD8⁺ cytotoxic T lymphocyte (Tc) can differentiate to at least five effector subsets with different cytokine producing phenotypes: IFN- γ ⁺ Tc1, IL-4⁺ Tc2, IL-9⁺ Tc9, IL-17A⁺ Tc17, and CD8⁺ regulatory T cell (Treg) [14]. The infiltration of these different Tc cell subsets in inflammatory site is presumably mediated by a complex interaction of cytokines, adhesion molecules, and chemokines [14]. Although an increased accumulation of CD8⁺ T cells in sinonasal mucosa of CRS patients has been documented by us and others [4, 15-17], the functional relevance of CD8⁺ T cells in the pathogenesis of different phenotypic CRS remain undefined.

In this study, we investigated the cytokine producing features of CD8⁺ T cells in CRSwNP and the association between Tc cell subset polarization and granulocyte activation in eosinophilic and non-eosinophilic CRSwNP. We also tested the classic cytotoxic activity of CD8⁺ T cells in NPs. We found here that compared with their

counterparts in peripheral blood, nasal CD8⁺ T cells were less cytotoxic with diminished perforin and granzyme B expression. However, CD8⁺ T cells were revealed to be an important but underappreciated source of inflammatory cytokine production in addition to CD4⁺ Th cells, and distinct polarization of Tc cell subsets was associated with different and biased granulocyte inflammation in eosinophilic and non-eosinophilic CRSwNP.

Methods

Subjects

This study was approved by the Ethics Committee of Tongji Hospital of Huazhong University of Science and Technology and conducted with written informed consent from every patient. Seventy-six control subjects, 84 patients with eosinophilic CRSwNP, and 93 patients with non-eosinophilic CRSwNP were enrolled in this study. Details of subjects' characteristics are included in Table S1 in the Online Supplement of this article. CRSwNP was diagnosed according to European EAACI Position Paper on Rhinosinusitis and Nasal Polyps and American guidelines [2, 3]. CRSwNP was classified as eosinophilic when percent tissue eosinophils exceeded 10% of total infiltrating cells as defined by our previous report [4]. Control subjects were those undergoing septoplasty because of anatomical variations and without other sinonasal diseases. Polyp tissues from middle meatus of CRSwNP patients and inferior turbinate mucosa from controls were obtained during surgery. Not all samples

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were included in every study method because of the limited amount of some samples. Atopic status was defined by skin prick test to a standard panel of common inhalant allergens that was performed in accordance with the WHO guideline [18]. The diagnosis of asthma was based on history and physician's diagnosis according to Global Initiative for Asthma [19]. Oral glucocorticoid and intranasal steroid spray were discontinued at least 3 months and 1 month before surgery, respectively. None had received antileukotrienes or immunotherapy. Subjects who had an antrochoanal polyps, cystic fibrosis, fungal sinusitis, primary ciliary dyskinesia, or gastroesophageal reflux disease were excluded from the study.

Immunohistochemistry and immunofluorescence

Hematoxylin-eosin and immunohistochemical staining of paraffin tissue sections and the cell counting were conducted as previously reported [4]. Consecutive sections were stained to define the relationship between CD8⁺ T cells and the expression of forkhead box P3 (FOXP3), perforin and granzyme B [6]. Coexpression of CD8 and IL-5 was detected by immunofluorescence staining [20]. Additional information regarding immunohistochemistry and immunofluorescence is provided in the Online Supplement.

Flow cytometric analysis

Nasal mucosal mononuclear cells (NMCs) and peripheral blood mononuclear cells (PBMCs) were isolated from sinonasal mucosa and blood, respectively, as previously described [9, 16, 17]. CD8⁺ T and CD4⁺ T cells were stained with fluorescence-conjugated antibodies and flow cytometric analysis was conducted with a LSRII flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA) as mentioned elsewhere [9, 16, 17]. More information is provided in the Online Supplement.

Quantitative RT-PCR

Total RNA was extracted from sinonasal mucosa and isolated cells and reverse-transcribed to cDNA. Real-time PCR assay was performed with appropriate primers [4, 21]. Relative gene expression was calculated by using the 2^(-Delta Delta CT) method unless otherwise indicated [22]. More information is provided in the Online Supplement.

ELISA

Tissue samples were weighed and homogenized, and the supernatants were harvested as mentioned elsewhere [23]. Then, the protein levels of CXCL16, CCL17 and CCL20 in the supernatants were detected by commercial ELISA kits (R&D

system, Minneapolis, MN, USA). The data were expressed as protein levels per milliliter. More information is provided in the Online Supplement.

Anti-CD3-redirected killing assay

CD8⁺ T cells were purified from PBMCs and NMCs by immunomagnetic cell sorting with anti-CD8 antibody-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions [24]. The purity of CD8⁺ T cells was greater than 95%. The target cells, Fc receptor-bearing and Fas negative K562 erythroleukemic human cells (American Type Culture Collection, Manassas, VA, USA), were seeded in the presence of either anti-CD3 antibody (OrthoClone OKT3; Janssen-Cilag, Boulogne-Billancourt, France) or an IgG2a isotype-matched control antibody (Becton Dickinson Biosciences, San Jose, CA, USA) at a final concentration of 10 µg/mL. After co-culturing with CD8⁺ T cells, supernatants were collected and lactate dehydrogenase was measured using a LDH Cytotoxicity Assay Kit (Biyuntian, Shanghai, China) [24, 25]. More information is provided in the Online Supplement.

Statistical analysis

For continuous variables, results are shown as dot plots. Symbols represent individual samples; horizontal bars represent median and error bars show interquartile

range. The Kruskal-Wallis H test was used to assess significant intergroup variability and Mann-Whitney U 2-tailed test was used for between-group comparison. The spearman test was used to determine correlations. Significance was accepted at $P < 0.05$.

RESULTS

The distribution of CD8⁺ T cells in sinonasal mucosa

Immunohistochemistry study showed that CD8⁺ T cells predominately infiltrated in lamina propria of sinonasal mucosa, while some of them could also be found in epithelium (Fig. 1, A). In comparison with controls, both eosinophilic and non-eosinophilic polyps showed increased accumulation of CD8⁺ T cells in both lamina propria and epithelium (Fig. 1).

Tc cell subsets in sinonasal mucosa

The cytokine producing features of CD8⁺ T cells and the polarization of Tc cell subsets in sinonasal mucosa were investigated by flow cytometry and the gating strategy is showed in Fig. 2, A. Consistent with the findings of immunohistochemistry, flow cytometric analysis discovered an increased percentage of total CD8⁺ T cells in NMCs from both eosinophilic and non-eosinophilic polyps compared with that in controls (Fig. 2, B). Moreover, in all studied groups, NMCs

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demonstrated a CD4/CD8 ratio that was significantly different from PBMCs, with relatively more CD8⁺ T cells and fewer CD4⁺ T cells in sinonasal mucosa (Fig. 2, B). Compared with those in controls, increased frequencies of Tc1 (IFN- γ ⁺), Tc2 (IL-4⁺) and Tc17 (IL-17A⁺) cell subset in NMCs were found in both eosinophilic and non-eosinophilic polyps (Fig. 2, C). Nevertheless, when comparing the difference between eosinophilic and non-eosinophilic polyps, a further increase of Tc1 and Tc17 cell subset frequency was found in non-eosinophilic polyps, whereas a more exaggerated up-regulation of Tc2 cell subset was noted in eosinophilic polyps (Fig. 2, C). Coincidentally, eosinophilic CRSwNP demonstrated an increased Tc2/Tc17 and a decreased Tc1/Tc2 ratio compared with non-eosinophilic CRSwNP (Fig. 2, D). We further studied the relative contribution of CD4⁺ and CD8⁺ T cells to the IFN- γ , IL-4, and IL-17A production in total CD3⁺ T cells. We found that in cytokine positive CD3⁺ T cells, the frequency of nasal CD8⁺ T cells producing IFN- γ or IL-4 was greater than or similar to that of CD4⁺ T cells producing the same cytokine in different study groups (Fig. 3, A). While, the frequency of nasal CD8⁺ T cells producing IL-17A was less than or comparable to that of CD4⁺ T cells producing IL-17A in IL-17A positive CD3⁺ T cells (Fig. 3, A). To further clarify the actual amounts of cytokines produced by the CD4⁺ and CD8⁺ T cells, we analyzed the median fluorescence intensity (MFI) of each cytokine in cytokine positive CD8⁺ T cells and CD4⁺ T cells. We found that the MFI of IFN- γ and IL-4 expressing nasal CD8⁺ T cells was greater than or similar to that of CD4⁺ T cells in different study groups (Fig. 3, B and Fig. S1). As to IL-17A, the MFI of nasal CD8⁺ T cells was less

than or comparable to that of CD4⁺ T cells (Fig. 3, B and Fig. S1). In contrast to the findings in sinonasal mucosa, no significant difference in the percentage of total CD8⁺ T cells or Tc cell subset was found in PBMCs among different groups (Fig. S2, A). No matter whether it was in tissue or in blood, Tc1 was the predominant Tc cell subset in all studied groups (Fig. S2, B and Fig. S3).

In order to define the change of CD8⁺ Treg in CRSwNP, we did the immunohistochemical staining using consecutive tissue sections. We found that the percentage of FOXP3⁺CD8⁺ cells in total CD8⁺ T cells was significantly decreased in both eosinophilic and non-eosinophilic polyps compared with that in controls (Fig. S4).

The correlation between Tc cell subset polarization and biased granulocyte inflammation in sinonasal mucosa

In line with our previous report [6], the number of mucosal eosinophils was significantly increased in eosinophilic CRSwNP compared with that in non-eosinophilic CRSwNP and controls, whereas the number of myeloperoxidase (MPO) positive neutrophils was increased in both eosinophilic and non-eosinophilic polyps as compared with controls, with a further greater increase in non-eosinophilic polyps (Fig. 4, A). Interestingly, we found that the frequency of Tc1 and Tc17 cell subset positively correlated with the number of neutrophils, whereas the frequency of Tc2 cell subset positively correlated with the number of eosinophils, in sinonasal

mucosa (Fig. 4, B). Nevertheless, we failed to find any correlation between the number of MPO positive neutrophils and the frequency of Tc2 cell subset ($r = 0.194$, $P = 0.264$), or between the number of eosinophils and the frequency of Tc1 ($r = 0.204$, $P = 0.232$) and Tc17 ($r = 0.106$, $P = 0.539$) cell subset.

Given the importance of IL-5 in initiating and exaggerating eosinophilic inflammation, we further detected the expression of IL-5 by CD8⁺ T cells in sinonasal mucosa by immunofluorescence. Similar to the IL-4⁺ Tc2 cells, we discovered that the frequency of IL-5⁺CD8⁺ T cells in total CD8⁺ T cells was significantly increased in eosinophilic polyps compared with that in controls and non-eosinophilic polyps (Fig. S5). In addition, we found that the frequency of IL-5⁺CD8⁺ T cells positively correlated with the number of eosinophils (Fig. S5), but not the number of neutrophils ($r = 0.174$, $P = 0.331$), in sinonasal mucosa.

The granzyme B and perforin expression and cytotoxicity of CD8⁺ T cell in NPs

As shown in Figure 5, A and C, although granzyme B immunoreactivity could be detected in sinonasal mucosa, we failed to find significant cellular colocalization of granzyme B and CD8 immunoreactivity. A small portion (around 20%) of granzyme B positive cells were CD8⁺ T cells and most of CD8⁺ T cells (around 95%) did not show granzyme B immunoreactivity (Fig. 5, A and C). No significant difference in the number of granzyme B⁺CD8⁺ T cells could be found among different study groups given the small number of granzyme B⁺CD8⁺ T cells detected in nasal tissues (Fig. 5,

C). As to perforin expression, no obvious perforin immunoreactivity could be detected in sinonasal mucosa (Fig. 5, B). In order to confirm our immunohistochemical findings, we further did flow cytometric analysis of perforin and granzyme B expression in CD8⁺ T cells in polyp tissues and peripheral blood. Despite the obvious perforin and granzyme B expression in peripheral CD8⁺ T cells, no significant granzyme B or perforin expression was observed in most nasal CD8⁺ T cells (Fig 6, A and B). No significant difference in the percentage of granzyme B⁺CD8⁺ T cells or perforin⁺CD8⁺ T cells could be discovered among different study groups no matter whether it was in peripheral blood or in nasal tissues (Fig.6, A and B, and Fig, S6). Consistently, we further found that the cytotoxic activity of CD8⁺ lymphocytes in polyps was significantly reduced compared with their peripheral counterparts (Fig.6, C). Since similar expression pattern of granzyme B and perforin in CD8⁺ T cells was found in eosinophilic and non-eosinophilic polyps, we did not stratify CRSwNP subjects into eosinophilic and non-eosinophilic group in cytotoxic activity study.

The expression of CD8⁺ T cell subset relevant chemokines and chemokine receptors in sinonasal mucosa

Finally, we investigated major chemokine-chemokine receptor elements that are potentially responsible for infiltration of different Tc cell subsets in sinonasal mucosa using real-time PCR assay. Compared with those seen in controls, CXCL16, CCL17 and CCL20 mRNA expression was significantly elevated in both eosinophilic and

non-eosinophilic polyps (Fig. 7, A). Nevertheless, no significant difference in CCL2, CCL3, CCL4, CCL5, CXCL9, or CXCL10 mRNA expression was demonstrated among different study groups (Fig. 7, A). Further ELISA study confirmed the increased expression of CXCL16 and CCL20 in both eosinophilic and non-eosinophilic polyps, and up-regulated expression of CCL17 in eosinophilic polyps, compared with controls at protein level (Fig. S7). The mRNA expression levels of CXCL16, CCL17, and CCL20 were positively correlated with the percentages of Tc1, Tc2, and Tc17 cell subset in sinonasal mucosa, respectively (Fig. 7, B). However, we did not find any correlation between Tc1, Tc2 and Tc17 cell subset infiltration and the expression of other chemokines (data not shown). The corresponding receptor for CXCL16, CXCR6, was up-regulated in mRNA level in both eosinophilic and non-eosinophilic polyps in comparison with controls, and the mRNA expression of CCR4, the receptor for CCL17, was increased in eosinophilic polyps compared with controls and non-eosinophilic polyps (Fig. S8, A). The expression of CXCR6 and CCR4 was positively associated with Tc1 and Tc2 cell subset infiltration in sinonasal mucosa, respectively (Fig. S8, B). Nevertheless, no significant increase of CCR6, the receptor for CCL20, was found in polyps, and no significant correlation between CCR6 expression and Tc17 cell subset infiltration in sinonasal mucosa was discovered either (Fig. S8).

DISCUSSION

Although CD4⁺ lymphocytes are well known to contribute to the pathology of CRS, the role of CD8⁺ lymphocytes in the progression of CRS has been poorly investigated. Previous studies have demonstrated an increased accumulation of CD8⁺ T cells, particularly effector memory CD8⁺ T cells with conventional $\alpha\beta$ -T-cell receptor and $\alpha\beta$ -CD8 coreceptor phenotype, in CRS [4, 17]; however, the function relevance of these CD8⁺ T cells in CRS remains undefined.

In this study, we first confirmed increased infiltration of CD8⁺ T cells in CRSwNP [4]. The similar increase of CD8⁺ T cells in eosinophilic and non-eosinophilic polyps and enhanced CD8/CD4 ratio in sinonasal mucosa compared with that in peripheral blood indicate a potential importance of CD8⁺ T cells in the pathophysiology of CRSwNP [15]. CD8⁺ T cells can form functionally similar subsets which exhibit similar cytokine profiles as Th cell subsets. More and more evidence suggest that CD8⁺ T cells might regulate allergic and immunologic diseases including asthma, chronic obstructive pulmonary disease, rheumatoid arthritis and psoriasis through releasing type 1, type 2, and type 17 cytokines [26-29]. In this study, we have provided a detailed description of cytokine-producing phenotypes of CD8⁺ Tc cells and polarization of Tc cell subsets in Chinese CRSwNP with different types of inflammation by flow cytometry. We found that Tc1 was the predominant Tc cell subset in sinonasal mucosa and peripheral blood. Similar to total CD8⁺ T cells, the percentages of Tc1, Tc2 and Tc17 cells were all increased in eosinophilic and non-eosinophilic polyps as compared with those of control nasal mucosa. Importantly, This article is protected by copyright. All rights reserved.

when comparing the difference between eosinophilic and non-eosinophilic NPs, a Tc2 skewed response in eosinophilic polyps and a Tc1/Tc17 dominated reaction in non-eosinophilic polyps were discovered. This biased Tc cell subset polarization is quite similar to that of Th cell subset found in eosinophilic and non-eosinophilic NPs [4]. Although a fundamental role in immune regulation has been well recognized for Th cell subsets [4, 7, 8], recent studies have shown that Tc2 cells are also able to induce lung eosinophilia and bronchial hyperresponsiveness and increased number of Tc2 cells has been found in sputum of patients with atopic asthma [30]. On the contrary, Tc1 and Tc17 cells have been found to facilitate neutrophil recruitment to inflammatory sites through inducing CXCL8 expression or promoting CCL3 function [31, 32]. In line with these studies, we found here that the Tc1 and Tc17 cell subset polarization correlated with neutrophilic inflammation, whereas Tc2 cell subset domination was associated with eosinophilic inflammation in sinonasal mucosa, suggesting that Tc cell subsets may contribute to the inflammatory reactions in CRS in a similar manner as Th cell subsets. In our study, in addition to detect IL-4⁺ Tc cells by flow cytometry, we also observed IL-5 expression by CD8⁺ T cells by immunofluorescence. We found the IL-5⁺CD8⁺ T cells were also accumulated in eosinophilic polyps and correlated with eosinophilic inflammation in sinonasal mucosa, further supporting a role of type 2 cytokines produced by CD8⁺ T cells in propagating eosinophilic inflammation in upper airways. Moreover, we found that CD8⁺ T cells produced comparable amount of type 1, type 2, and type 17 cytokines as CD4⁺ T cells did. These findings strongly suggest that in addition to CD4⁺ T cells,

CD8⁺ T cells should be considered as a significant source of the inflammatory cytokines in NPs and underline a previously underappreciated role of Tc cell subset in shaping inflammation pattern in CRSwNP.

The best characterized function of CD8⁺ T cell or Tc1 subpopulation is to clear the intracellular pathogens [33]. They can kill cells bearing target antigens by releasing cytotoxic molecules, such as granzyme B and perforin, into the immunological synapse and by secreting proinflammatory cytokine IFN- γ and TNF- α [10, 34]. However, in this study, we were surprised to find that there was very limited expression of perforin and granzyme B in nasal mucosal CD8⁺ T cells and polyp CD8⁺ T cells presented a poor cytolytic activity compared with their counterparts in peripheral blood. These findings were confirmed by several complementary techniques, including immunohistochemistry, flow cytometry, and cytotoxic activity analysis, and suggest that CD8⁺ T cells may not function as classic cytotoxic cells in NPs. However, the pathophysiological relevance of low cytotoxic CD8⁺ T cells in nasal mucosa is currently unclear. There are several possible mechanisms that could induce low granzyme B and perforin expression in CD8⁺ T cells in nasal mucosa. 1) CD8⁺ T cells may react to viral or other intracellular infection and exhibit active and continuous degranulation, thus resulting in the loss of their granzyme B and perforin content. Our previous study has shown a high prevalence of viruses in nasal epithelial cells from controls and CRSwNP patients [35]. However, we did not find a significant difference in the mRNA expression of CD107a, a marker of degranulation [36], in CD8⁺ T cells purified from sinonasal mucosa and peripheral blood (Fig. S9, A).

Therefore, CD8⁺ T cells in nasal mucosa are less likely to undergo a continuous degranulation; nevertheless, we acknowledge that the surface expression of CD107a on CD8⁺ T cells needs to be determined in future to confirm this hypothesis. 2) Early differentiated (CD27⁺CD28⁺) or transitory (CD27⁻CD28⁺) CD8⁺ T cells have low cytotoxic activity and such kinds of cells have been identified in synovial fluid of patients with rheumatoid arthritis [37]. However, Pant et al have reported that CD8⁺ T cells in nasal mucosa were mainly effector memory (CD45RA⁻CD27⁻CD62L⁻) CD8⁺ T cells compared with those in peripheral blood [17]. We also found that the mRNA expression of CD27 and CD28 was significantly lower in CD8⁺ T cells isolated from nasal tissues compared with those isolated from peripheral blood (Fig. S9, B), suggesting that most of nasal CD8⁺ T cells are unlike early differentiated or transitory cells in contrast to CD8⁺ T cells in peripheral blood. 3) In response to chronic infection or inflammation, CD8⁺ T cells may lose their effector function including cytokine production ability via programmed cell death protein 1 (PD-1) mediated exhaustion pathway [38]. In contrast, in this study, we found an up-regulated cytokine production in nasal CD8⁺ T cells compared with peripheral CD8⁺ T cells (Fig. S10), disfavoring an exhausted phenotype of nasal CD8⁺ T cells. However, the involvement of PD-1/PD-1 ligand pathway in the phenotype modulation of nasal CD8⁺ T cells needs further studies. 4) The local immune environment in nasal mucosa may modulate the function of CD8⁺ T cells. Indoleamine-2,3-dioxygenase (IDO) induced by local inflammatory cytokines has been shown to down-regulate the expression of perforin and granzyme B in CD8⁺ T cells [39]. A significant expression of IDO has

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been demonstrated in epithelial cells and infiltrated leucocytes in CRSwNP [40]. In this study, we found that the mRNA expression level of IDO was significantly increased in sinonasal mucosa compared with that in peripheral blood (Fig. S11), suggesting that IDO may drive the loss of perforin and granzyme B expression in CD8⁺ T cells in nasal mucosa under physiological or pathological pressure. Obviously, the regulatory effect of IDO on cytotoxic activity of CD8⁺ T cells in nasal mucosa requires further investigations.

The chemokine-chemokine receptor interaction is a major factor determining the infiltration of immune cells at inflammatory site. In order to elucidate the potential mechanisms underlying the Tc cell subset polarization in CRSwNP, we explored the expression of several relevant chemokines. Consistent with the enhanced infiltration of various Tc cell subsets in both eosinophilic and non-eosinophilic CRSwNP, we found that the expression of CXCL16, CCL17, and CCL20 was elevated in both types of CRSwNP. CXCL16 is a chemokine mainly secreted by macrophage and can guide the migration of activated Tc1 cells through binding to its receptor CXCR6 expressed on Tc1 cells [41, 42]. CCR4 expressed on type 2 T lymphocytes (Th2 and Tc2 cells) interacts with its ligand CCL17, thus playing an important role in the recruitment of Th2 and Tc2 cells [43]. Th17 and Tc17 cells home to a CCL20-enriched environment relaying on CCR6-CCL20 interaction [44]. We found here that the expression of CXCL16/CXCR6, CCL17/CCR4, and CCL20 significantly correlated with Tc1, Tc2, and Tc17 cell subset infiltration in sinonasal mucosa, respectively. The lack of association between CCR6 and Tc17 cell subset may be due to the broad expression

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pattern of CCR6 on immune cells other than T cells [9]. Therefore, our results indicate that CXCL16/CXCR6, CCL17/CCR4, and CCL20/CCR6 interaction may contribute to the accumulation of Tc1, Tc2, and Tc17 cell subset in polyp tissues, respectively. Besides the chemokine and chemokine receptor mediated trafficking of differentiated Tc cell subsets to NPs, it is also possible that polarization of Tc cell subsets occurs at the local inflammatory site, which can re-enforce the local immune responses [14]. For example, the high levels of IL-4 in eosinophilic NPs may facilitate the differentiation of naive CD8⁺ T cells into Tc2 cell subset in nasal mucosa [14].

In addition to Tc1, Tc2, and Tc17 cell subset, we found here that the percentage of CD8⁺FOXP3⁺ Tregs was significantly decreased in eosinophilic and non-eosinophilic polyps, which was consistent with the findings reported by Pant et al [45]. It has been demonstrated that CD8⁺ Tregs could inhibit the proliferation of CD4⁺ and CD8⁺ T cells and their cytokine production, but did not alter the expression of granzyme B or perforin in CD8⁺ effector T cells [46], suggesting that a defect of CD8⁺ Tregs more likely contributes to the exaggerated inflammation in CRSwNP.

In summary, we reported here that although nasal CD8⁺ T cells may not primarily function as classic cytotoxic cells, they are potent and underappreciated producer of type 1, 2, and 17 cytokines in NPs. The different Tc cell subset polarization may underlie the distinctly biased granulocyte inflammation in eosinophilic and non-eosinophilic CRSwNP. The interaction of CXCL16/CXCR6, CCL17/CCR4, and CCL20/CCR6 potentially contribute to the accumulation of specific Tc cell subsets in CRSwNP. Our findings provide evidence that CD8⁺ T cells

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may contribute substantially to the pathogenesis of CRSwNP through releasing pathogenic cytokines and that classic models of CRS focusing primarily on the pathogenic role of CD4⁺ T cells may be incomplete. In addition, since CD8⁺ T lymphocytes are potentially less sensitive than CD4⁺ T cells to glucocorticoid therapy [47], further pharmaceutical studies targeting CD8⁺ T cell population may offer an opportunity to achieve better control of CRSwNP.

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FIGURE LEGENDS

Figure 1. CD8⁺ T cells in sinonasal mucosa detected by immunohistochemistry. (A)

Representative photomicrographs of sinonasal tissue sections showing

immunohistochemical staining of CD8⁺ T cells. Original magnification × 400. (B)

Quantification of CD8⁺ T cells in sinonasal mucosa. Eos CRSwNP, eosinophilic

chronic rhinosinusitis with nasal polyps; Non-Eos CRSwNP, non-eosinophilic chronic rhinosinusitis with nasal polyps.

Figure 2. Flow cytometric analysis of CD8⁺ T cells and different T cytotoxic (Tc) cell subsets in sinonasal mucosa. (A) Gating strategy and representative flow plots. CD3⁺CD8⁺ Tc cells were characterized based on the cytokine expression of IFN- γ (Tc1), IL-4 (Tc2), or IL-17A (Tc17). (B) The percentage of total CD8⁺ T cells in nasal mucosal mononuclear cells (NMCs) and the ratio of CD4/CD8 in sinonasal mucosa and peripheral blood. (C) The percentages of Tc1, Tc2, and Tc17 cells in NMCs. (D) The ratios of Tc1/Tc2, Tc1/Tc17, and Tc2/Tc17 cell subset in NMCs. Eos CRSwNP, eosinophilic chronic rhinosinusitis with nasal polyps; Non-Eos CRSwNP, non-eosinophilic chronic rhinosinusitis with nasal polyps.

Figure 3. The relative contribution of CD4⁺ and CD8⁺ T cells to cytokine expression in sinonasal mucosa studied by flow cytometry. (A) The percentages of IFN- γ , IL-4, and IL-17A positive CD4⁺ and CD8⁺ T cells in CD3⁺ T cells with corresponding cytokine expression in sinonasal mucosa. (B) The median fluorescence intensity (MFI) of IFN- γ , IL-4, and IL-17A positive CD4⁺ and CD8⁺ T cells in sinonasal mucosa. Eos CRSwNP, eosinophilic chronic rhinosinusitis with nasal polyps; Non-Eos CRSwNP, non-eosinophilic chronic rhinosinusitis with nasal polyps.

Figure 4. (A) The numbers of myeloperoxidase (MPO) positive neutrophils and eosinophils in sinonasal mucosa. (B) The association between different Tc cell subsets and eosinophilic and neutrophilic inflammation in sinonasal mucosa. Eos CRSwNP, eosinophilic chronic rhinosinusitis with nasal polyps; Non-Eos CRSwNP, non-eosinophilic chronic rhinosinusitis with nasal polyps; HPF, high power field; NMCs, nasal mucosal mononuclear cells.

Figure 5. The expression of granzyme B and perforin in CD8⁺ T cells studied by immunohistochemistry. (A and B) Representative photomicrographs of (A) CD8 and granzyme B, and (B) CD8 and perforin immunohistochemical staining of consecutive sections of nasal polyp tissues from a patient with non-eosinophilic chronic rhinosinusitis with nasal polyps. Few cells could be identified presenting CD8 and granzyme B co-expression and no obvious immunoreactivity of perforin could be found in nasal polyps. In perforin immunostaining, lymphoma tissues served as positive control. (C) The number of granzyme B⁺CD8⁺ cells, the percentage of granzyme B expressing CD8⁺ T cells, and the proportion of CD8⁺ cells in total granzyme B⁺ cells in sinonasal mucosa. Eos CRSwNP, eosinophilic chronic rhinosinusitis with nasal polyps; Non-Eos CRSwNP, non-eosinophilic chronic rhinosinusitis with nasal polyps; HPF, high power field.

Figure 6. The expression of granzyme B (GZMB) and perforin (PRF1) in CD8⁺ T cells studied by flow cytometry and the cytotoxic activity analysis of CD8⁺ T cells.

(A) Gating strategy and representative flow plots of granzyme B expression in samples from a patient with eosinophilic chronic rhinosinusitis with nasal polyps and the percentages of granzyme B⁺CD8⁺ T cells in total CD8⁺ T cells in nasal tissues and peripheral blood. (B) Gating strategy and representative flow plots of perforin expression in samples from a patient with non-eosinophilic chronic rhinosinusitis with nasal polyps and the percentages of perforin⁺CD8⁺ T cells in total CD8⁺ T cells in nasal tissues and peripheral blood. (C) Redirected killing of Fas negative K562 cells by freshly purified CD8⁺ T lymphocytes from nasal polyps and peripheral blood in the presence of anti-CD3 or an IgG2a isotype-matched control at a final concentration of 10 µg/mL. Eos CRSwNP, eosinophilic chronic rhinosinusitis with nasal polyps; Non-Eos CRSwNP, non-eosinophilic chronic rhinosinusitis with nasal polyps; PBMCs, peripheral blood mononuclear cells; NMCs, nasal mucosal mononuclear cells.

Figure 7. (A) The mRNA expression of CD8⁺ T cell subset relevant chemokines in sinonasal mucosa. (B) The association of mRNA expression of CXCL16, CCL17 and CCL20 with the infiltration of Tc1, Tc2, and Tc17 cell subset in sinonasal mucosa, respectively. Eos CRSwNP, eosinophilic chronic rhinosinusitis with nasal polyps; Non-Eos CRSwNP, non-eosinophilic chronic rhinosinusitis with nasal polyps; NMCs, nasal mucosal mononuclear cells.











