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Group 2 innate lymphoid cells are increased in nasal polyps in patients with eosinophilic chronic rhinosinusitis

Ichiro Tojima [a,*], Hideaki Kouzaki [a], Shino Shimizu [a], Takao Ogawa [a], Masahiko Arikata [a], Hirohito Kita [b], Takeshi Shimizu [a]

[a] Department of Otorhinolaryngology, Shiga University of Medical Science, Otsu, Japan
[b] Division of Allergic Diseases, Department of Internal Medicine, and the Department of Immunology, Mayo Clinic, Rochester, MN, United States

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ABSTRACT

ILC2s represent a critical innate cellular source of type 2 cytokines and may play important roles in various diseases. We examined the role of ILC2s in the pathogenesis of two subgroups of CRSwNP: ECRS and non-ECRS. We analyzed the prevalence of ILC2s in sinonasal tissues and in blood from patients with ECRS, non-ECRS, CRSsNP, and control. The prevalence of ILC2s in nasal tissues was higher in patients with ECRS as compared to those with non-ECRS or CRSsNP. The prevalence of blood ILC2s was not different between patients with ECRS and non-ECRS. The prevalence of blood ILC2s was higher in patients with allergic rhinitis and elevated serum IgE levels. *Alternaria*-induced IL-33 secretion was increased in nasal epithelial cells derived from patients with ECRS as compared to those from patients with non-ECRS or CRSsNP. ILC2s may be involved in the pathogenesis of CRSwNP, in particular in patients with tissue eosinophilia (i.e., ECRS).

1. Introduction

Innate lymphoid cells (ILCs) are emerging as important effector cells of the innate immune system, which may play important roles in pathogen clearance, tissue remodeling, and immune homeostasis. Among ILCs, group 2 innate lymphoid cells (ILC2s) are a recently identified subset that produces large amounts of type 2 cytokines, such as IL-5 and IL-13. Epithelium-derived IL-33, IL-25, and tymic stromal lymphopoietin (TSLP) are likely to be the key cytokines for induction of the production of type 2 cytokines by ILC2s [1]. Recent studies suggest that ILC2s may play important roles in innate allergic inflammation [2–4].

Chronic rhinosinusitis (CRS) is characterized by persistent symptomatic inflammation of the nasal and paranasal mucosa that lasts longer than 12 weeks [5].CRS can be further classified into 2 major subtypes: CRS without nasal polyps (CRSsNP) and CRS with nasal polyps (CRSwNP) [5]. In the US and in European countries, CRSsNP presents as a predominant infiltration of neutrophils and Th1- or Th17-type cytokines, whereas CRSwNP is characterized by eosinophilic infiltration and Th2-type cytokines. However, in Asian countries, CRSwNP may represent both eosinophilic and neutrophilic phenotypes [6,7]. Recently, the Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis Study (JESREC Study) classified CRSwNP into two subtypes: eosinophilic CRS (ECRS) and non-ECRS. ECRS represents marked eosinophilia in nasal polyps and is associated with greater clinical and radiological severity, high morbidity with bronchial asthma, and higher risk of polyp recurrence, as compared to non-ECRS [8].

In our previous study, IL-33, IL-25, and TSLP were detected at higher levels in epithelial cells of nasal polyps from ECRS patients as compared to those from non-ECRS patients [9]. Human ILC2s were first identified in nasal polyp tissues, and increased numbers of ILC2s were found in nasal polyps [3]. However, the functional importance of ILC2s in the pathogenesis of CRS is not clearly understood. In addition, only a few reports examined the ILC2s in peripheral blood in association with allergic diseases [10,11]. To fill this gap in our knowledge, in the present study, we analyzed the prevalence of ILC2s in sinonasal mucosa and nasal polyps and in peripheral blood from patients with ECRS, as compared to patients with non-ECRS; patients with CRSsNP and those without CRS were also used as controls. Our findings suggest that ILC2s are increased in nasal polyps of patients with ECRS as compared to those with non-ECRS. The increased prevalence of blood ILC2s was associated with the presence of allergic rhinitis and elevated serum IgE levels.

Abbreviations: ILC2s, group 2 innate lymphoid cells; CRS, chronic rhinosinusitis; CRSwNP, chronic rhinosinusitis with nasal polyps; CRSsNP, chronic rhinosinusitis without nasal polyps; ECRS, eosinophilic chronic rhinosinusitis; UTs, uncinate tissues; NPs, nasal polyps; AR, allergic rhinitis; FACS, fluorescence-activated cell sorting, PBMCs, peripheral blood mononuclear cells.

* Corresponding author at: Department of Otorhinolaryngology, Shiga University of Medical Science, Seta-Tsukinowa, Otsu, Shiga 520-2192, Japan.
E-mail address: itirotz@hotmail.com (I. Tojima).

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Furthermore, airway epithelial cells from patients with ECRS produced larger quantities of IL-33 when stimulated *in vitro* with allergen as compared to those produced from non-ECRS.

2. Materials and methods

2.1. Study subjects

Patients with sinonasal diseases were recruited from the department of Otorhinolaryngology at Shiga University of Medical Science hospital according to the protocol approved by the Institutional Review Board of Shiga University of Medical Science (ethics approval number 25–36). Informed consent was obtained from all participants. Sinonasal tissues of CRS patients such as uncinate tissues (UTs) and nasal polyps (NPs) were obtained during endoscopic sinus surgery, and peripheral blood samples were collected preoperatively. The diagnosis of CRS was made based on clinical, endoscopic, and radiographic criteria as described by Fokkens et al. [5] in the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS) 2012. Control subjects who did not have CRS but did have asthma, allergic rhinitis (AR), or hypertrophic rhinitis were also recruited. A detailed list of the patients' characteristics is presented in Table 1. Patients with CRS with nasal polyps (CRSwNP) were divided into ECRS and non-ECR according to a published clinical scoring system (JESREC score), and mucosal eosinophilia that was defined as higher than 70 per high-power field (HPF) [8]. The JESREC score consists of bilateral disease sites (3 points), nasal polyps (2 points), CT shadow: ethmoid > maxillary sinus (2 points), and blood eosinophilia: 2–5% (4 points), 5–10% (8 points), or >10% (10 points), and patients with scores higher than 11 points were defined as ECRS. All CRS patients had previously failed conservative medical treatments and saline irrigations. The patients were not treated with systemic corticosteroids within 4 weeks before the surgery. Patients with respiratory epithelial adenomatoid hamartoma (REAH), post-operative sinus cyst, fungal sinusitis, or CRS with sinonasal tumor were excluded from the study. The diagnosis of asthma was based on criteria of the Global Initiative for Asthma, published in 2006 [12]. Allergic rhinitis (AR) was diagnosed based on "Practical Guideline for the Management of Allergic Rhinitis in Japan," published in 2009 [13].

Patients were assigned to four groups: ECRS ($n = 14$), non-ECRS ($n = 16$), CRSsNP ($n = 16$), and control subjects ($n = 17$: 13 patients with allergic rhinitis with or without asthma, 4 patients with hypertrophic rhinitis) (Table 1). The percentage of peripheral blood eosinophils was significantly higher in ECRS patients (8.9 ± 0.8%), compared with non-ECRS patients (5.7 ± 1.3%), CRSsNP patients (4.1 ± 0.7%), or control subjects (5.3 ± 1.2%).

2.2. Isolation of ILC2s from peripheral blood and sinonasal tissues

Heparinized peripheral blood was layered over an equal volume of Histopaque 1083 (Sigma-Aldrich; St Louis, MO) and centrifuged according to the manufacturer's instructions. Peripheral blood mononuclear cells (PBMCs) were collected from the interface between Histopaque and plasma. PBMCs were washed and resuspended in RPMI 1640 media (Wako; Osaka, Japan) containing 10% fetal bovine serum (FBS) (bioewest; Nuaillé, France), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Wako).

Sinonasal tissues were cut into fine pieces and digested for 30–45 min at 37 °C with Liberase TM (125 μg/mL) and DNase I (200 μg/mL) (both from Roch Diagnostics GmbH; Mannheim, Germany). Alternatively, cells were isolated by mechanical disruption of the tissues with the gentleMACS Dissociator and Tumor Dissociation Kit (Miltenyi Biotec Inc.; Auburn, CA) following the procedure recommended by the manufacturers. The cell suspensions were filtered through a 70 μm nylon mesh. The remaining cells were treated with ACK lysing buffer (Lonza) to lyse red blood cells.

Fluorescence-activated cell sorting (FACS) was used to identify ILC2s following the strategy used by Mjösberg et al. [3]. Briefly, PBMCs or total cell suspensions from sinonasal tissues were stained with the following antibody cocktail: fluorescein isothiocyanate (FITC)-labeled antibodies to CD3, CD14, CD16, CD19, CD20, CD56 (Lineage cocktail), phycoerythrin (PE)-cyanine 7 (Cy7)-labeled antibody to CD45, PE-labeled antibody to CD127, and Alexa Fluor®-labeled antibody to CRTH2 (BioLegend; San Diego, CA). ILC2s were identified as lineage$^-$ CD45$^+$ CD127$^+$ CRTH2$^+$ cells. The prevalence of ILC2s was calculated as the numbers of ILC2s divided by the numbers of total lineage$^-$ CD45$^+$ cells.

2.3. ILC2s culture

In some experiments, ILC2s were sorted from sinonasal and peripheral blood specimens. Sorted ILC2s (1.0×10^3 cells/mL) were cultured in 96-well round-bottom plates in RPMI 1640 containing 10% autologous serum at 37 °C with 5% CO_2 for 8 days with or without IL-33 (10 ng/mL, R&D System; Minneapolis, MN) in the presence or absence of IL-2 (50 ng/mL, R&D System). The concentrations of IL-5 and IL-13 in cell-free supernatants were determined using ELISA kits (R&D System).

2.4. Epithelial cell culture

Nasal epithelial cells were obtained by scratching UTs of patients with CRSsNP or NPs of patients with ECRS or non-ECRS by using curettes (ASI; Springville, UT). Cells were seeded in supplemented basal epithelial growth medium (Lonza Corp.; Walkersville, MD) and were cultured in a type I collagen-coated 75-cm^2 flask (Iwaki; Tokyo, Japan) in a humidified incubator at 37 °C with 5% CO_2. Once confluent, nasal epithelial cells were subcultured and seeded onto a type I collagen-coated 24-well tissue culture plate (Iwaki). After the cells were grown to confluency, *Alternaria* extract (100 μg/mL, Greer Laboratories; Lenoir, NC) was added, and the cells were incubated for 2 h. Cell-free supernatants were collected and stored at −20 °C until use. The concentration of IL-33 was determined using an ELISA kit (R&D System).

Table 1
Clinicopathological characteristics of patients with CRSsNP and of ECRS and non-ECRS subgroups of CRSwNP.
AIA, aspirin-induced asthma.

	Control subjects	Patients with CRSsNP	Patients with CRSwNP	
			Non-ECRS	ECRS
	($n = 17$)	($n = 18$)	($n = 16$)	($n = 14$)
Age (years) mean ± SEM	36.8 ± 3.5	56.2 ± 4.4	50.9 ± 4.4	55.5 ± 3.5
Sex, male/female	10/7	10/6	10/6	7/7
Asthma (AIA), *n*	4 (0)	2 (0)	4 (0)	11 (2)
Allergic Rhinitis, *n*	11	11	9	7
% of blood eosinophil	5.3 ± 1.2	4.1 ± 0.7	5.7 ± 1.3	8.9 ± 0.8
Serum total lgE (IU/ml)	220 ± 60	206 ± 55	192 ± 55	217 ± 37
Lund–Mackay score	0	5.1 ± 0.9	12.6 ± 1.3	16.8 ± 1.0
JESREC score	0	5.0 ± 1.0	9.4 ± 1.2	15.6 ± 0.5