



Contents lists available at ScienceDirect

Allergology International

journal homepage: <http://www.elsevier.com/locate/alit>

Original Article

Increased CD69 expression on activated eosinophils in eosinophilic chronic rhinosinusitis correlates with clinical findings

Yasutaka Yun ^{a, b}, Akira Kanda ^{a, c, *, 1}, Yoshiki Kobayashi ^{a, c}, Dan Van Bui ^a, Kensuke Suzuki ^a, Shunsuke Sawada ^a, Kazuyasu Baba ^{a, b}, Masao Yagi ^a, Mikiya Asako ^{a, c}, Haruka Okazaki ^d, Hiroki Ikeda ^e, Shigeki Kawamura ^f, Akihiko Nakamura ^g, David Dombrowicz ^h, Koichi Tomoda ^a, Hiroshi Iwai ^a

^a Department of Otorhinolaryngology, Head & Neck Surgery, Kansai Medical University, Osaka, Japan

^b Department of Otorhinolaryngology, Takeda General Hospital, Kyoto, Japan

^c Allergy Center, Kansai Medical University, Osaka, Japan

^d Okazaki ENT Clinic, Osaka, Japan

^e Ikeda ENT Clinic, Wakayama, Japan

^f Kawamura ENT Clinic, Osaka, Japan

^g Nakamura ENT Clinic, Osaka, Japan

^h University of Lille, Inserm, CHU Lille, Institut Pasteur de Lille, U1011-EGID, Lille, France

ARTICLE INFO

Article history:

Received 8 May 2019

Received in revised form

19 November 2019

Accepted 21 November 2019

Available online xxx

Keywords:

Asthma

CD69

Chronic rhinosinusitis

Eosinophil

Nasal polyp

Abbreviations:

CRS, chronic rhinosinusitis;

CRSwNP, chronic rhinosinusitis with nasal

polyp; CRSsNP, chronic rhinosinusitis

without nasal polyp; ECRS, eosinophilic

chronic rhinosinusitis; NECRS, non-

eosinophilic chronic rhinosinusitis;

NP, nasal polyp; UT, uncinate process tissue;

PB, peripheral blood; JESREC, Japanese

Epidemiological Survey of Refractory

Eosinophilic Chronic Rhinosinusitis;

MBP, major basic protein; ECP, eosinophilic

cationic protein; EDN, eosinophilic-derived

neurotoxin; EPX, eosinophil peroxidase;

CD, cluster of differentiation; NK, natural

killer; MFI, mean fluorescence index

ABSTRACT

Background: Eosinophilic chronic rhinosinusitis (ECRS) is a subtype of chronic rhinosinusitis associated with asthma. CD69 is an important marker of activation for eosinophils. But, whether a correlation exist between the CD69 expression on eosinophils and clinical findings is unclear.

Methods: We performed quantitative PCR and/or flow cytometry using tissue and purified eosinophils from the blood and nasal polyps of 12 patients with ECRS and from 8 patients without ECRS (controls). We assessed clinical findings including nasal polyp (NP) scores, sinus CT findings, and pulmonary function test results, and examined their possible association with the CD69 expression. We also performed CD69 cross-linking experiments in mouse eosinophils to investigate the functional role of CD69.

Results: Levels of cytokine mRNAs (IL-4, -5, -10, and -13) were significantly higher in purified NP eosinophils and tissues from patients with ECRS than the levels of those in controls. The expressions of major basic protein (MBP), eosinophilic cationic protein (ECP), eosinophilic-derived neurotoxin (EDN), eosinophil peroxidase (EPX) in cytotoxic granules, and CD69 mRNA were significantly higher in purified eosinophils from NPs than in those from blood. We also found a correlation between expression of CD69 and clinical findings. Moreover, we found EPX release from mouse eosinophils following CD69 cross-linking.

Conclusions: These data suggest that increased CD69 expression by eosinophils is not only a biomarker for nasal obstruction and pulmonary dysfunction, but also a potential therapeutic target for patients with ECRS and asthma.

Copyright © 2020, Japanese Society of Allergy. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

* Corresponding author. Department of Otorhinolaryngology, Head and Neck Surgery, Kansai Medical University, 2-5-1, Shinmachi, Hirakata City, Osaka 573-1010, Japan.
E-mail address: akanda@hirakata.kmu.ac.jp (A. Kanda).

Peer review under responsibility of Japanese Society of Allergy.

¹ This author shares first authorship.

<https://doi.org/10.1016/j.alit.2019.11.002>

1323-8930/Copyright © 2020, Japanese Society of Allergy. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Chronic rhinosinusitis (CRS) is a heterogeneous disease classified into two types based on the presence (CRSwNP) or absence (CRSSNP) of nasal polyps (NP).¹ Approximately, 80% of patients with CRSwNP have infiltration of eosinophils into the NPs.² Patients who exhibit CRS with eosinophilia (or eosinophilic chronic rhinosinusitis, ECRS) are diagnosed based on the Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis (JESREC) scoring system.³ ECRS is characterized by ethmoid-predominant sinusitis with eosinophilic infiltration. Kobayashi *et al.* and Ame-link *et al.* reported that 74% of patients with asthma have ECRS,⁴ and that 53% of those with severe asthma and 26.5% of those with mild-to-moderate asthma have NPs.⁵ Consequently, ECRS and asthma share macroscopic pathological and histological characteristics,^{6,7} and require comprehensive care according to the united airway concept.⁸

In patients with ECRS with asthma, eosinophils play a critical role in the pathogenesis of the diseases. Eosinophils must migrate from the bloodstream to the site of inflammation to exert injury as effector cells by releasing cytokines, cytotoxic granules, and chemokines.^{9–11} During this process, eosinophils express activation markers such as CD69, CD44, L-selectin, CD54, CD162, and CD11b.^{12,13} CD69 is a type II transmembrane protein, a member of the C-type lectin family, and a very early activation antigen (VEA).¹⁴ This molecule is expressed on infiltrated leukocytes such as T cells, B cells, NK cells, neutrophils, and eosinophils during chronic airway inflammation.^{15–17} In terms of CD69 regulation in eosinophils, Walsh *et al.* found that GM-CSF, IL-3, or IL-5 induce CD69 expression on peripheral blood eosinophils, but not in untreated ones.¹⁸ Reports have shown that signaling through CD69 modulates the differentiation of T helper cells and induces platelet degranulation and aggregation.^{14,18} Although several CD69 ligands have been isolated, including galectin-1 and myosin light chain 9 and 12 (MyI9/12),^{14,19} the ligand on eosinophils has not been identified.²⁰ Whether CD69 is expressed on eosinophils in the NPs and in peripheral blood (PB) of patients with ECRS patients, and whether a correlation exist between CD69 expression on NP eosinophils and clinical findings of patients with ECRS (such as NP score, sinus CT findings, and pulmonary function) remains unclear.

For this report, we developed an approach for obtaining a high number of purified eosinophils from NPs, because conventional protocols lead to poor numbers. We also measured cytokine production, levels of cytotoxic granules, and CD69 expression on purified eosinophils, and determined correlations between their expression levels and clinical findings. Moreover, we investigated the functional role of CD69 signaling in mouse eosinophils.

Methods

Patients

We recruited patients from the Otorhinolaryngology and Head and Neck surgery of the Takeda General Hospital and Kansai Medical University during a two-year period (2017–2018). The diagnoses of patients with ECRS and non-ECRS (NECRS) patients were based on those in the JESREC study. ECRS was diagnosed by 11

points or higher JESREC as reported by Fujieda *et al.*²¹ Moreover, a final diagnosis of ECRS was made by examination of NP under a microscope; mean number of eosinophils in three fields were >70 cells in the high-power field (400×).²¹ By contrast, NECRS were defined as both <11 score by JESREC and <70 eosinophils in the tissue.²¹ In all, 12 patients with ECRS and 8 controls (NECRS) participated in the study. We obtained NPs and uncinate process tissues (UT) during endoscopic sinus surgeries. Peripheral blood (PB) samples were collected before surgical operations. A respiratory physician diagnosed asthma based on the patient's history of wheezing responsive to a bronchodilator. No systemic corticosteroids were administered to the patients at least 4 weeks prior to their surgery. We used NP scores to evaluate the severity of NPs in each patient,²² and we defined sinus CT findings according to the Lund–Mackay scale.²³ The examination of pulmonary function was performed using SPIRO SIFT SP-770COPD (Fukuda Denshi, Tokyo, Japan) measuring the following parameters: the ratio of peak expiratory flow (%PEF), ratio of predicted forced expiratory volume in 1 s to forced vital capacity (FEV_{1.0}%), percentage of predicted mean forced expiratory flow between 25 and 75% of forced vital capacity (%FEF_{25–75}). Table 1 and Supplementary Table 1 present patient characteristics.

The local ethics committees at the Takeda General Hospital (No 2016-002) and at the Kansai Medical University (No.1313) approved this study. We obtained informed consents from all participants.

Mice

We used the following mouse strains: IL-5 transgenic (Tg) mice (BALB/c background), obtained from the co-author Dr. D. Dom-browicz (Institut Pasteur de Lille, Lille, France). All mice were housed at 21°C–23 °C with 40%–60% humidity in animal facilities with a 12-h light/dark cycle and were provided food and water ad libitum. All animal experiments were performed using protocols approved by the Kansai Medical University Animal Ethics Committee (18-082).

Human and mouse eosinophils preparation

We separated human granulocytes from PB using the Ficoll–Paque technique (GE healthcare, Uppsala, Sweden). After lysing red blood cells, eosinophils were isolated by using a cell sorter, Aria III (BD science, New Jersey, USA). Detailed protocols for the human eosinophil purification and isolation from NP and mouse eosinophils purification from splenocytes of IL-5 Tg mice are shown the Supplementary Methods and Supplementary Figure 1.

Flow cytometric analysis

We performed flow cytometry experiments (Aria III and CantII, BD Biosciences, New Jersey, USA) using the FlowJo software (BD Biosciences, New Jersey, USA).

We stained dissociated cells from human PB or NP with antibodies against CD3, CD14, CD16b, CD19, CD45, CD68, Siglec8, CD69, and/or control antibodies for 30 min at 4 °C. For CD69 mean fluorescence index (MFI) ratio on surface expression was calculated according as described.²⁴

$$\text{CD69 MFI ratio} = \frac{(\text{MFI of anti CD69 reacted cells on NP eosinophils or T cells} - \text{MFI of isotype})}{(\text{MFI of anti CD69 reacted cells on PB eosinophils or T cells} - \text{MFI of isotype})}$$

Table 1
Subject characteristics.

	non-ECRS	ECRS	P value
Patients, n	8	12	
Age (y), median	45.5 ± 19.7	58.7 ± 10.2	
Male/female, n	3/5	9/3	
Blood eosinophils counts (/μl)	245.5 ± 151.9	374.8 ± 192.6	
Total IgE	358.5 ± 461.7	761.2 ± 1332.8	
Asthma, n	1	10	P < 0.05
NP, n	0	12	
Tissue eosinophils counts (/HPF)		227.1 ± 146.6	

The purified mouse eosinophils from mouse spleens were stained with Gr-1, CD45, SiglecF, CD69, and/or control antibodies for 30 min at 4 °C. Eosinophils were identified by gated

CD45⁺SiglecF⁺Gr-1^{int} (Supplementary Fig. 2). Supplementary Table 2 lists detailed information regarding the antibodies for cell sorting.

Immunofluorescence staining and quantitative PCR

We stained tissue sections with antibodies against siglec8, CD69 and control antibodies overnight at 4 °C. Then, we incubated them with an appropriate secondary antibody for 1 h at room temperature (as listed in the Supplementary Table 2). Tissues were counterstained with Hoechst dye and mounted using Fluorescence Mounting Medium (Dako, CA, USA). We captured images and analyzed them using a LSM 700 scope (Carl Zeiss, Jene, Germany).

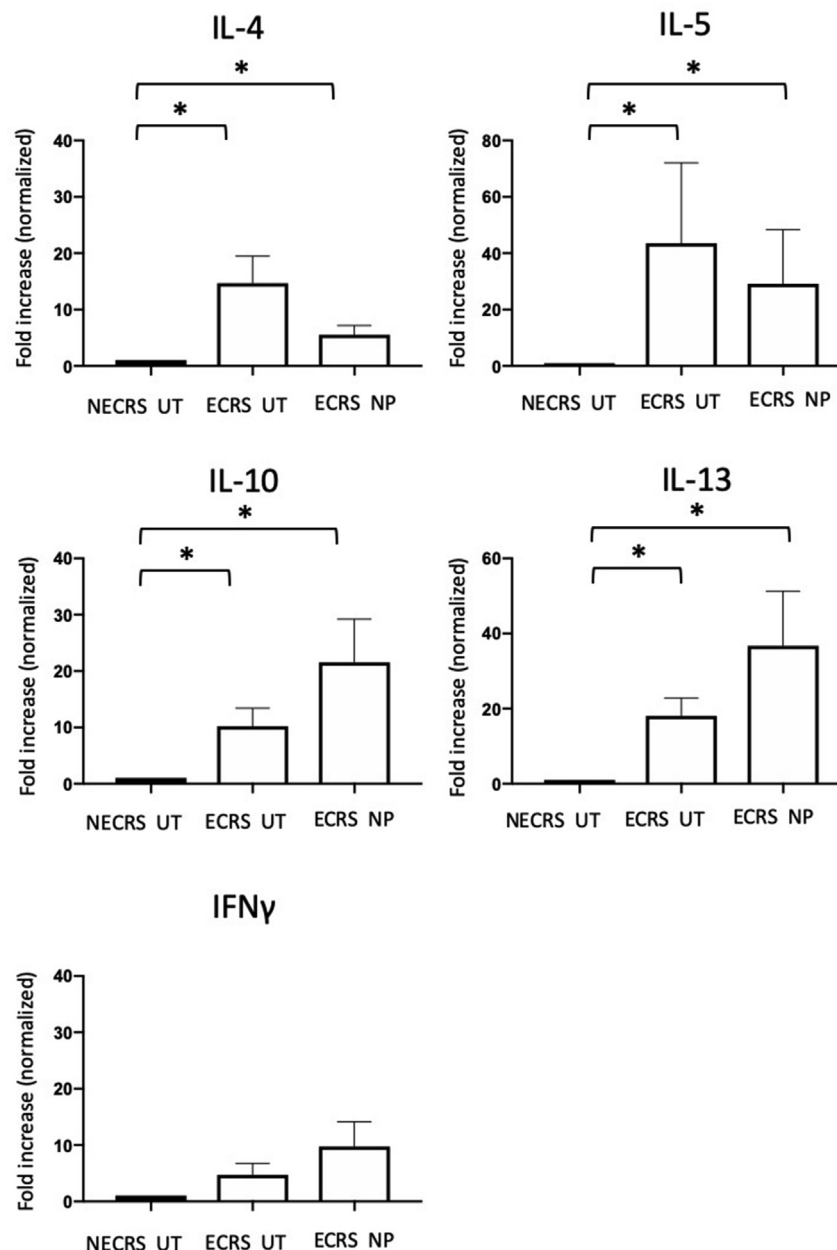


Fig 1. mRNA expression of cytokines in tissues of patients with ECRS and controls. Quantitative PCR analysis of IL-4, IL-5, IL-10, IL13, and IFN- γ mRNA expression levels in tissues of patients with ECRS and in controls. NECRS, non-eosinophilic chronic rhinosinusitis, controls; ECRS, eosinophilic chronic rhinosinusitis; UT, uncinate process tissue; NP, nasal polyp. Relative gene expression levels were calculated by the comparative C_T method with UT of controls as the reference. Data are expressed as means $\pm$ SEM (ECRS and controls are n = 12, 8). P values determined by the Mann–Whitney U-test (*P < 0.05).

We performed quantitative PCRs (qPCRs) following the extraction of mRNA from tissues and eosinophils. Relative gene expression was calculated using the comparative C_T method.²⁵ [Supplementary Table 3](#) lists primer sequences. Detailed protocols for immunofluorescence staining and qPCR are shown in the supplementary data.

EPX releasing from mouse eosinophils by cross-linking of CD69

We resuspended eosinophils (0.4 M/200ul per well) purified from mouse spleens in PBS and incubated them with 1–100 µg/ml Armenian Hamster anti-mouse CD69 mAb (Novus Biologicals, CO, USA) or with 10 µg/ml Armenian Hamster IgG (BioLegend, CA, USA) as control antibody for 1 h at 4 °C. We then transferred the eosinophils into a 24-well plate coating with 20 µg/ml Goat anti-Armenian Hamster IgG (Jackson ImmunoResearch, PA, USA) and incubated for 2 h at 37 °C. After centrifuge plate, we transferred the supernatants into a microtiter plate and analyzed them using an EPX detection kit as per the manufacturer's instructions (Cell Technology, CA, USA). Chemiluminescence was immediately measured with a luminometer, EnSpire (PerkinElmer, Mass, USA).

Statistical analysis

We presented data as means $\pm$ SEM and evaluated group differences for statistical significance using the Mann–Whitney U-test. We performed correlation analyses using the Spearman rank correlation coefficient. We considered all P values <0.05 as statistically significant for all tests. All statistical analyses were performed using GraphPad Prism ver.8 (GraphPad Software, CA, USA).

Results

Expression of cytokine mRNAs in tissue from controls and patients with ECRS

To investigate the cytokine profiles in the UTs and NPs of patients with ECRS, and in the UTs of controls, we quantified cytokine mRNAs by qPCR. The expressions of IL-4, IL-5, IL-10, and IL-13 mRNA in the UTs of patients with ECRS were significantly higher

than those in UTs from controls ([Fig. 1](#)). We observed similar results in terms of cytokine expressions in the NPs of patients with ECRS and UTs of controls ([Fig. 1](#)). Although the expressions of IFN γ mRNA among UT from patients with ECRS, NP from patients with ECRS, and UT from controls were all similar, the expressions were 4.7- and 9.8-fold higher in the UT and NP of patients with ECRS than in the UT of controls.

Character of purified eosinophils from PB and NP

To investigate differences in cytokine production between purified eosinophils from PB and NP, we modified a conventional protocol for NP eosinophil purification. As shown in [Supplementary Figure 3](#), the number and percentage of CD45⁺ dissociated cells from NP using the tumor dissociation kit was significantly higher than the number and percentage of the conventional protocol: while the tumor dissociation kit yielded $1.93 \pm 1.07 \times 10^7$ /g total cells with $14\% \pm 9\%$ CD45⁺ cells, we only obtained $0.76 \pm 0.6 \times 10^7$ /g total cells and $7\% \pm 4\%$ CD45⁺ cells after the conventional protocol. Moreover, the % viability between the two protocols was comparable: $76\% \pm 8\%$ viability using the tumor dissociation kit and $75\% \pm 16\%$ viability using the conventional protocol. After tissues dissociation, $0.79\% \pm 0.2\%$ eosinophils and $1.7\% \pm 0.6\%$ T cells from NPs were obtained by cell sorter. Using qPCR, we found significant differences in the levels of cytokines (IL-4, -5, -10, and -13) and cytotoxic granules (MBP, ECP, EDN, and EPX) between purified eosinophils from PB of controls and NP of patients with ECRS ([Fig. 2A, B](#)). Interestingly, we found significant differences in levels of MBP, EDN, and EPX between purified eosinophils from PB and NP of patients with ECRS ([Fig. 2B](#) and [Supplementary Fig. 4](#)); however, their cytokine expressions were similar ([Fig. 2A](#)).

Expression of CD69 on infiltrating eosinophils

Next, we examined CD69 expression on eosinophils using qPCR and flow cytometry. As shown in [Figure 3A](#), infiltrating eosinophils isolated from the NP of patients with ECRS had significantly higher expression of CD69 mRNA than the eosinophils from PB. According to our flow cytometry analysis results, the MFI of CD69 was significantly increased on eosinophils from NP compared with that

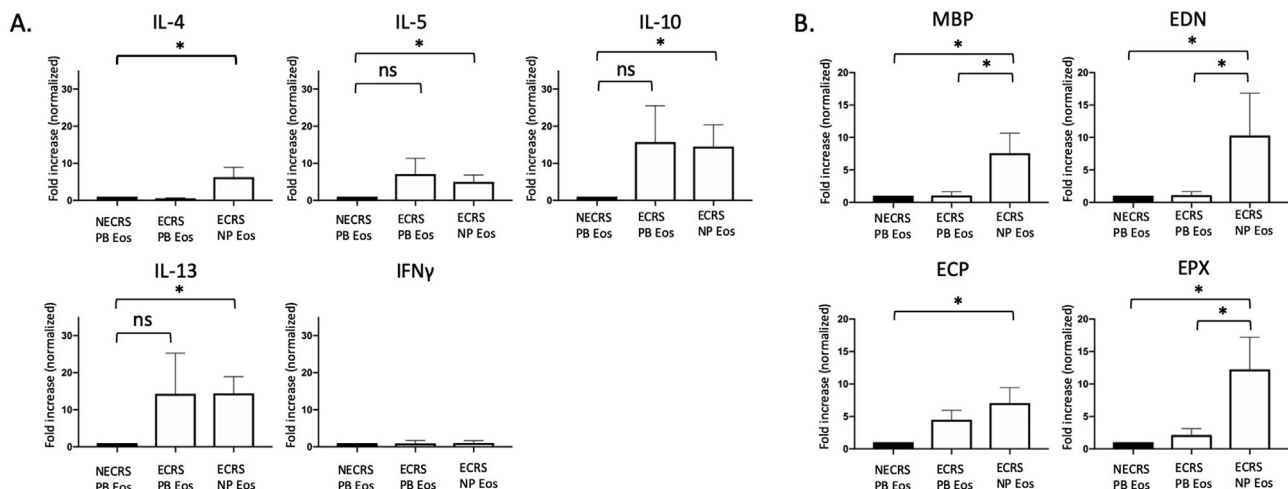


Fig 2. mRNA expression of cytokines and cytotoxic granules on purified eosinophils from peripheral blood (PB) and nasal polyps (NP). **(A)** Quantitative PCR analysis of IL-4, IL-5, IL-10, IL13, and IFN- γ mRNA expression levels on purified eosinophils from patients with ECRS and controls. **(B)** Quantitative PCR analysis of MBP, EDN, ECP, and EPX mRNA expression levels on purified eosinophils from patients with ECRS and controls. PB Eos, Eosinophils from peripheral blood; NP Eos, eosinophils from nasal polyp. Relative gene expression levels were calculated by the comparative C_T method with PB of NECRS as the reference. We expressed data as means $\pm$ SEM (ECRS and controls are $n = 8, 5$). P values determined by the Mann–Whitney U-test (* $P < 0.05$).

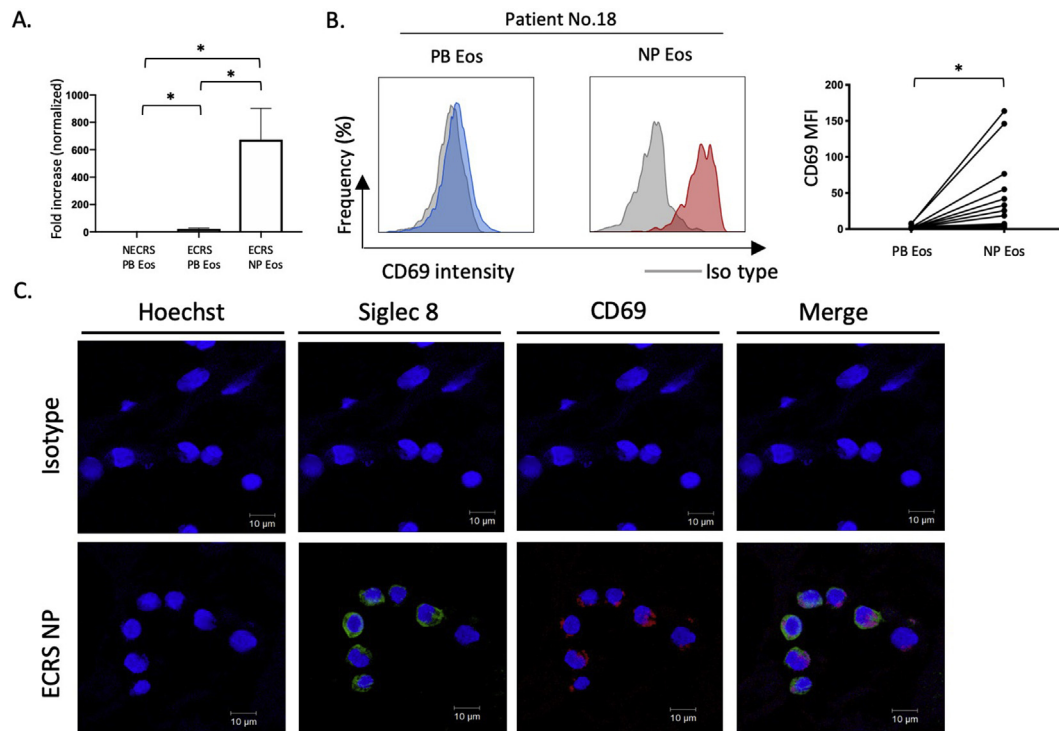


Fig 3. Eosinophils infiltrating the nasal polyps of patients with ECRS were activated. (A) Quantitative PCR analysis of CD69 mRNA expression levels from peripheral blood (PB) eosinophils and nasal polyp (NP) eosinophils from patients with ECRS. Relative gene expression levels were calculated by the comparative C_T method with PB of controls as the reference. P values determined by the Mann–Whitney U-test (* $P < 0.05$, NECRS and ECRS are $n = 5, 8$). (B) Histograms of CD69 expression on eosinophils in NP (red) and PB (blue) from patients with ECRS. Representative data from patient #18. The gray bar is isotype control. MFI of CD69 comparing eosinophils in PB to those in NPs. Result shown as MFI subtracting corresponding to the isotype control. P values determined by the Mann–Whitney U-test (* $P < 0.05$, $n = 12$). (C) Immunofluorescence images of fixed NP sections stained with antibodies against CD69 and Siglec8, and corresponding isotype controls. Representative data from patient #18. 400 $\times$, Scale bar 10 μ m.

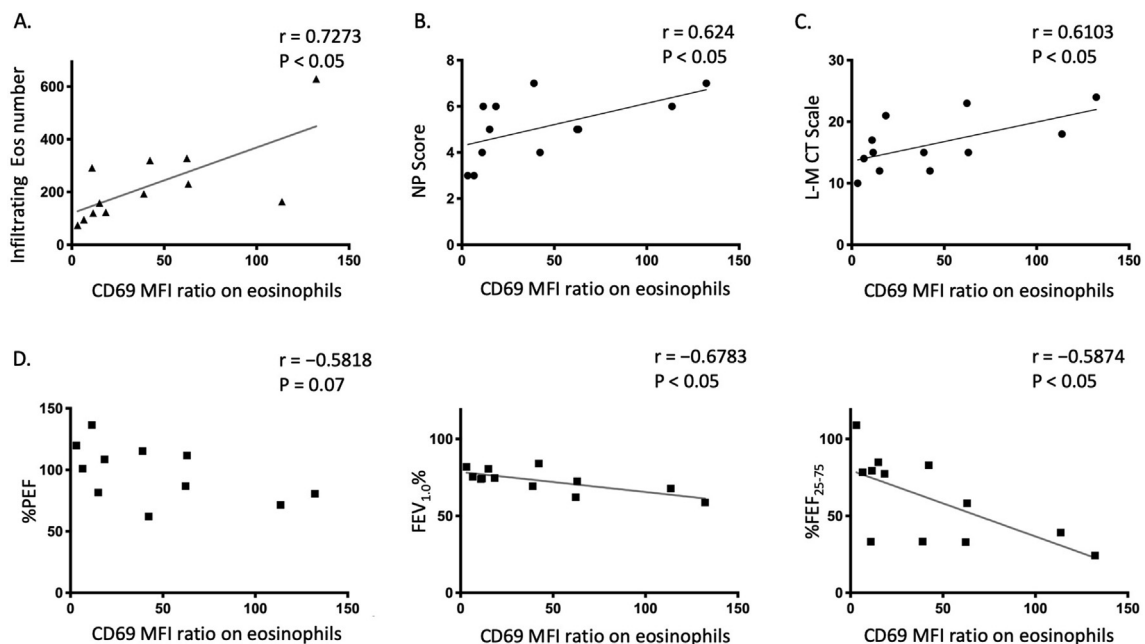


Fig 4. Eosinophil activation correlated with clinical findings and pulmonary function results. (A) Positive correlation between CD69 MFI ratio on eosinophils with numbers of infiltrating eosinophils to NPs of patients with ECRS ($P < 0.05$, $r = 0.7273$, $n = 12$). (B) Positive correlation between CD69 MFI ratio on eosinophils with nasal polyp scores ($P < 0.05$, $r = 0.624$, $n = 12$). (C) Positive correlation between CD69 MFI ratio on eosinophils with Lund–Mackay CT scale ($P < 0.05$, $r = 0.6103$, $n = 12$). (D) Negative correlation between CD69 MFI ratio on eosinophils with $FEV_{1.0}\%$ and $\%FEF_{25-75}$ (reflecting asthma severity), but not with $\%PEF$ ($FEV_{1.0}\%$: $P < 0.05$, $r = -0.6783$, $n = 12$, $\%FEF_{25-75}$: $P < 0.05$, $r = -0.5874$, $n = 12$). We expressed correlations as Spearman's rank correlation coefficients. PB Eos: Eosinophils from peripheral blood. NP Eos: eosinophils from nasal polyp.

in PB eosinophils from the same patients (Fig. 3B). We found similar results regarding CD69 expression on eosinophils using immunofluorescence (Fig. 3C).

Correlation between CD69 MFI on eosinophils and clinical findings

We found that the CD69 MFI ratio on eosinophils was correlated with the number of eosinophils infiltrating NPs (Fig. 4A). We also found similar results in terms of the NP score (Fig. 4B) and sinus CT findings as measured by the Lund–Mackay scale (Fig. 4C). Moreover, we found that the CD69 MFI ratio on eosinophils was negatively correlated with pulmonary functions such as FEV_{1.0}% and %FEF_{25–75} but not %PEF (Fig. 4D). By contrast, we found no significant correlations between CD69 MFI ratios on T cells and clinical findings (Supplementary Fig. 5). In addition, we found the number of infiltrating eosinophils to NPs and clinical findings correlated negatively with the %FEF_{25–75} (Supplementary Fig. 6).

Functional role of CD69 signaling in mouse eosinophils

To investigate the functional role of CD69 signaling, we analyzed EPX release from mouse eosinophils following cross-linking to CD69, since obtaining sufficient numbers of human eosinophils during the protein assays is difficult. We found CD69 ($6.1\% \pm 0.3\%$) on mouse eosinophils purified from splenocytes (Fig. 5A), but not on PB eosinophils. Moreover, cross-linking of CD69 to mouse eosinophils from spleen induced EPX release in a dose dependent manner (Fig. 5B).

Discussion

In this study, we found significantly higher mRNA expressions of the Treg-associated cytokine (IL-10) and Th2 cytokines (IL-4, IL-5, and IL-13) in both UT and NP tissues from patients with ECRS compared with the same expressions in controls. In addition, we observed increased expression of the Th1 cytokine (IFN γ) in 4 by 12 patients with ECRS, compared with NECRS. As demonstrated by our results, Tomassen *et al.* used a cluster analysis of biomarkers to show that CRS is a heterogeneous inflammatory disease, and they suggested that patients tend to have endotypes corresponding to diverse inflammatory profiles containing Th1 and Th2 cytokines.²⁶

To better characterize eosinophils into the inflamed sites, we also developed a purification protocol using a cell sorter following a density gradient technique. Our protocol resulted in a 4-fold increase in the number of CD45⁺ cells compared to the number obtained after the conventional protocol, with comparable cell viabilities. We found only one other study demonstrating eosinophil purification from NPs in patients with ECRS: A report by Miyake *et al.*, in which authors directly purified eosinophils from dissociated cells from NPs using a cell sorter without density gradient technique. However, they did not mention the total corrected cell numbers or the cell viability.²⁷

Next, we also found high mRNA expression of cytokines in the purified eosinophils from NPs in ECRS patients, supporting the existence of a difference in cytokine expression in the tissues compared with the expressions in controls. These data suggest that eosinophils may be one of the sources of cytokines in ECRS patients as well as ILCs.²⁸ Moreover, we found a significant difference in mRNA levels of the eosinophil-derived cytotoxic granules (MBP, EDN, and EPX) between eosinophils in the PB and NP from patients with ECRS. Although the cytokine levels between these groups were similar, higher levels of cytotoxic granules indicate eosinophil activation, since eosinophil-specific granules are associated with airway remodeling during the allergic disorders.²⁹ However, Miyata reported similar granule proteins (EDN, ECP, and EXP) contained in eosinophils from both NPs and PB.²⁷ This discrepancy between mRNA and protein in eosinophils may result from type of eosinophil in each experiment. Eosinophils come in two different types (hypodense and normodense eosinophils), the former and later reflect released and contained rich granules, respectively.³⁰ Since the ratio of hypodense eosinophils (reflecting activated eosinophils) exists dominantly during allergic inflammations,^{31–33} it is possible that the protein of granules between these eosinophils be similar. By contrast, our results, showing high mRNA expressions of these cytotoxic granules in the tissue eosinophils, suggests hypodense but not normodense eosinophils, reflecting activation.

Interestingly, we also found over 30-fold increase in CD69 expression on eosinophils between NPs and PB from ECRS. Similarly, we found upregulated CD69 protein expression. Thus, the degree of CD69 expression on eosinophils may be used as a biomarker for eosinophilic inflammation. Then, we also investigated the association between the CD69 MFI ratio on eosinophils

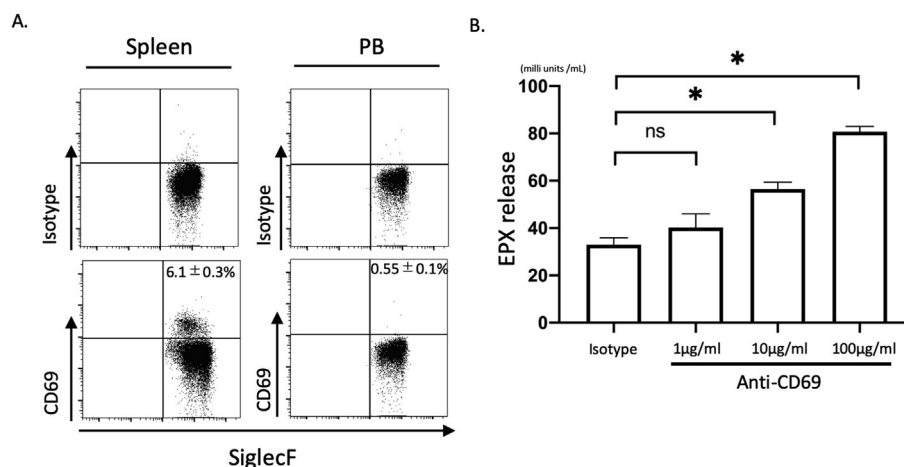


Figure 5. CD69 expression and EPX release by cross-linking of CD69. (A) Expression of CD69 on eosinophils purified from mouse spleen IL-5 Tg mouse and peripheral blood eosinophils. (B) Indicates EPX release after cross-linking of CD69 on mouse eosinophils purified from mouse spleen IL-5 Tg (n = 4). We expressed data as means $\pm$ SEM. P values determined by the Mann–Whitney U-test (*P < 0.05).

and the severity of symptoms among patients with ECRS having asthma, and we found that expression of CD69 (as an activation marker) in eosinophils from NPs was positively correlated with the NP score and sinus CT findings, and negatively correlated with pulmonary function (as measured by FEV_{1.0}% and %FEF_{25–75}). By contrast, we could not find any correlation between the CD69 MFI ratio on T cells and these clinical findings. In ECRS, FEV_{1.0}% is a noninvasive marker of airway remodeling during asthma,³⁴ and %FEF_{25–75} is associated with bronchial hyperresponsiveness in asthma.^{35,36} Studies reported that the FEV_{1.0}% and %FEF_{25–75}, but not the %PEF, were lower in patients with ECRS than in controls.^{37,38} Therefore, CD69 expression on the eosinophils (but not in T cells isolated from the NP of patients with ECRS) is a potential biomarker for the severity of patients with ECRS.

Moreover, we found EPX release after cross-linking of CD69 onto mouse eosinophils, suggesting that the signaling contributes to development of remodeling during the eosinophilic airway inflammation. By contrast, Walsh *et al.* reported that CD69 signaling induced apoptosis, but not EPX release.¹⁸ The discrepancies between their and our results could be due to differing incubation times; they analyzed results after 48 h and we after 2 h. Hosokawa *et al.* showed that airway inflammation and airway hyper-responsiveness (AHR) were diminished in CD69 deficient mice, and systemic treatment with anti-CD69 antibody in an asthma mouse model improved symptoms compared with treatment in naïve animals and treatment with isotype control antibodies.³⁹ These findings suggest that CD69 plays a role as a marker of activation, and that it regulates airway inflammation.⁴⁰

In conclusion, we found that activated eosinophils from the NPs of patients with ECRS expressed CD69, but not eosinophils from the PB of the same patients. In addition, CD69 expression on eosinophils was significantly correlated with the number of eosinophils infiltrating NPs, the NP score, sinus CT findings, and measurements of pulmonary function such as FEV_{1.0}% and %FEF_{25–75}. Moreover, we found that CD69-expressing eosinophils released EPX following activation (cross-linking to CD69). Thus, our results suggest that CD69 is a biomarker for the severity of nasal obstructions and for the degree of pulmonary dysfunction as well as a novel therapeutic target for patients with ECRS and asthma.

Acknowledgments

This research was supported by grants from the Japanese MEXT (Ministry of Education, Culture, Sports, Science and Technology: KAKENHI, no. 15K10793, no. 16K08951, no. 19K09860, and no. 19K18749); the Fund of Academic Society for Research in Otolaryngology, Kansai Medical University; a research grant D2 from Kansai Medical University; The branding program as a world-leading research university on intractable immune and allergic diseases. The authors would like to thank Enago (www.enago.jp) for the English language review.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2019.11.002>.

Conflict of interest

The authors have no conflict of interest to declare.

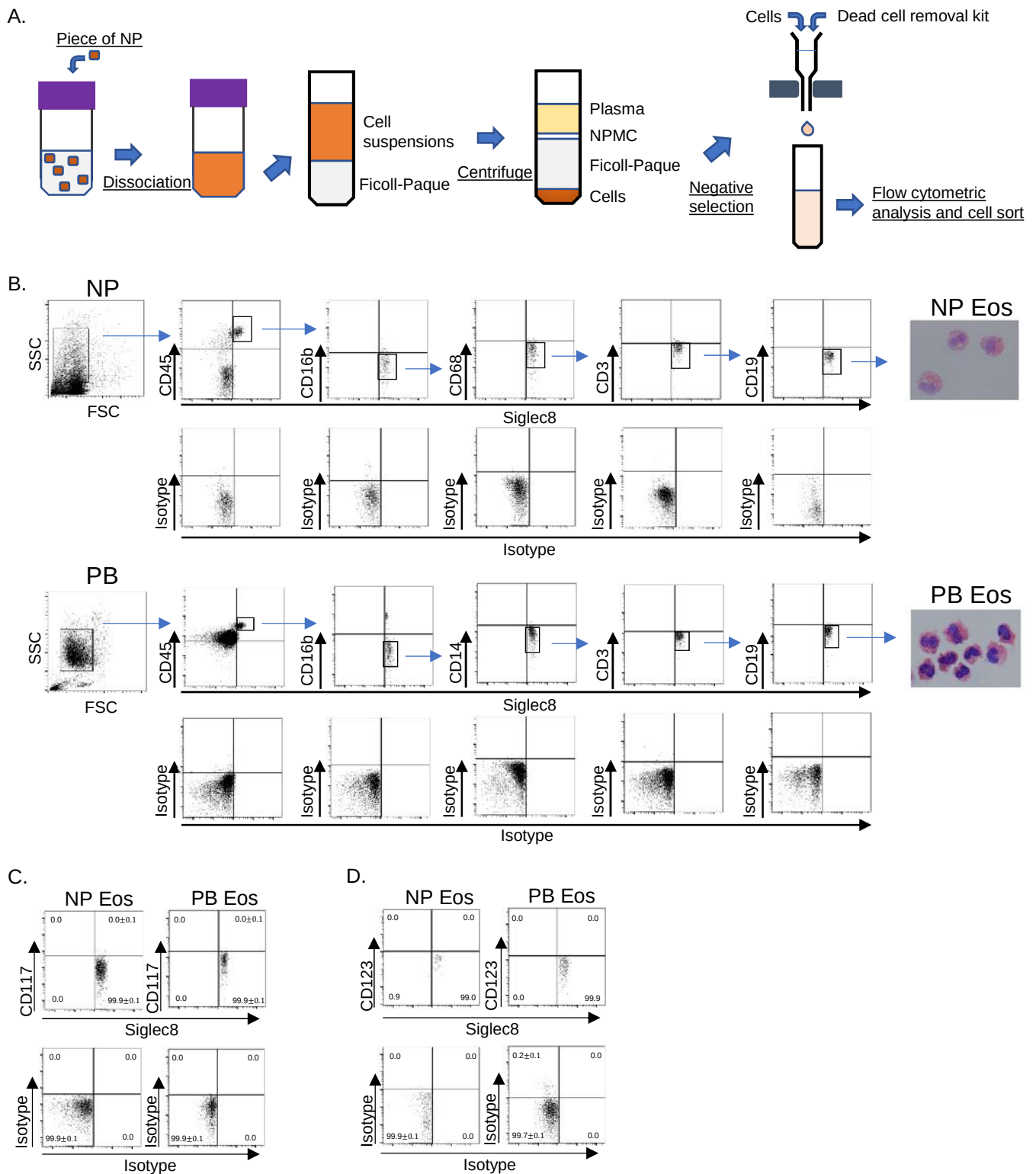
Author's contributions

Conceived and designed the experiments: YY, and AK. Performed the experiments: YY, YK, HO, DVB, MA, HI, SK, and AK. Analyzed the data: YY, HO, and AK. Wrote the paper: YY, and AK. All authors read and approved the final manuscript.

References

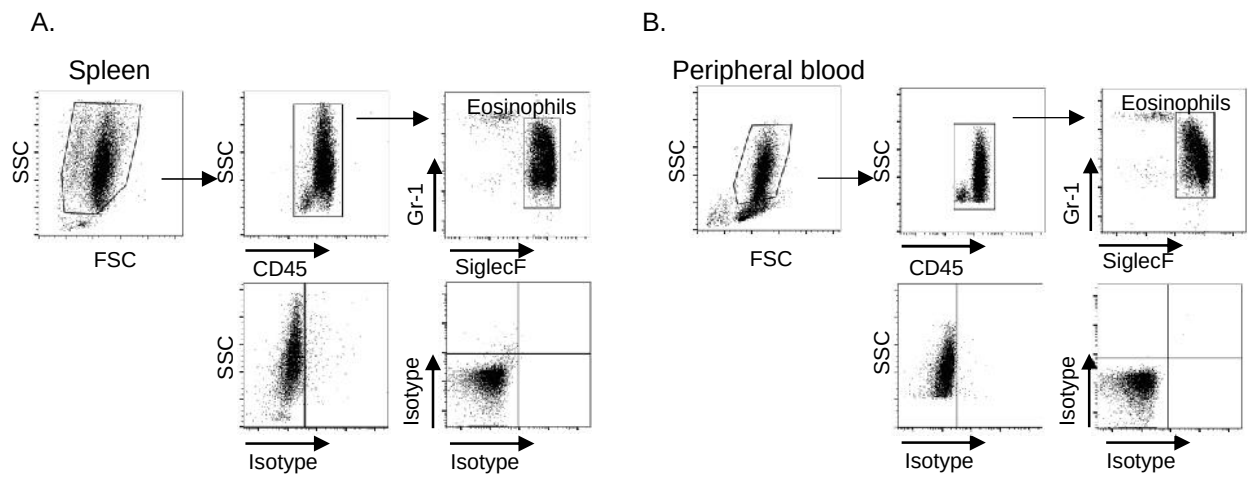
- Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. European position paper on rhinosinusitis and nasal polyps 2012. *Rhinology* 2012;**23**:3 (preceding table of contents, 1–298).
- Wang ET, Zheng Y, Liu PF, Guo LJ. Eosinophilic chronic rhinosinusitis in East Asians. *World J Clin Cases* 2014;**2**:873–82.
- Tokunaga T, Sakashita M, Haruna T, Asaka D, Takeno S, Ikeda H, et al. Novel scoring system and algorithm for classifying chronic rhinosinusitis: the JESREC Study. *Allergy* 2015;**70**:995–1003.
- Kobayashi Y, Asako M, Ooka H, Kanda A, Tomoda K, Yasuba H. Residual exhaled nitric oxide elevation in asthmatics is associated with eosinophilic chronic rhinosinusitis. *J Asthma* 2015;**52**:1060–4.
- Amelink M, de Groot JC, de Nijs SB, Lutter R, Zwirderman AH, Sterk PJ, et al. Severe adult-onset asthma: a distinct phenotype. *J Allergy Clin Immunol* 2013;**132**:336–41.
- Kanda A, Kobayashi Y, Asako M, Tomoda K, Kawauchi H, Iwai H. Regulation of interaction between the upper and lower airways in united airway disease. *Med Sci* 2019;**7**. <https://doi.org/10.3390/medsci7020027>.
- Koyama T, Kariya S, Sato Y, Gion Y, Higaki T, Haruna T, et al. Significance of IgG4-positive cells in severe eosinophilic chronic rhinosinusitis. *Allergol Int* 2018;**68**:216–24.
- Kobayashi Y, Yasuba H, Asako M, Yamamoto T, Takano H, Tomoda K, et al. HFA-BDP metered-dose inhaler exhaled through the nose improves eosinophilic chronic rhinosinusitis with bronchial asthma: a Blinded, Placebo-controlled Study. *Front Immunol* 2018;**9**:2192.
- Hogan SP, Rosenberg HF, Moqbel R, Phipps S, Foster PS, Lacy P, et al. Eosinophils: biological properties and role in health and disease. *Clin Exp Allergy* 2008;**38**:709–50.
- Maeda Y, Tsuda T, Takeda Y, Koyama S, Hayama Y, Nojima S, et al. SEMA4A promotes eosinophil survival and contributes to eosinophil-mediated allergic diseases. *Allergol Int* 2019;**68**:274–6.
- Noyama Y, Okano M, Fujiwara T, Kariya S, Higaki T, Haruna T, et al. IL-22/IL-22R1 signaling regulates the pathophysiology of chronic rhinosinusitis with nasal polyps via alteration of MUC1 expression. *Allergol Int* 2017;**66**:42–51.
- Shah SA, Ishinaga H, Takeuchi K. Pathogenesis of eosinophilic chronic rhinosinusitis. *J Inflamm* 2016;**13**:11.
- Johansson MW. Activation states of blood eosinophils in asthma. *Clin Exp Allergy* 2014;**44**:482–98.
- Cibrian D, Sanchez-Madrid F. CD69: from activation marker to metabolic gatekeeper. *Eur J Immunol* 2017;**47**:946–53.
- Martin P, Gomez M, Lamana A, Matesanz Marin A, Cortes JR, Ramirez-Huesca M, et al. The leukocyte activation antigen CD69 limits allergic asthma and skin contact hypersensitivity. *J Allergy Clin Immunol* 2010;**126**:355–65. 65.e1–3.
- Julius P, Luttmann W, Knoechel B, Kroegel C, Matthys H, Virchow Jr JC. CD69 surface expression on human lung eosinophils after segmental allergen provocation. *Eur Respir J* 1999;**13**:1253–9.
- Hartnell A, Robinson DS, Kay AB, Wardlaw AJ. CD69 is expressed by human eosinophils activated in vivo in asthma and in vitro by cytokines. *Immunology* 1993;**80**:281–6.
- Walsh GM, Williamson ML, Symon FA, Willars GB, Wardlaw AJ. Ligation of CD69 induces apoptosis and cell death in human eosinophils cultured with granulocyte-macrophage colony-stimulating factor. *Blood* 1996;**87**:2815–21.
- Kimura MY, Hayashizaki K, Tokoyoda K, Takamura S, Motohashi S, Nakayama T. Crucial role for CD69 in allergic inflammatory responses: CD69-Myl9 system in the pathogenesis of airway inflammation. *Immunol Rev* 2017;**278**:87–100.
- Hayashizaki K, Kimura MY, Tokoyoda K, Hosokawa H, Shinoda K, Hirahara K, et al. Myosin light chains 9 and 12 are functional ligands for CD69 that regulate airway inflammation. *Sci Immunol* 2016;**1**:eaaf9154.
- Fujieda S, Imoto Y, Kato Y, Ninomiya T, Tokunaga T, Tsutsumiuchi T, et al. Eosinophilic chronic rhinosinusitis. *Allergol Int* 2019;**68**:403–12.
- Meltzer EO, Hamilos DL, Hadley JA, Lanza DC, Marple BF, Nicklas RA, et al. Rhinosinusitis: developing guidance for clinical trials. *J Allergy Clin Immunol* 2006;**118**:S17–61.
- Lund VJ, Mackay IS. Staging in rhinosinusitis. *Rhinology* 1993;**31**:183–4.
- Marquez ME, Deglesne PA, Suarez G, Romano E. MFI ratio estimation of ZAP-70 in B-CLL by flow cytometry can be improved by considering the isotype-matched antibody signal. *Int J Lab Hematol* 2011;**33**:194–200.
- Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. *Nat Protoc* 2008;**3**:1101–8.
- Tomassen P, Vandeplas G, Van Zele T, Cardell LO, Arebro J, Olze H, et al. Inflammatory endotypes of chronic rhinosinusitis based on cluster analysis of biomarkers. *J Allergy Clin Immunol* 2016;**137**:1449–56.e4.
- Miyata J, Fukunaga K, Kawashima Y, Watanabe T, Saitoh A, Hiroaki T, et al. Dysregulated fatty acid metabolism in nasal polyp-derived eosinophils from patients with chronic rhinosinusitis. *Allergy* 2019;**74**:1113–24.
- Kato A. Immunopathology of chronic rhinosinusitis. *Allergol Int* 2015;**64**:121–30.
- Rosenberg HF, Dyer KD, Foster PS. Eosinophils: changing perspectives in health and disease. *Nat Rev Immunol* 2013;**13**:9–22.

30. Legrand F, Woerly G, Driss V, Capron M. Innate immune function of eosinophils: from antiparasite to antitumor cells. *Methods Mol Biol* 2008;**415**:215–40.
31. Conesa A, Tassinari P, Rivera H, De Sanctis JB, Bianco N, Aldrey O. Hypodense eosinophils: characterization of surface molecule expression. *Allergy Asthma Proc* 2002;**23**:117–24.
32. Frick WE, Sedgwick JB, Busse WW. Hypodense eosinophils in allergic rhinitis. *J Allergy Clin Immunol* 1988;**82**:119–25.
33. Kuo HP, Yu TR, Yu CT. Hypodense eosinophil number relates to clinical severity, airway hyperresponsiveness and response to inhaled corticosteroids in asthmatic subjects. *Eur Respir J* 1994;**7**:1452–9.
34. Barisione G, Crimi E, Bartolini S, Saporiti R, Copello F, Pellegrino R, et al. How to interpret reduced forced expiratory volume in 1 s (FEV1)/vital capacity ratio with normal FEV1. *Eur Respir J* 2009;**33**:1396–402.
35. Alberts WM, Ferris MC, Brooks SM, Goldman AL. The FEF25–75% and the clinical diagnosis of asthma. *Ann Allergy* 1994;**73**:221–5.
36. Contoli M, Bousquet J, Fabbri LM, Magnussen H, Rabe KF, Siafakas NM, et al. The small airways and distal lung compartment in asthma and COPD: a time for reappraisal 2010;**65**:141–51.
37. Tanaka S, Hirota T, Kamijo A, Ishii H, Hatsushika K, Fujieda S, et al. Lung functions of Japanese patients with chronic rhinosinusitis who underwent endoscopic sinus surgery. *Allergol Int* 2014;**63**:27–35.
38. Uraguchi K, Kariya S, Makihara S, Okano M, Haruna T, Oka A, et al. Pulmonary function in patients with eosinophilic chronic rhinosinusitis. *Auris Nasus Larynx* 2018;**45**:476–81.
39. Miki-Hosokawa T, Hasegawa A, Iwamura C, Shinoda K, Tofukuji S, Watanabe Y, et al. CD69 controls the pathogenesis of allergic airway inflammation. *J Immunol* 2009;**183**:8203–15.
40. Weller PF, Spencer LA. Functions of tissue-resident eosinophils. *Nat Rev Immunol* 2017;**17**:746–60.



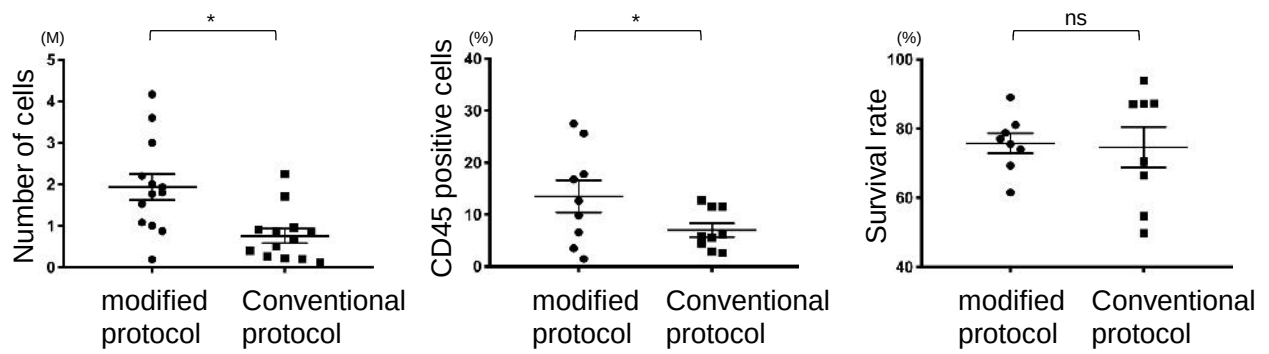
Supplementary Figure 1. Modified protocol to purify eosinophils from nasal polyps (NPs).

(A) We dissociated NPs using a human tumor dissociation kit. After dissociating the NPs, the buffer diluted cell samples (the cell suspensions) were separated using the Ficoll-Paque technique by centrifuge at 300 g for 30 min. The 2nd layer contained the NP mononuclear cells, and the lowest layer contained granulocytes and eosinophils. To eliminate dead cells, we resuspended the lower layer in a suspension of the Dead Cell Removal kit. We applied the cell suspensions onto magnetic columns, and collected the effluent containing the live cells, following cell sort. (B) We then used a flow cytometer to identify eosinophils using a gating strategy. We showed representative data from one of the patients with ECRS. Eosinophils from NPs were gated as cell populations with CD45+siglec8+CD68-CD16b-CD3-CD19-. Eosinophils from PB were gated as cell populations with CD45+siglec8+CD14-CD16b-CD3-CD19-. Magnification, 400 $\times$. (C)(D) Isolated eosinophils from NP and PB were stained using CD117, CD123 and corresponding isotypes. NP Eos: eosinophils from nasal polyp. PB Eos: eosinophils from peripheral blood.



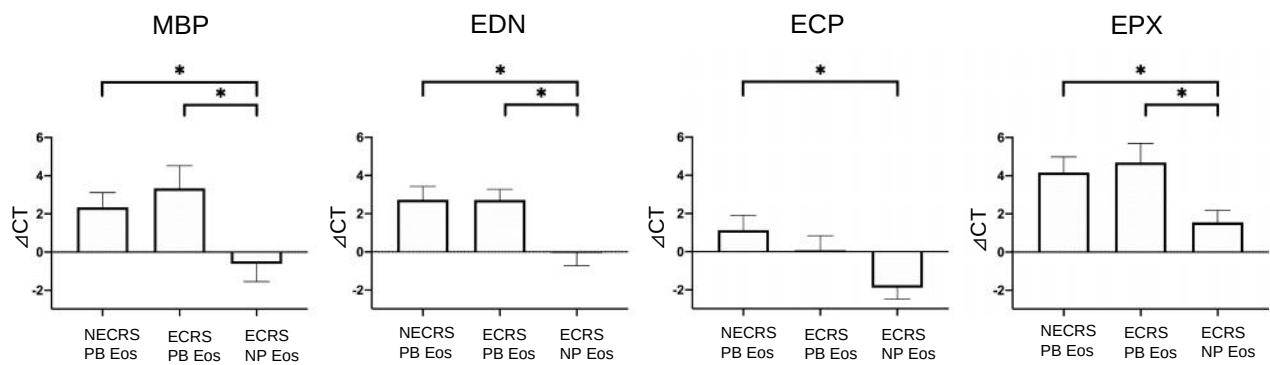
Supplementary Figure 2. Gating strategy for mouse eosinophils

(A)(B) Gating strategy for the identification of mouse eosinophils from spleen and PB using flow cytometry. Mouse eosinophils were gated as cell populations with $CD45^{+}siglecF^{+}Gr-1^{int}$.



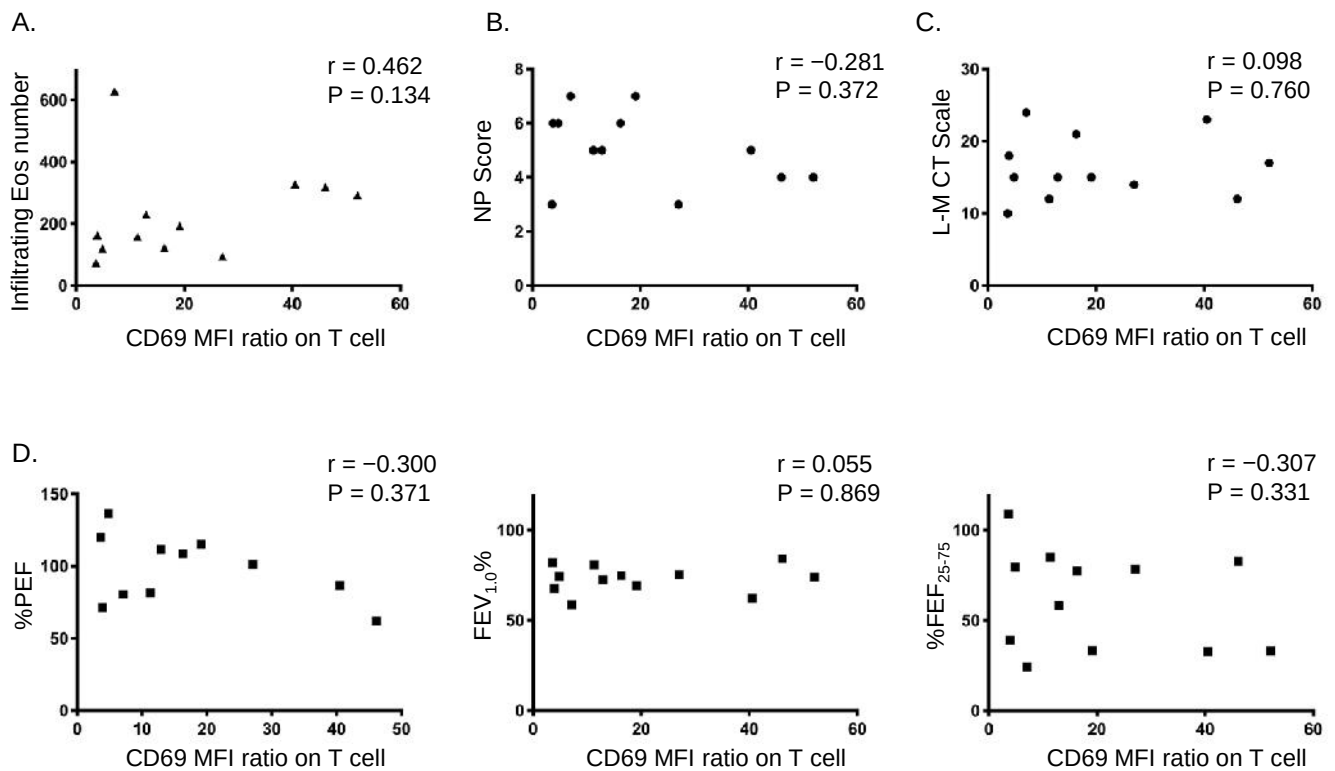
Supplementary Figure 3. Comparison between modified and conventional protocols

We compared both protocols in terms of dissociation cell numbers, CD45 positive cells, and cell survival rates. Data are expressed as means $\pm$ SEM (n = 12). P-values were determined by the Mann–Whitney U-test.



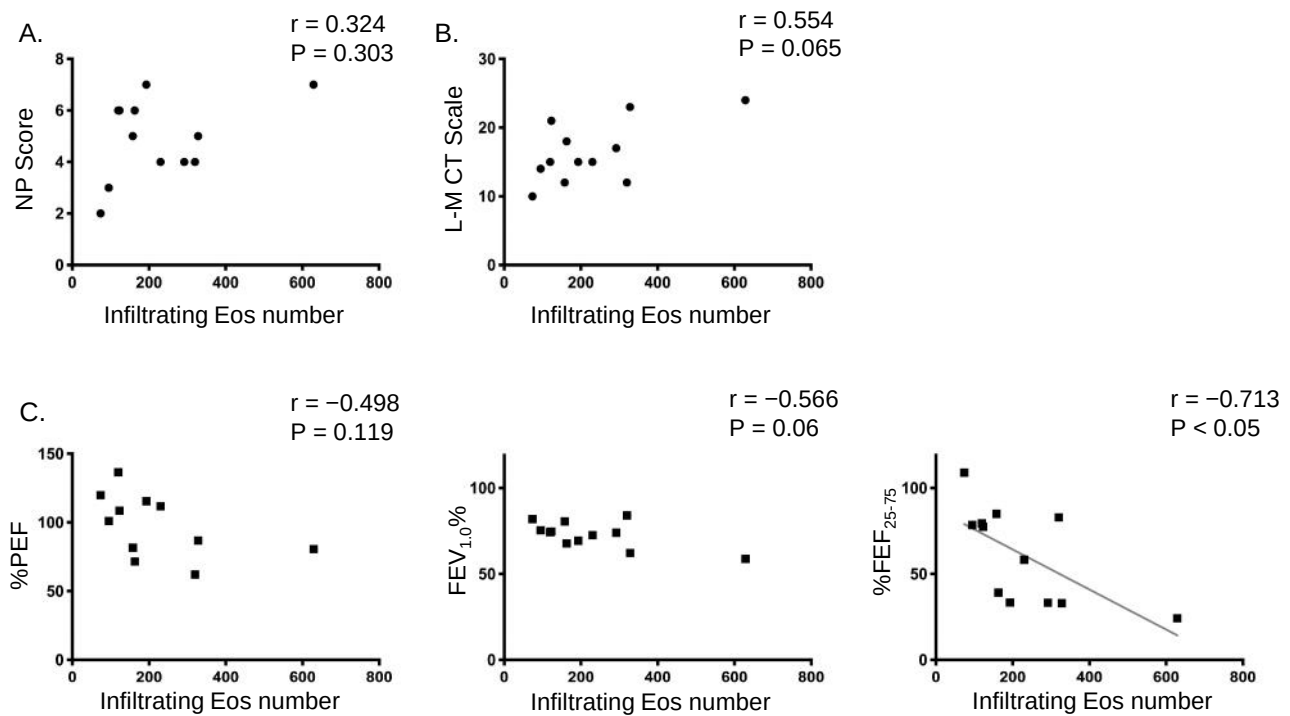
Supplementary Figure4. mRNA expression of cytotoxic granules on purified eosinophils from peripheral blood (PB) and nasal polyps (NP)

Delta Ct values distribution graphs of MBP, EDN, ECP, and EPX mRNA on purified eosinophils from patients with ECRS and controls. PB Eos: Eosinophils from peripheral blood. NP Eos: eosinophils from nasal polyp. We expressed data as means $\pm$ SEM (ECRS and controls are n = 8, 5). P values determined by the Mann–Whitney U-test (*P < 0.05).



Supplementary Figure 5. Comparison of T cell activation and clinical findings

(A-D) We found no correlations among CD69 MFI ratios on T cells, infiltrating number of eosinophils, and clinical findings. We assessed correlations using the Spearman's rank correlation coefficient.



Supplementary Figure 6. Correlation between number of infiltrating eosinophils and clinical findings

(A)(B) We found no correlation between the number of NP infiltrating eosinophils and the NP scores or sinus CT findings (as defined by Lund-Mackay CT scale). (C) The number of NP infiltrating eosinophils correlated with the %FEF₂₅₋₇₅. While other pulmonary functions lacked correlations. We applied Spearman's rank correlation coefficients to assess correlations.

Supplementary Methods

Title :

Increased CD69 expression on activated eosinophils in eosinophilic chronic rhinosinusitis correlates with clinical findings

Author:

Yasutaka Yun, Akira Kanda, Yoshiki Kobayashi, Dan Van Bui, Kensuke Suzuki, Shunsuke Sawada, Kazuyasu Baba, Masao Yagi, Mikiya Asako, Haruka Okazaki, Hiroki Ikeda, Shigeki Kawamura, Akihiko Nakamura, David Dombrowicz, Koichi Tomoda, and Hiroshi Iwai

Human and mouse eosinophil purification

For eosinophil purifications from nasal polyps (NP), as shown in the **Supplementary Figure 1A**, we used a dissociator from a tumor dissociation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) or a conventional protocol using collagenases, hyaluronidases, and DNases. These dissociated cells were separated by granulocytes including eosinophils and other cells using density gradient centrifugation with Ficoll-Paque (1.077 mg/dl). We removed dead cells from separated granulocytes in the bottom layer using the Dead cell removable kit (Miltenyi Biotec, Bergisch Gladbach,

Germany). Then, we isolated eosinophils using a cell sorter. Eosinophils from NP and PB were identified by gated $CD45^+Siglec8^+CD68^-CD16b^-CD3^-CD19^-$ and $CD45^+Siglec8^+CD14^-CD16b^-CD3^-CD19^-$, respectively (**Supplementary Fig. 1B**). Isolated eosinophils from NP and PB did not contain mast cells or basophils, respectively (**Supplementary Fig. 1C, D**). To assess viability, or survival rate, we stained cells with 7-AAD and Annexin V. T cells from NP and PB were identified by gated $CD45^+CD3^+Siglec8^-CD68^-CD16b^-CD19^-$ and $CD45^+CD3^+Siglec8^-CD14^-CD16b^-CD3^-CD19^-$, respectively.

To purify eosinophils from splenocytes of IL-5 Tg mice (hyper eosinophilic mice) we followed a published protocol¹. Briefly, we used negative selection with biotin-conjugated anti-CD19, anti-CD90.2, and anti-CD8a antibodies (BD PharMingen, CA, U.S.A) and anti-biotin-conjugated magnetic beads. Purity was greater than 95%.

Immunofluorescence staining

NPs and UTs were fixed in 10% formaldehyde and embedded in paraffin. Sections were sliced at 4- μ m thicknesses. For antigen retrieval, sections were activated in Histo VT One solution (Nacalai Tesque, Kyoto, Japan) for 15 min at 121°C. Sections were then incubated with a blocking solution of phosphate-buffered saline containing 10% Goat serum (Sigma-Aldrich, Miss, U.S.A) and 0.5% tween-20 (Sigma-Aldrich, Miss, U.S.A)

for 1 hour at room temperature.

qPCR

mRNA was extracted using RNeasy Micro Kit (Qiagen, Hilden, Germany) or PureLink™ RNA Micro Kit (Invitrogen, CA, U.S.A). We performed reverse transcription to the first-strand cDNA using PrimeScript RT Master Mix (Takara, Shiga, Japan), and performed quantitative PCRs (qPCRs) using Rotor-Gene SYBR Green PCR Kit (Qiagen, Hilden, Germany) on a Rotor-Gene Q HRM (Qiagen, Hilden, Germany).

Supplementary Tables

Supplementary Table 1. Detail character information of NECRS and ECRS

No.	Age	sex	Phenotype	Tissue Eos (/HPF)	Eos (%)	Blood Eos (/μl)	OCS	INCS
1	21	M	NECRS	NA	6.0	468	-	+
2	49	M	NECRS	NA	2.9	240.7	-	+
3	67	F	NECRS	NA	5.4	297	-	+
4	49	F	NECRS	NA	4.7	408.9	-	+
5	62	M	NECRS	NA	3.1	161.2	-	+
6	70	F	NECRS	NA	1.3	41.6	-	+
7	31	F	NECRS	NA	7.4	325.6	-	+
8	15	F	NECRS	NA	0.5	21	-	+
9	73	M	ECRS	158	7.9	402.9	-	+
10	68	M	ECRS	120	3.0	123	-	+
11	51	F	ECRS	163	4.8	211.2	-	+
12	62	M	ECRS	320	4.6	211.6	-	+
13	40	F	ECRS	292	4.8	321.6	-	+
14	46	M	ECRS	123	10.3	700.4	-	+
15	70	M	ECRS	629	11.3	542.4	-	+
16	66	M	ECRS	328	9.6	662.4	-	+
17	68	M	ECRS	230	5.5	258.5	-	+
18	51	F	ECRS	193	8.3	581	-	+
19	56	M	ECRS	95	5.8	330.6	-	-
20	53	M	ECRS	74	3.1	151.9	-	+

NECRS: non-eosinophilic chronic rhinosinusitis, ECRS: eosinophilic chronic

rhinosinusitis, HPF: high power field, OCS: oral corticosteroids, INCS: intranasal

corticosteroids, NP: nasal polyp, Eos: eosinophils, NA: not applicable.

Supplementary Table 2. List of antibodies

Flow cytometric analysis	Clone	Supplier	Dilution	Isotype
Antibody				
mouse anti-human CD45 FITC	HI30	Invitrogen	1:200	mouse IgG1
mouse anti-human CD125 PE	A14	BD Biosciences	1:200	mouse IgG1
mouse anti-human CD68 PerCP	Y1/82A	BioLegend	1:200	mouse IgG2b
mouse anti-human CD14 PerCP	HCD14	BioLegend	1:200	mouse IgG1
mouse anti-human CD69 PE/Cy7	FN50	BioLegend	1:200	mouse IgG1
mouse anti-human Siglec8 APC	7C9	BioLegend	1:200	mouse IgG1
mouse anti-human CD16b APC-Vio770	REA589	Miltenyi Biotec	1:200	human IgG1
mouse anti-human CD3 BV421	UCHT1	BD Biosciences	1:200	mouse IgG1
mouse anti-human CD19 BV510	5E8	BD Biosciences	1:200	mouse IgG2b
Hamster anti-mouse CD69 PE	H1.2F3	BD Pharmingen	1:100	Ar Hamster IgG1
Rat anti-mouse Gr-1 PerCP	RB6-8C5	BD Pharmingen	1:200	Rat IgG2a
Rat anti-mouse SiglecF Alexa Fluor 647	E50-2440	BD Pharmingen	1:200	Rat IgG2a
Rat anti-mouse CD45 V500	30-F11	BD Horizon	1:200	Rat IgG2b
Immunofluorescence staining				
1st antibody				
Rabbit anti-human Siglec8	polyclonal	GeneTex	1:400	Rabbit IgG
mouse anti-human CD69	FN50	Biolegend	1:400	mouse IgG
2st antibody				
conjugated with CF TM 488A Goat Anti-rabbit IgG		Biotium	1:200	
conjugated with CF TM 647A Goat Anti-mouse IgG		Biotium	1:200	

Supplementary Table 3. Primer List

	Forward	Reverse
IL-4	5'-GCAGCTGATCCGATTCCTGA-3'	5'-CTCTGGTTGGCTTCCTTCAC-3'
IL-5	5'-AACTGTGCAAGGGGGTACTG-3'	5'-TCTCCGTCTTTCTTCTCCACA-3'
IL-10	5'-GAGATGCCTTCAGCAGAGTGAA-3'	5'-ATCTCAGACAAGGCTTGGCAAC-3'
IL-13	5'-CTGACAGCTGGCATGTACTGTG-3'	5'-TGTCTCGGACATGCAAGCTG-3'
IFN γ	5'-TTCAGCTCTGCATCGTTTTG-3'	5'-TCTTTTGGATGCTCTGGTCA-3'
MBP	5'-GAAAGATGGGGCTGTTGAGT-3'	5'-CTTCTCACCAGGAGGTAGCG-3'
EDN	5'-TCTCACAGGAGCTACAGCGCG-3'	5'-AACATGTTTGCTGGTGTCTGC-3'
ECP	5'-GCAGACAGACCAGGAAGGAG-3'	5'-GCAGACAGACCAGGAAGGAG-3'
EPX	5'-CCCGGTGGAATGGAGACAAA-3'	5'-TCGATGCCCCCTTCATACAC-3'
CD69	5'-CTGGACTTCAGCCCAAAATGC-3'	5'-TCAGTCCAACCCAGTGTTC -3'
GAPDH	5'-TTCACCACCATGGAGAAGGC-3'	5'-AGGAGGCATTGCTGATGATCT-3'

Reference

1. Kanda A, Driss V, Hornez N, Abdallah M, Roumier T, Abboud G, et al. Eosinophil-derived IFN-gamma induces airway hyperresponsiveness and lung inflammation in the absence of lymphocytes. *J Allergy Clin Immunol* 2009; 124:573-82, 82 e1-9.