

ORIGINAL ARTICLE

Rhinitis, Sinusitis, and Upper Airway Disease

A murine model of eosinophilic chronic rhinosinusitis using the topical application of a vitamin D3 analog

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Abstract

Background: Eosinophilic chronic rhinosinusitis (ECRS) is a chronic inflammatory disease, characterized by eosinophilic infiltration, T-helper type 2 (Th2-type) response, and olfactory dysfunction. A master regulator of Th2-type inflammation, thymic stromal lymphopoietin (TSLP), is important for basophil activation. TSLP-elicited basophils are a key factor in the pathogenesis of ECRS.

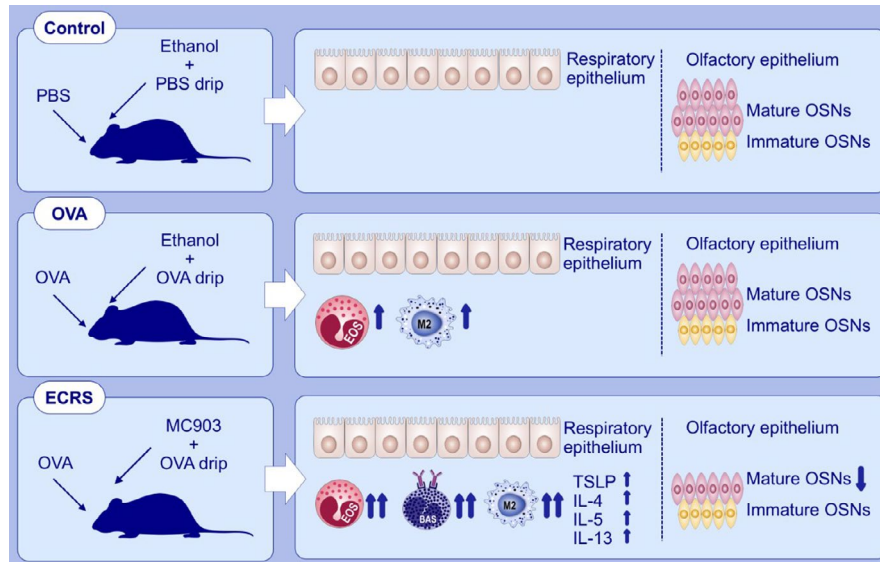
Methods: In order to elucidate the mechanisms of ECRS in humans, we aimed to establish a murine model of ECRS based on TSLP production in response to the topical application of MC903 (a vitamin D3 analog) and the subsequent TSLP-induced basophil activation. Histological analyses were performed to assess immune cell infiltration into the nasal mucosa and to explore the impact of eosinophilic inflammation on the olfactory epithelium. The status of Th2-type inflammation was evaluated by quantitative real-time PCR and enzyme-linked immunosorbent assay (ELISA).

Results: Eosinophils, basophils, and M2 macrophages increased significantly in the nasal mucosa of the mice treated with MC903 and ovalbumin (OVA), compared to those treated with OVA alone or the controls. Quantitative real-time PCR and ELISA revealed elevated expression of interleukin (IL)-4, IL-5, IL-13, TSLP, the chemokine CCL11, and CCL24 in the nasal mucosa of the ECRS mice. In parallel, thinned olfactory epithelium and decreased mature olfactory sensory neurons were observed in the ECRS mice.

Conclusions: Our model of ECRS displayed Th2-type inflammation in the sinonasal region, including both eosinophil infiltration and basophil infiltration. Additionally, olfactory epithelium turned out to be affected by eosinophilic inflammation. These features are consistent with the characteristics of the human ECRS.

KEYWORDS

basophil, eosinophilic chronic rhinosinusitis, olfactory epithelium, thymic stromal lymphopoietin



GRAPHICAL ABSTRACT

A murine model of eosinophilic chronic rhinosinusitis is established, in which skin treatment with vitamin D3 analog and ovalbumin (OVA) followed by intranasal OVA challenges cause severe eosinophilia in paranasal sinuses. Infiltration of basophils and M2 macrophages accompanied by increased levels of Th2 cytokines and TSLP in the nasal mucosa are demonstrated. Thinned olfactory epithelium and decreased mature olfactory sensory neurons are also observed.

1 | INTRODUCTION

Chronic rhinosinusitis (CRS) is one of the most prevalent chronic diseases, reportedly affecting approximately 12% of the general population.¹ It is defined as local inflammation of the sinonasal mucosa with symptoms including nasal congestion, olfactory disturbance, rhinorrhea, and facial pain that persist for at least 12 consecutive weeks.² Generally, CRS is divided into two subgroups based on endoscopic findings: CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP).³ In Europe and the United States, CRSwNP is characterized by T-helper type 2 (Th2-type) inflammation and eosinophil infiltration in nasal polyps.⁴ In East Asian countries, the extents of eosinophilia are variable, depending on which CRSwNP is divided into eosinophilic CRS (ECRS) and non-eosinophilic CRS (non-ECRS).⁵ ECRS is thought to be equivalent to CRSwNP in Europe and the United States.

In the Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis (JESREC) Study, a final diagnosis of ECRS is made by histological examination of nasal polyps under a microscope at 400 $\times$ (ocular lens, field number 22). When the mean number of eosinophils in three fields is 70 or more, a definite diagnosis of ECRS is established.⁶

ECRS is a multifactorial disease, whose pathogenesis has yet to be fully elucidated. Bachert's group demonstrated that nasal polyps contained Staphylococcal enterotoxin (SE)-specific IgE. They suggested that SE and SE-specific IgE were the pathogenic factors of ECRS.⁷ There are some murine models of ECRS in which SE, in combination

with ovalbumin (OVA) or house dust mite allergens, has been used as the inducer of eosinophilic inflammation.⁸⁻¹⁰ On the other hand, it is possible that an IgE-independent mechanism could also be associated with ECRS as reported recently. Thymic stromal lymphopoietin (TSLP) is a master regulator of Th2-type inflammation¹¹ and is associated with multiple allergic disorders including ECRS.¹²⁻¹⁵ The function of TSLP is not only activating dendritic cells and supporting IgE production but also promoting Th2-type inflammation by directly activating basophils¹⁶ and group 2 innate lymphoid cells (ILC2s).¹⁷ TSLP induces interleukin (IL)-5 and IL-13 production from ILC2s¹⁸ and IL-4 production from basophils.¹⁶ Moreover, basophil-derived IL-4 promotes ILC2 proliferation.¹⁹ From recent reports, it is possible that the TSLP-basophil axis might be involved in the pathogenesis of ECRS. Dogan et al²⁰ reported that the level of TSLP was elevated in the nasal tissue of ECRS patients. In addition, a single nucleotide polymorphism analysis showed that TSLP polymorphisms might be associated with ECRS.²¹ Furthermore, basophils have been reported to increase in the nasal polyps of ECRS patients.^{22,23} Recently, some reports showed that the topical application of MC903 (calcipotriol, an analog of vitamin D3) triggered keratinocytic TSLP production and basophil activation.^{18,24,25} A number of studies have employed the topical application of MC903 to develop murine models of various allergic diseases, including asthma, atopic dermatitis, and eosinophilic esophagitis.^{16,24,25}

In the present study, we concentrated on TSLP-elicited basophils and established a new murine model of ECRS, which is the first of its kind to focus on the TSLP-basophil axis.

2 | MATERIALS AND METHODS

2.1 | Mice

C57BL/6 male mice were purchased from CLEA Japan (Tokyo, Japan). All the mice were 8 weeks of age at the start of the experiment. They were housed in a temperature-controlled facility and maintained on a 12-hour light/dark cycle and were given with free access to diet and water. All procedures were approved by the University of Tokyo Animal Care Committee and conducted in accordance with the University of Tokyo guidelines for the handling and care of laboratory animals.

2.2 | Experimental protocol

The mice were divided into three groups, namely control, OVA, and ECRS. The procedure is depicted in Figure 1A. Briefly, the ECRS group mice were topically treated once daily with 2 nmol of MC903 (Tocris Bioscience) in 20 μ L of 100% ethanol along with 100 μ g of OVA in 20 μ L of phosphate-buffered saline (PBS) after tape tripping on the right ear for 14 days. Next, daily intranasal administration of 2400 μ g of OVA in 40 μ L of PBS was performed for 5 days. The OVA group received 100 μ g of OVA in 20 μ L of PBS together with 20 μ L of 100% ethanol without MC903 after tape tripping. This group was then challenged intranasally with OVA in a similar fashion to the ECRS group. The control group was given 20 μ L of PBS and 100% ethanol, followed by intranasal administration only with 40 μ L of PBS. All the mice were sacrificed at day 19.

2.3 | Histological analyses

Serial coronal sections (4 μ m thick) were cut at two levels: (a) anterior third of the level of naso-ocular distance, that is, one-third of

the distance from the nose tip to the anterior border of the eyes (Figure 1B); and (b) anterior border of the eyes (Figure 1C). To determine the degree of immune cell infiltration into the nasal mucosa, the number of infiltrated cells was manually counted, in the sections cut at the level of the anterior third of the naso-ocular distance. The number of immune cells per 200 μ m length of the nasal epithelium was counted in the following four areas: nasal septum, dorsal nasal concha, ventral nasal concha, and maxillary sinus,²⁶ which is shown in Figure 1B. Then, the mean cell count for each immune cell was calculated. To analyze any change in the olfactory epithelium (OE), we used the sections at the level of the anterior boarder of the eyes. The OE thickness and the number of OMP-positive cells per 100 μ m length were determined in three areas of the nasal septum: superior, middle, and inferior. Each area is shown in Figure 1C.

2.4 | Statistical analyses

Statistical analyses were conducted by SPSS statistical software (SPSS Inc). The Kruskal-Wallis ANOVA followed by the Dunn-Bonferroni test was used to assess the difference between the control, OVA, and ECRS groups. The Mann-Whitney *U* test was used for comparison between right side and left side. Data were plotted as means $\pm$ standard error. A *P* value of $<.05$ was considered significant.

Detailed materials and methods are fully described in Supporting Information of this article.

3 | RESULTS

3.1 | MC903 + OVA treatment induces nasal tissue eosinophilia

Eosinophil infiltration into the nasal mucosa was quantified by Sirius Red staining.²⁷ The numbers of eosinophils in the right side

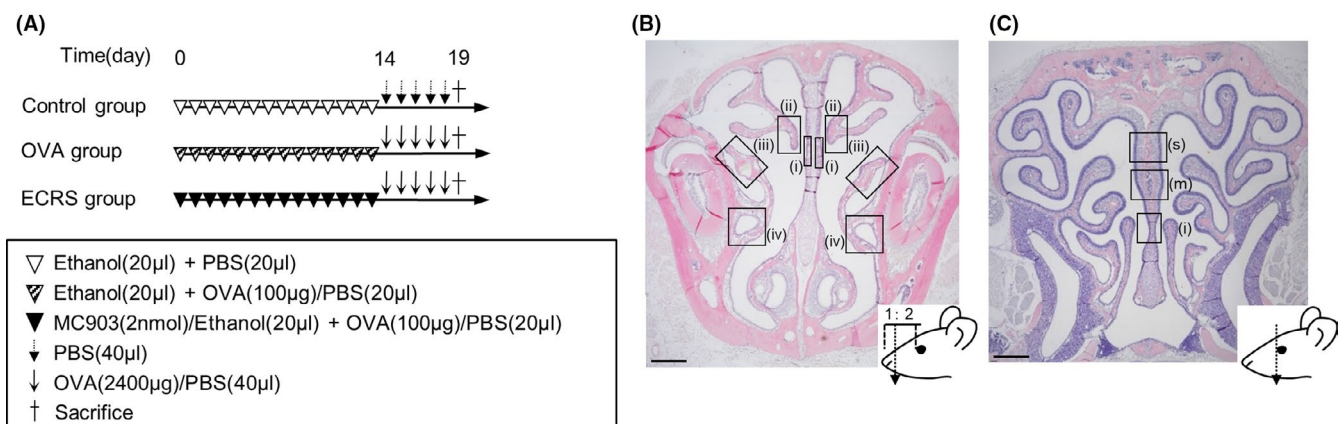


FIGURE 1 Protocol of the ECRS model and images for analysis. Three groups of mice were treated with either MC903 and OVA, OVA alone, or only vehicle (A). The images and levels of cutting positions for evaluation of immune cells and olfactory epithelium are shown in (B) and (C), respectively. The areas for cell count are the nasal septum (i), dorsal nasal concha (ii), ventral nasal concha (iii), and maxillary sinus (iv). The areas for evaluation of the olfactory epithelium are the (s) superior, (m) middle, and (i) inferior nasal septum. Scale bar = 500 μ m. ECRS, eosinophilic chronic rhinosinusitis; OVA, ovalbumin; PBS, phosphate-buffered saline

(OVA-treated side) of the nasal septum, dorsal nasal concha, ventral nasal concha, and maxillary sinus were individually calculated, totally averaged, and closely compared between the groups. Within each group, left-right differences in eosinophil count were also compared. Representative images of the dorsal nasal concha are shown in Figure 2A. Those of other areas are described in Figure S1A of this article. In the ECRS group, the number of eosinophils in each area and the average eosinophil count of the entire four areas increased significantly compared with the control group. Moreover, the eosinophil counts of all the four areas, averagely and individually, were meaningfully higher in the right compared with the left side in the ECRS group. Similarly, in the OVA group, eosinophil counts in the nasal septum, in the maxillary sinus, and on average appeared to be significantly higher in the right side vs the left side. These results are summarized in Figure 2B.

3.2 | Basophils are elevated in the nasal tissue of MC903 + OVA-treated mice

Immunostaining with anti-mcpt8 antibody was employed to identify infiltrated basophils,¹⁹ which were subsequently counted to draw comparisons between groups in the same manner as performed for eosinophils. Representative images of the dorsal nasal concha are shown in Figure 2C. Those of other areas are described in Figure S1B of this article. Compared with eosinophils, a lower number of basophils were observed in the nasal mucosa, which is consistent with the characteristics of nasal polyps in ECRS patients.²² The number of basophils in the nasal septum, in the dorsal nasal concha, in the ventral nasal concha, and on average were significantly elevated in the ECRS group compared with the control group and the OVA group. Left-right comparison of the number of basophils in the ECRS group showed that basophil counts, not only specifically in the dorsal and ventral nasal concha but also averagely, were significantly larger in the right side than the left side. These results are summarized in Figure 2D.

3.3 | Macrophages increase in the nasal tissue of MC903 + OVA-treated mice

Immunostaining with anti-F4/80 antibody was carried out to detect infiltrated macrophages,²⁸ which were then counted and compared between different groups in a similar fashion to eosinophils.

Representative images of the dorsal nasal concha are shown in Figure 2E. Those of other areas are described in Figure S1C of this article. In comparison with the control group, the ECRS group demonstrated a significantly greater number of macrophages in the four areas, both averagely and individually. The number of macrophages in the dorsal nasal concha was also significantly elevated in the ECRS group compared with the OVA group. Upon side comparison of the number of macrophages in the ECRS group and in the OVA group, average and individual macrophage counts of the four areas turned out to be significantly higher in the right side than the left side for both groups. These results are summarized in Figure 2F.

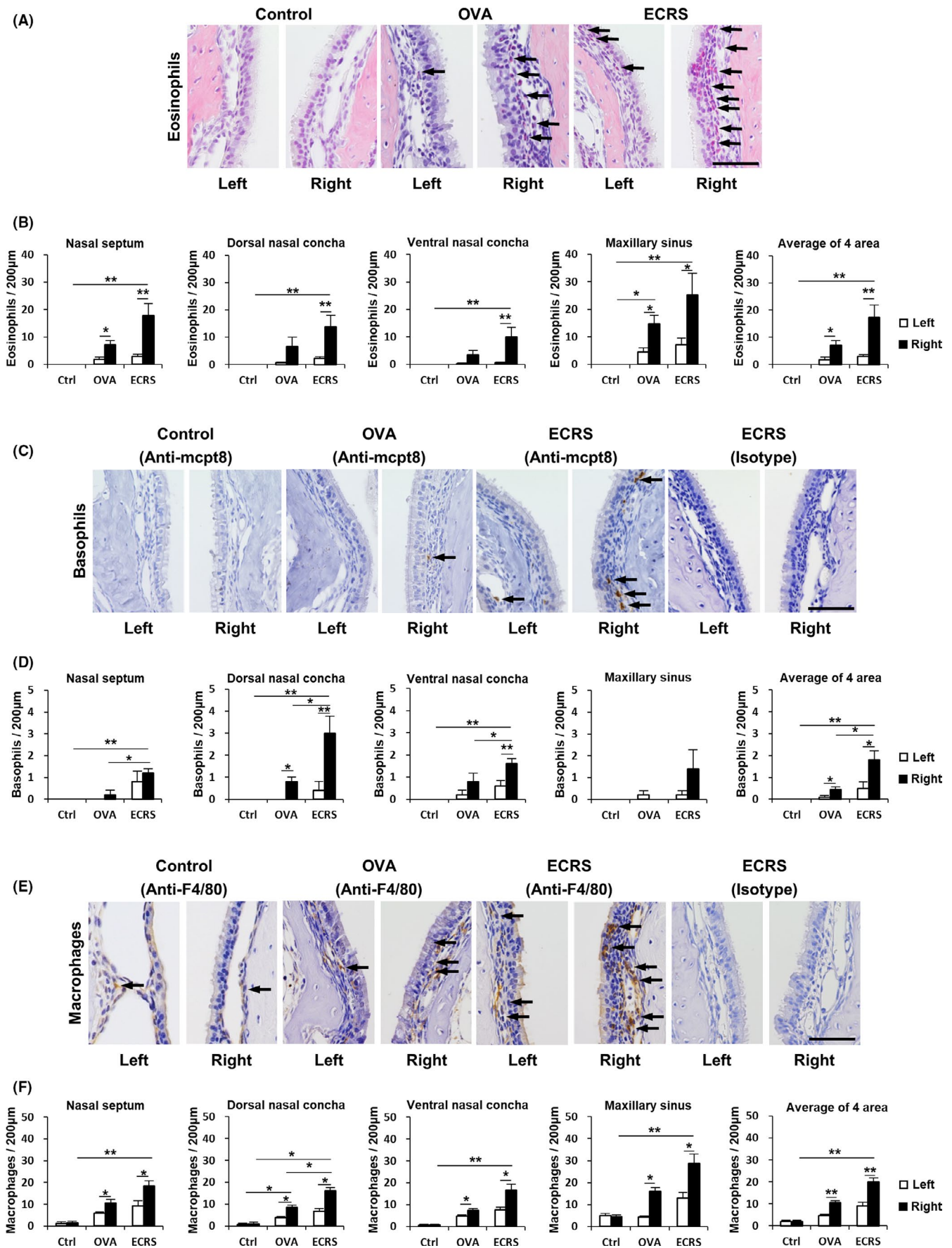
3.4 | M2 Macrophages are elevated in the nasal tissue of ECRS mice

M2 macrophage infiltration into the nasal mucosa was evaluated by immunofluorescence staining with anti-CD163 and anti-F4/80 antibody,²⁹ which were counted and compared between the groups in the same manner as performed for other immune cells. Representative images of the maxillary sinus are shown in Figure 3A. Images of other areas are presented in Figure S2 of this article. In comparison with the control group, the ECRS group demonstrated a significantly greater number of M2 macrophages in the four areas, both averagely and individually. The number of M2 macrophages in the nasal septum and on average was also significantly elevated in the ECRS group, compared with the OVA group. Upon side comparison of the number of macrophages in the ECRS group and in the OVA group, average and individual macrophage counts of the four areas turned out to be significantly higher in the right side than the left side for both groups. These results are summarized in Figure 3B.

3.5 | MC903 + OVA intranasal treatment induces Th2-type inflammation

Gene expression levels of Th2 cytokines, epithelial cytokines including TSLP, and eosinophil-recruiting chemokines in the nasal mucosa were analyzed by real-time quantitative PCR. The expression levels of *IL4* were significantly elevated in the ECRS group, compared with both the control and OVA groups. Furthermore, significantly higher expression levels of *IL5*, *IL13*, *TSLP*, *CCL11*, and *CCL24* were detected in the ECRS group than the control group. These results are summarized in Figure 4.

FIGURE 2 (A) Sirius red-stained sections, showing eosinophil infiltration into dorsal nasal concha of mice in—from left to right—the control, OVA, and ECRS groups (scale bar = 50 mm). (B) Comparison of eosinophil counts in the four described areas across the groups. (C) Immunostained or isotype control-stained sections, showing basophil infiltration into dorsal nasal concha of mice (scale bar = 50 mm). (D) Comparison of basophil counts in the four described areas among the groups. (E) Immunostained or isotype control-stained sections, showing macrophage infiltration into dorsal nasal concha of mice (scale bar = 50 mm). (F) Comparison of macrophage counts in the above-described areas among the groups. Statistical analysis was performed using the Kruskal-Wallis ANOVA followed by the Dunn-Bonferroni test, with $P < .05$ (*) and $P < .01$ (**) representing statistical significance. Error bars shown represent the standard error of the mean ($n = 5$). Data are representative of two independent experiments. Ctrl, control; OVA, ovalbumin; ECRS, eosinophilic chronic rhinosinusitis



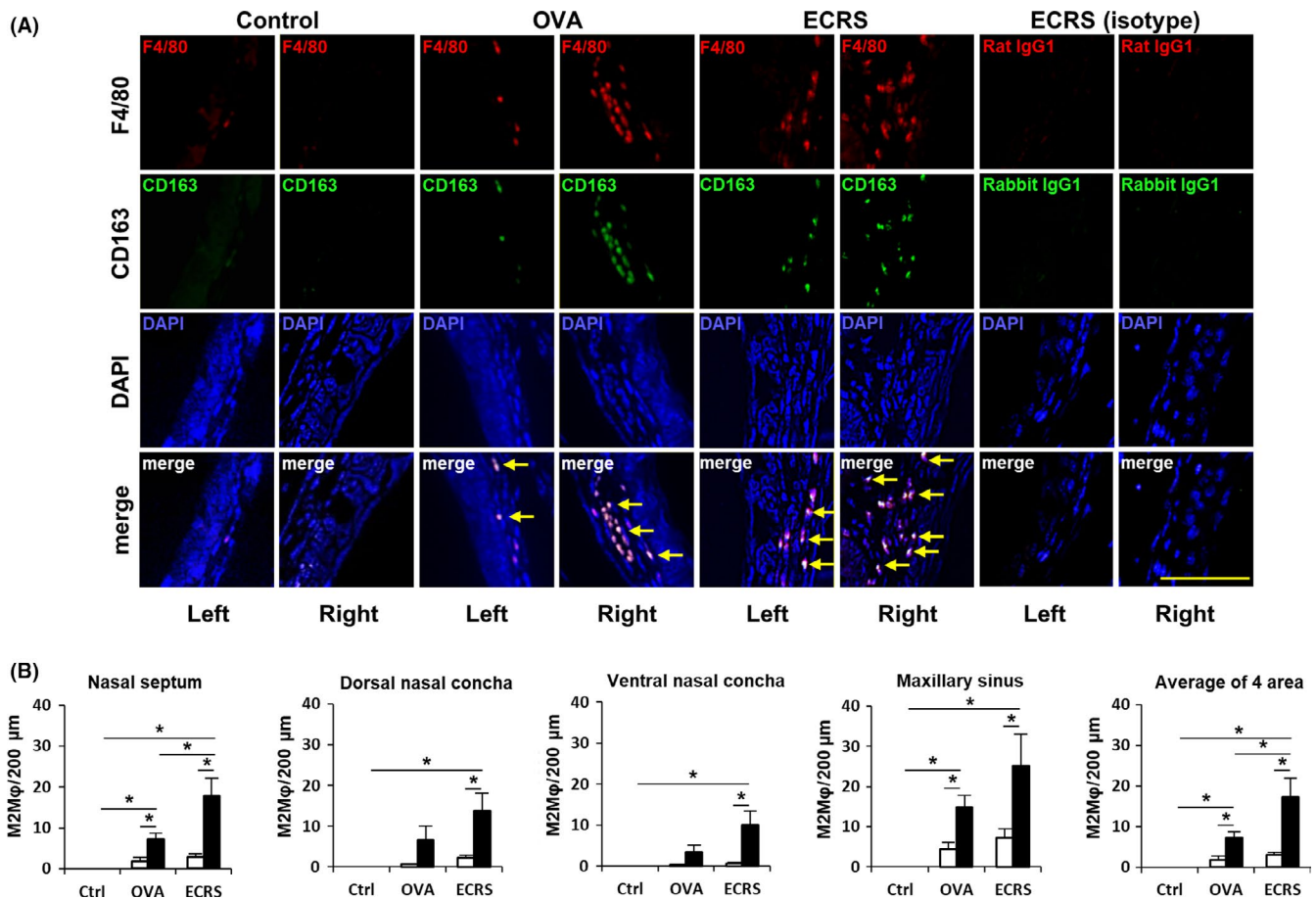


FIGURE 3 (A) Immunostained or isotype control-stained sections, showing M2 macrophage infiltration into maxillary sinus of mice (scale bar = 50 mm). (B) Comparison of M2 macrophage counts in the four described areas among the groups. Statistical analysis was performed using the Kruskal-Wallis ANOVA followed by the Dunn-Bonferroni test, with $P < .05$ (*) and $P < .01$ (**) representing statistical significance. Error bars shown represent the standard error of the mean. The number of animals per group was 5. Data are representative of two independent experiments. Ctrl, control; OVA, ovalbumin; ECRS, eosinophilic chronic rhinosinusitis

The protein levels of the cytokines and chemokines in the nasal mucosa were confirmed by enzyme-linked immunosorbent assay (ELISA). The levels of IL-4, IL-5, IL-13, TSLP, and CCL24 in the nasal mucosa were significantly elevated in the ECRS group, compared with the control group and the OVA group. In addition, significantly higher expression level of CCL11 was detected in the nasal mucosa of the ECRS group than those of the control group. These results are summarized in Figure 5A. To confirm that TSLP overproduced in the right ear leaked into blood circulation, the protein levels of TSLP in the serum (day 10 and day 19) and the right ear tissue were also evaluated by ELISA. The levels of TSLP in the serum and the right ear tissue were significantly elevated in the ECRS group, compared with both the control and OVA groups. These results are summarized in Figure 5B,C.

3.6 | MC903 + OVA treatment affects olfactory mucosa structure

The impact of treatment with MC903 and OVA on the OE was analyzed histologically. Upon examination of the right sides, a

significantly larger reduction in the thickness of the OE lining the superior and inferior areas and in the average OE thickness noticed in the ECRS group than the control and OVA groups. Left-right comparison within the ECRS group revealed that the thickness of the OE lining the superior and inferior areas as well as the average OE thickness significantly decreased in the right side compared with the left side. Representative images of the superior area are shown in Figure 6A, and the results are summarized in Figure 6B. Images of middle and inferior area are described in Figure S3A of this article.

Observation of the right sides indicated that the number of OMP-positive cells, mature OSNs in the OE lining the superior and inferior areas as well as the average OMP-positive cell count declined significantly in the ECRS group compared with the control and OVA groups. Besides, left-right comparison within the ECRS group demonstrated a significantly decreased number of OMP-positive cells in the OE of the superior and inferior areas and, on average, in the right side vs the left side. Representative images of the superior area are shown in Figure 6C, and the results are summarized in Figure 6D. Images of middle and inferior area are described in Figure S3B of this article.

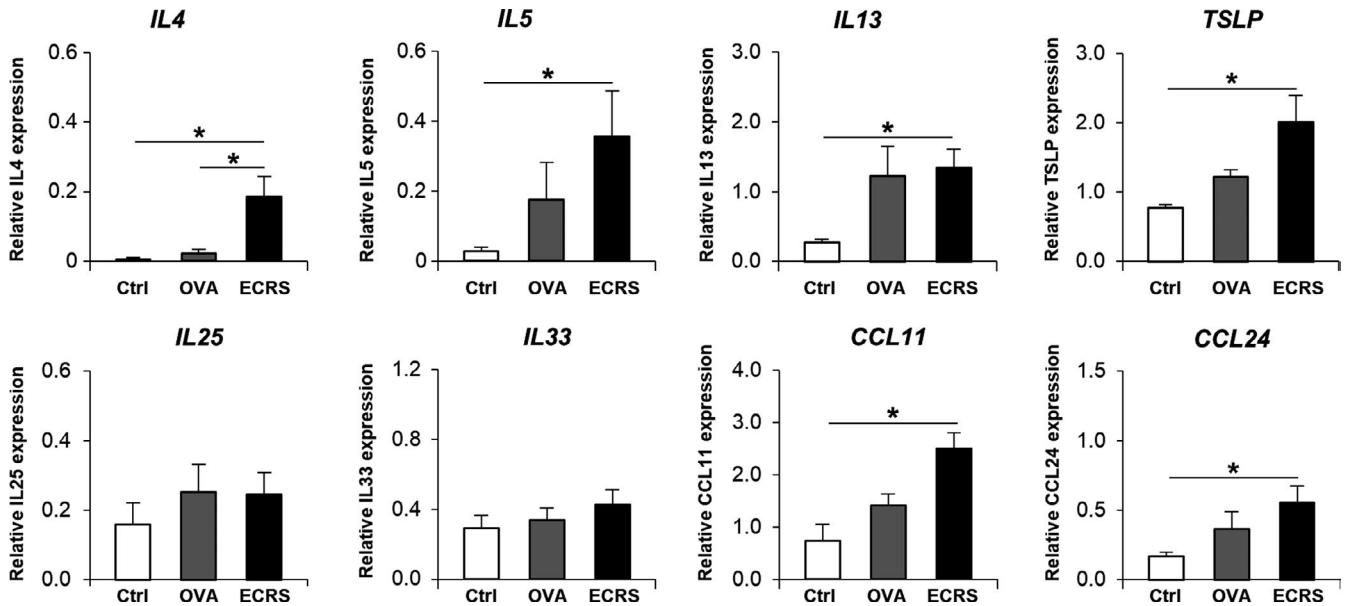
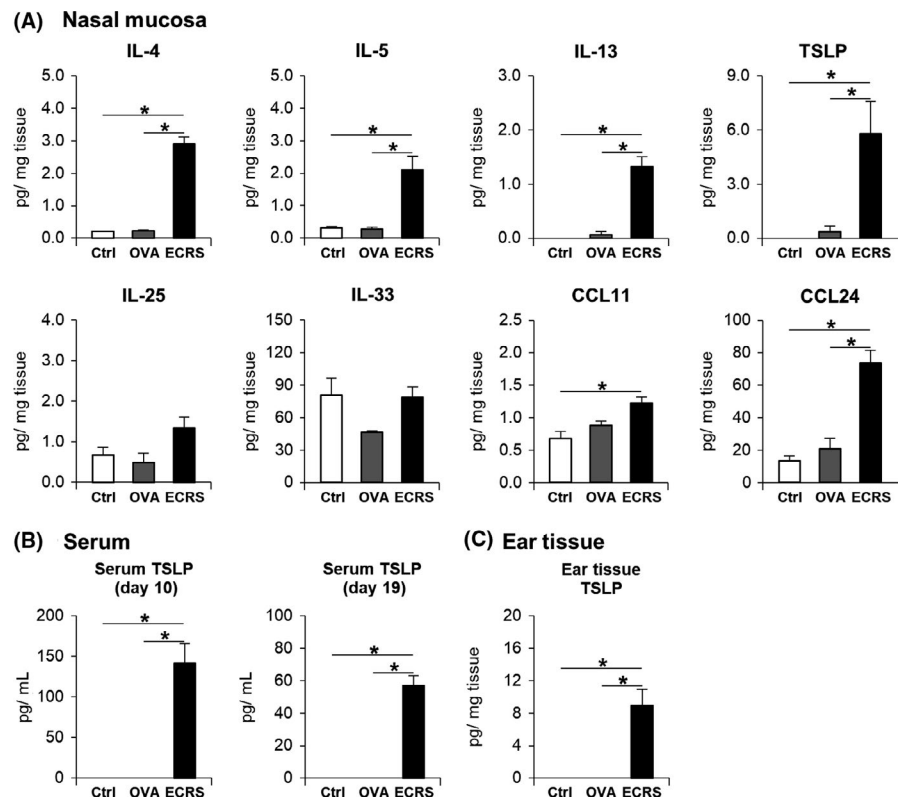


FIGURE 4 Gene expression of cytokines and chemokines in the respiratory nasal mucosa. Statistical analysis was performed using the Kruskal-Wallis ANOVA followed by the Dunn-Bonferroni test, where $P < .05$ (*) was deemed to represent statistical significance. Error bars shown represent the standard error of the mean. The number of animals per group was 5. Data are representative of two independent experiments. Ctrl, control; OVA, ovalbumin; ECRS, eosinophilic chronic rhinosinusitis

FIGURE 5 (A) Protein level expression of cytokines and chemokines in the nasal mucosa. (B) Protein level expression of TSLP in the serum. (C) Protein level expression of TSLP in the right ear. Statistical analysis was performed using the Kruskal-Wallis ANOVA followed by the Dunn-Bonferroni test, where $P < .05$ (*) was deemed to represent statistical significance. Error bars shown represent the standard error of the mean. The number of animals per group was 5. Ctrl, control; OVA, ovalbumin; ECRS, eosinophilic chronic rhinosinusitis



4 | DISCUSSION

In the present study, we described a new murine model in which topical skin treatment with MC903 and OVA followed by intranasal OVA challenges caused severe eosinophilic inflammation in

paranasal sinuses. We also demonstrated the presence of basophils and M2 macrophages in the nasal mucosa of the model. In addition, we showed that both gene and protein expression levels of Th2 cytokines, TSLP, and eosinophil-recruiting chemokines in the nasal mucosa of the mice treated with MC903 and OVA were

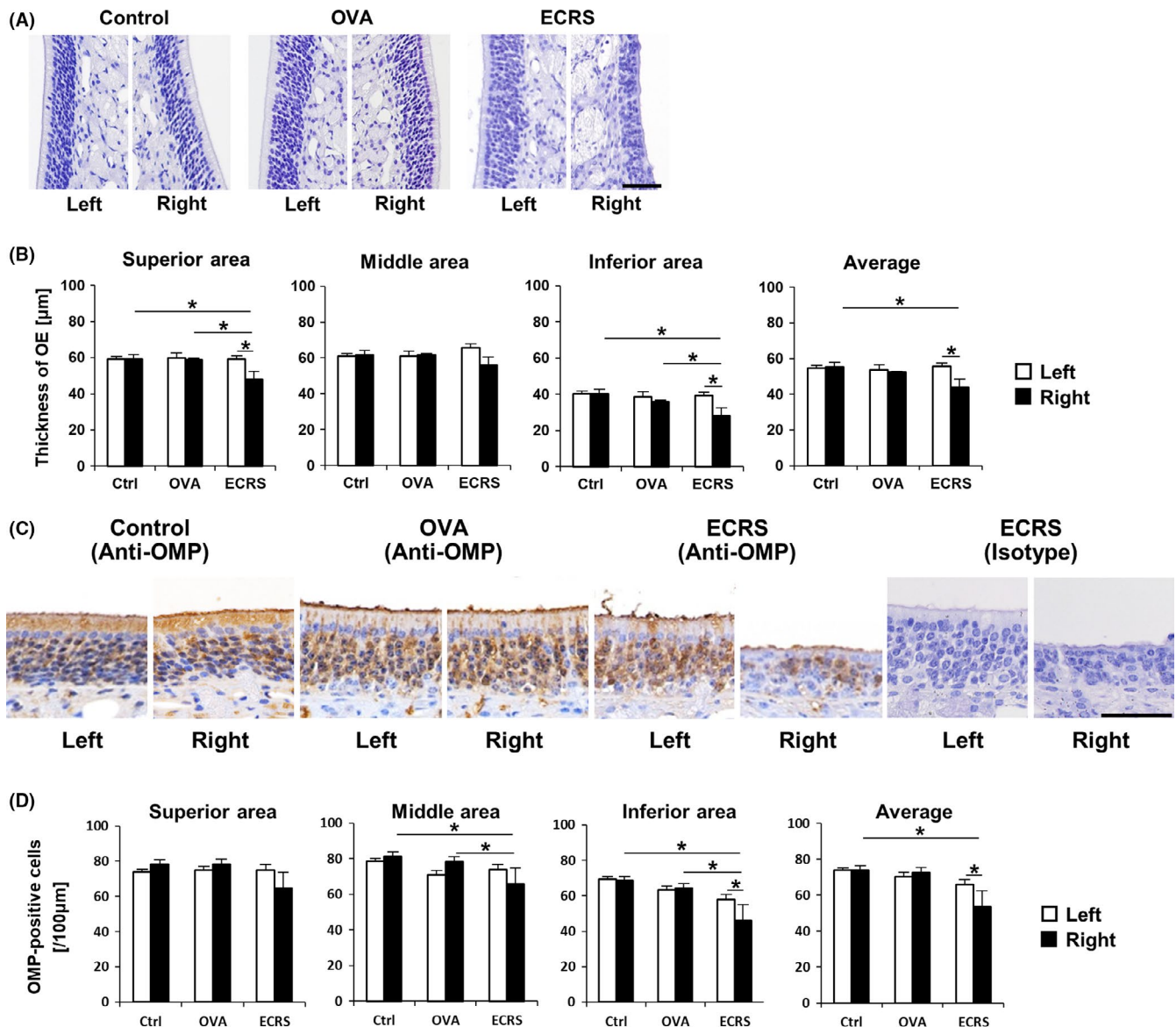


FIGURE 6 (A) Sirius red-stained sections, showing OE thickness in the superior areas of the nasal septum (scale bar = 100 μm). (B) Comparison of OE thickness in the three described areas between the studied groups. (C) Immunostained sections, showing mature OSNs in the superior area of the nasal septum (scale bar = 50 μm). (D) Comparison of the number of mature OSNs in the three described areas among the studied groups. Statistical analysis was performed using the Kruskal-Wallis ANOVA followed by the Dunn-Bonferroni test, where a $P < .05$ (*) was regarded as statistically significant. Error bars shown represent the standard error of the mean. The number of animals per group was 5. Data are representative of two independent experiments. OE, olfactory epithelium; OSNs, olfactory sensory neurons; Ctrl, control; OVA, ovalbumin; ECRS, eosinophilic chronic rhinosinusitis

higher than those in the mice treated with OVA alone and the controls. Furthermore, MC903 and OVA treatment induced thinner OE and decreased OMP-positive cells. In other words, this model exhibited characteristics consistent with clinical features of ECRS, such as basophil and M2 macrophage infiltration,^{22,30} more severe Th2-type inflammation than allergic rhinitis,³¹ and olfactory dysfunction.³² To our knowledge, this is the first murine model to show infiltration of both eosinophils and basophils in the nasal mucosa following the topical application of a vitamin D3 analog.

Basophils account for only 0.1%–1% of all peripheral blood leukocytes and share some common features with mast cells,³³ such as

cell surface expression of Fc epsilon receptor 1 (Fc ϵ RI) and release of chemical mediators including histamine followed by cross-linking of IgE. Because of this similarity and their minority status, the role of basophils had been underestimated for a long time. Recently, new aspects of basophils were revealed. TSLP-elicited basophils serve as the major source of IL-4, which plays versatile roles in Th2-type inflammation.³⁴ Basophil-derived IL-4 not only promotes Th2 cell differentiation, but also controls ILC2-mediated eosinophilic inflammation.^{18,19,35} Thus, various functions of basophils other than degranulation triggered by the cross-linking of allergen-specific IgE are important for Th2-type inflammation.

The vitamin D3 analog MC903 is known to induce TSLP production from keratinocytes when applied topically.¹⁶ MC903 has been used to establish murine models of diseases involving Th2-type inflammation, such as eosinophilic esophagitis,¹⁶ atopic dermatitis,²⁴ and bronchial asthma.²⁵ In a model of eosinophilic esophagitis, which is the prototype of the ECRS model in the present study, IgE-deficient mice showed tissue eosinophilia to the same extent as that in wild-type mice. On the other hand, TSLP-deficient mice and basophil-depleted mice showed a marked reduction in tissue eosinophilia.¹⁸ These results reflected that TSLP and basophils, rather than IgE, mainly contributed to Th2-type inflammation in the model using MC903. Although the vitamin D3 analog is a useful reagent for inducing production of TSLP at the skin with reduced barrier function in various murine models,^{16,24,25} it has the dual nature, both pro- and anti-inflammatory properties when applied to the respiratory epithelium.³⁶ Because the barrier dysfunction of the skin and production of the TSLP are important steps of allergic diseases, such as bronchial asthma, allergic rhinitis, and food allergy,^{37,38} skin-barrier defects and overproduction of TSLP may be also one of the aggravating factors of ECRS. However, the role of vitamin D3 itself in the nasal sinuses of the ECRS patients remains to be investigated in the future.

In the present study, mice treated with MC903 and OVA showed higher basophil infiltration in the nasal mucosa and elevated protein expression of TSLP, as compared to those treated with OVA alone and the controls. In addition, the number of basophils in each region was lower than that of eosinophils. These results accord with the clinical features of ECRS. Although the TSLP produced by the keratinocytes of the auricles leaked into blood circulation and acted in the nasal cavity in the present model, the results of quantitative real-time PCR and ELISA showed that TSLP level in the nasal mucosa was also elevated, most likely through the following mechanism. Since IL-13 is reported to induce TSLP production from epithelial cells,³⁹ locally increased IL-13 probably induced TSLP production in the nasal mucosa, leading to a positive feedback between TSLP and IL-13. That is, the increased level of TSLP in the nasal tissue following treatment with MC903 activated basophils, facilitated basophil-derived IL-4 production, and consequently promoted Th2-type inflammation.

The OVA group mice also showed eosinophil infiltration in the nasal mucosa, albeit less remarkable than the ECRS group mice. Because the mice were treated with topical OVA for 14 days, followed by intranasal OVA challenge, this group is considered to be a model of allergic rhinitis based on epicutaneous sensitization.^{40,41}

Thinned OE and decreased mature OSNs were observed in our ECRS model. These are also important findings because olfactory dysfunction is a common clinical feature of the human ECRS.³² In some other murine models of ECRS, changes in the olfactory system are also observed. Khalmuratova et al⁴² reported thinned OE in their model of ECRS. Kanda et al¹⁰ revealed hyposmia in their model of ECRS by food buried food tests. The exact mechanism for olfactory nerve disorder by nasal eosinophilic inflammation remains to be elucidated. Mori et al⁴³ suggested that IL-4 and IL-13

cytokines could contribute to pathological mechanisms resulting in the loss of dopaminergic neurons in Parkinson's disease; they also reported that IL-13R α 1 and IL-4R α were detected in mature OSNs. Although further investigation is necessary, there is a possibility that elevated IL-4 and IL-13 may have some impact on the homeostasis of OSNs.

Distribution of immune cells is interesting. The density of eosinophils and macrophages was higher in the maxillary sinus than in the other regions of the nasal passage. These results are different from the clinical features of the human ECRS, where ethmoid regions rather than the maxillary sinus have dominant eosinophilic inflammation. Anatomically, the region around the dorsal nasal concha in mice, which is equivalent to the human ethmoid sinus, is not composed of many cells unlike human sinus. Therefore, this region in mice may be relatively resistant to airflow obstruction and local inflammation.

There are several limitations to the current study that should be recognized. First, formation of nasal polyps was not evident in the presented model, although subepithelial thickening of the respiratory nasal mucosa with eosinophil infiltration was seen. ECRS is a chronic inflammatory disease, and nasal polyposis is thought to occur over time. In the murine models of ECRS reported before, study periods of more than 80 days were necessary.^{8,9,44} In the present study, however, the eosinophilic inflammation of the nasal mucosa occurred after treatment for only 19 days. For establishing a model closer to ECRS, treatments over longer time may be essential. Nonetheless, the short-term protocol is also a strength of our model that makes it possible to conduct a variety of studies on ECRS in a short time. Second, this model does not focus on mechanisms other than the basophil-TSLP axis that are responsible for the human ECRS. As reported previously, ECRS is probably a multifactorial disease.⁶ *Staphylococcus aureus* (*S aureus*) is one of the most well-known causative microorganisms of ECRS. Enterotoxins produced by *S aureus* not only induce SE-specific IgE but also serve as a superantigen, enhancing the release of Th2 cytokines from T cells.⁷ *Aspergillus fumigatus* is another causative antigen, which induces IL-33 release from sinonasal epithelial cells and enhances Th2-type inflammation.⁴⁵ Recently, the imbalance between the coagulation cascade and the fibrinolysis cascade in the nasal mucosa has been reported to predispose to the development of nasal polyps.^{46,47} Yet, the TSLP-basophil axis remains to be considered one of the important factors involved in the pathogenesis of ECRS. The murine model in the present study is a useful tool for investigating eosinophilic inflammation in the sinonasal mucosa based on the TSLP-basophil axis.

In conclusion, we established a new murine model of ECRS by the topical application of MC903. In the model, the nasal mucosa was infiltrated by not only eosinophils but also basophils. The ECRS mice also demonstrated elevated gene expression of molecules related to Th2-type inflammation in the nasal mucosa, as compared to the mice treated with OVA alone. Additionally, thinned OE and decreased mature OSNs were observed in the model. These results are consistent with current knowledge regarding ECRS.

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CONFLICT OF INTEREST

Dr Kagoya, Dr Kondo, Dr Kishimoto-Urata, Dr Shimizu, Dr Kikuta, and Dr Yamasoba have nothing to disclose.

AUTHOR CONTRIBUTIONS

RK, KK, and MKU conducted the experiment. KK, YS, SK, and TY gave technical support and conceptual advice. RK designed studies, interpreted the results, and wrote the article. KK and TY made helpful revisions of the manuscript.

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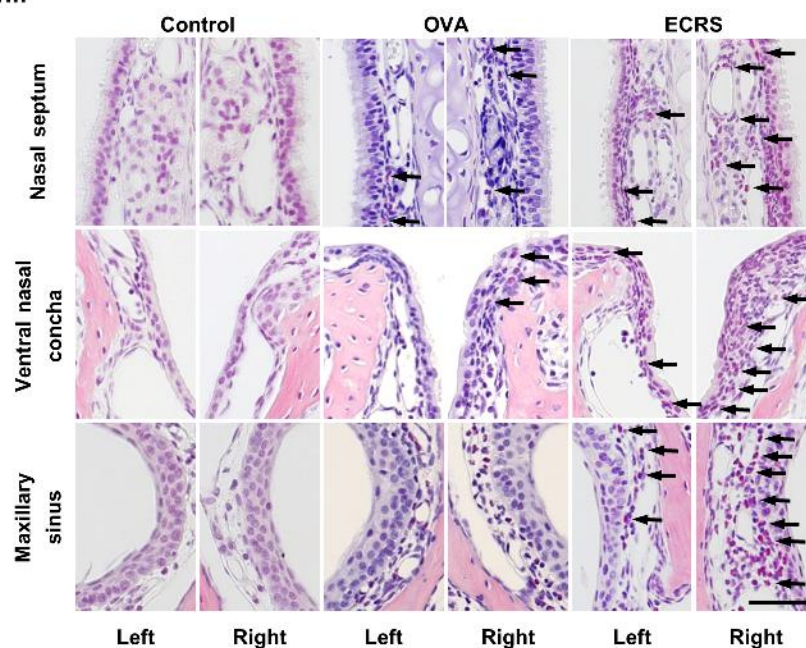
SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Figure S1

(A) Eosinophil



(B) Basophil

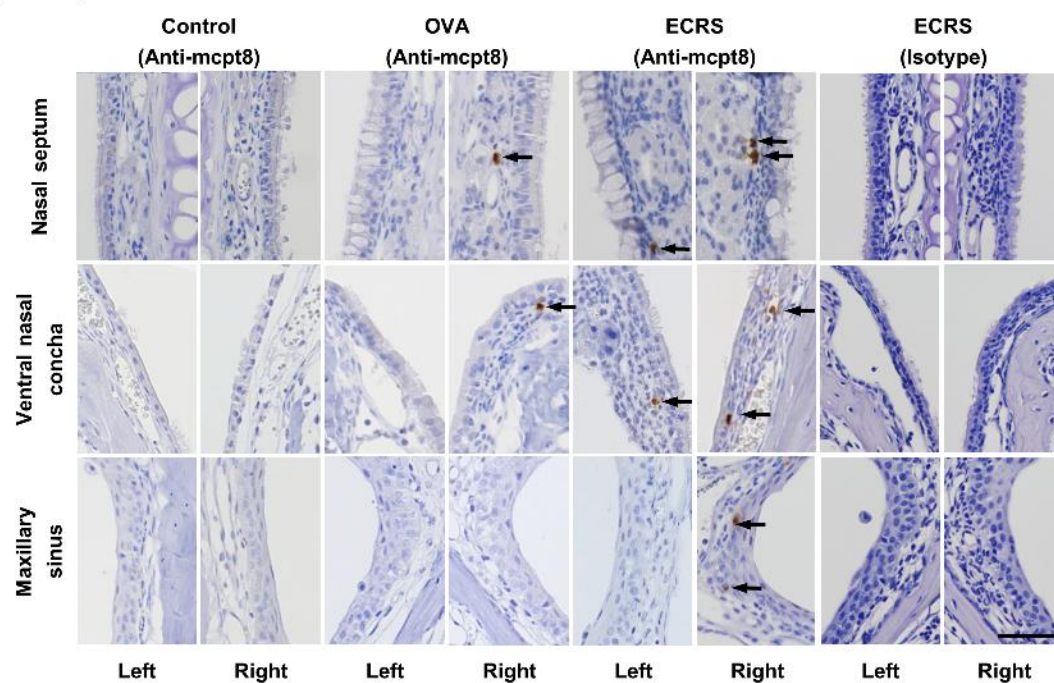


Figure S1

(C) Macrophage

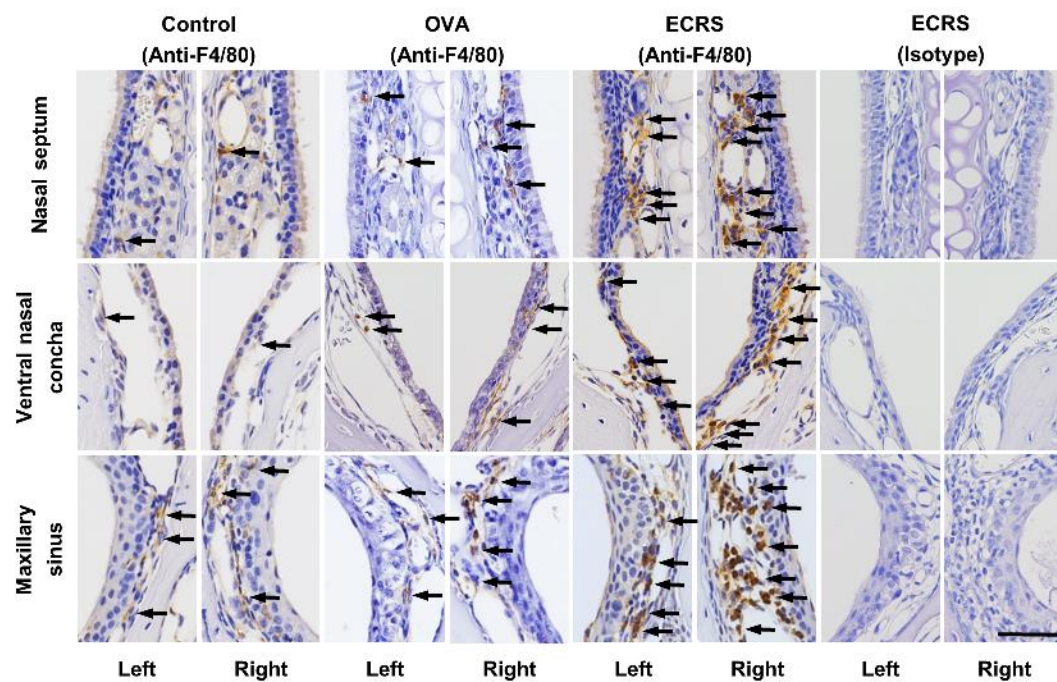
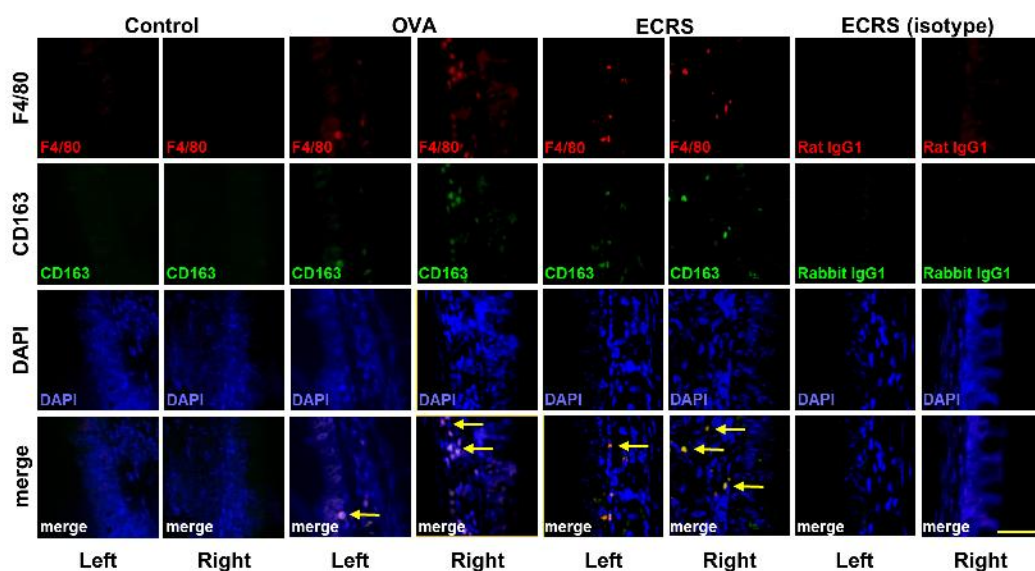


Figure S2

(A) M2 Macrophage (Nasal septum)



(B) M2 Macrophage (Dorsal nasal concha)

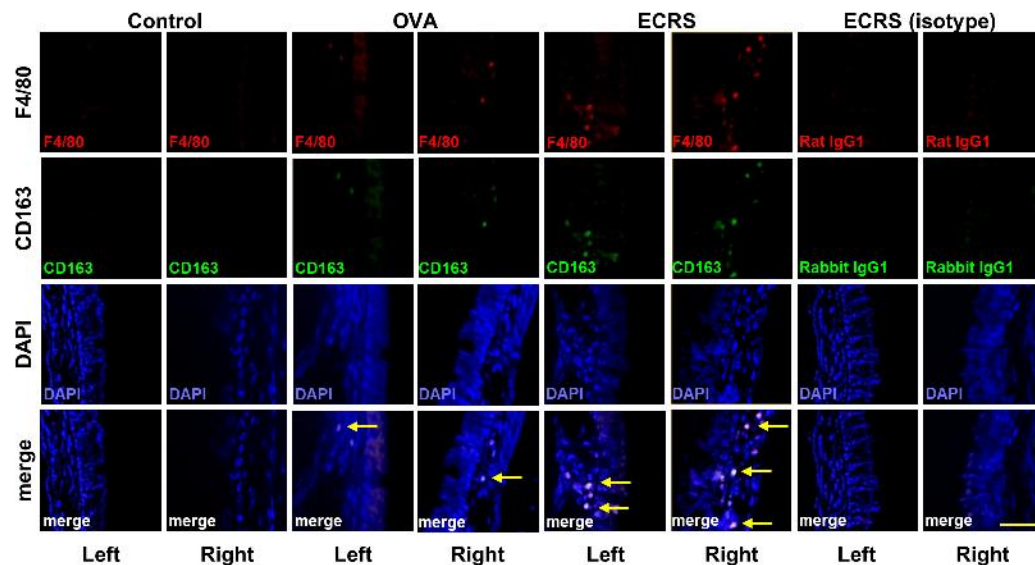


Figure S2

(C) M2 Macrophage (Ventral nasal concha)

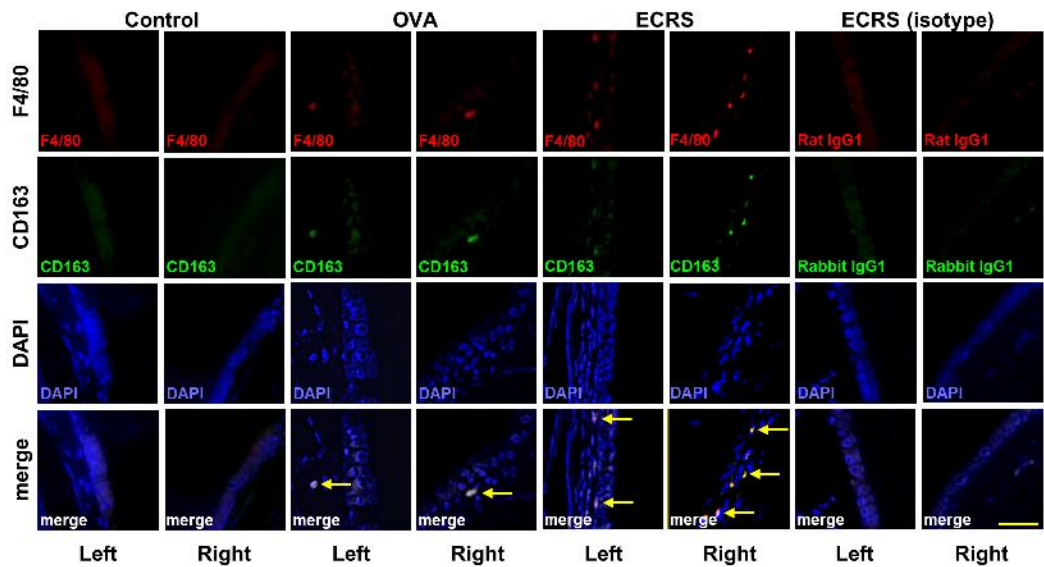
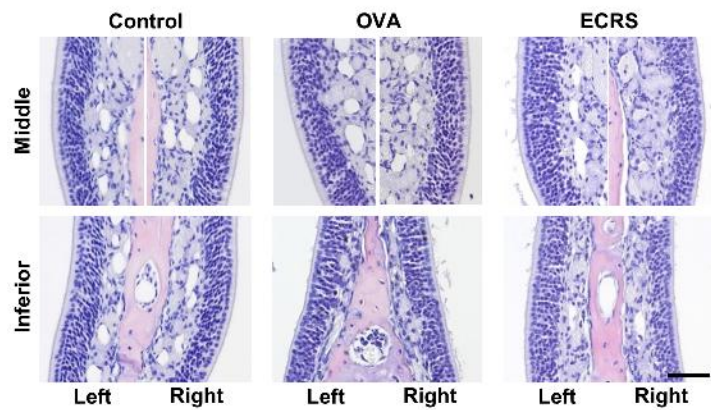
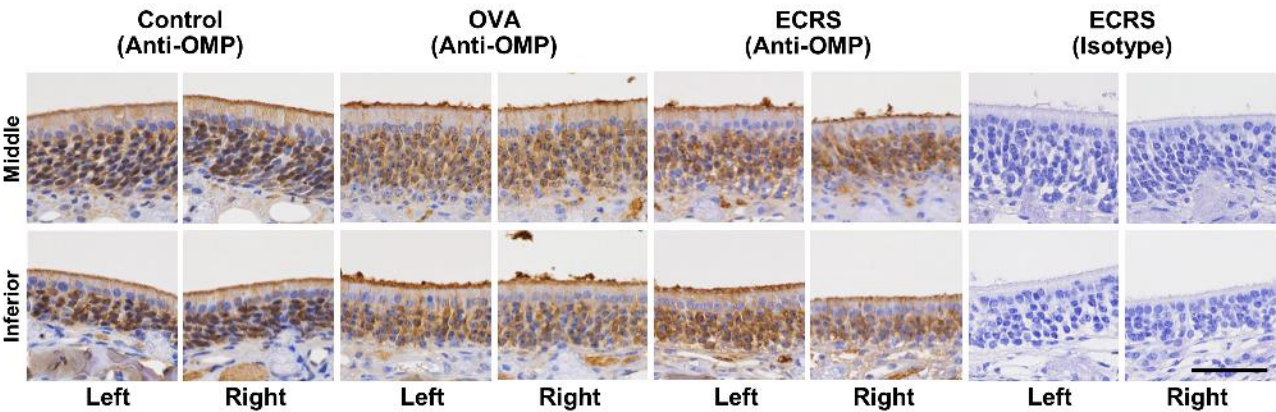


Figure S3

(A) Thickness of olfactory epithelium



(B) OMP-positive cells in olfactory epithelium



Supporting information 1

This appendix is provided by the authors to give readers additional information about their work.

1 | Supplementary methods

1.1 | Mice

C57BL/6 male mice were purchased from CLEA Japan (Tokyo, Japan). All the mice were 8 weeks of age at the start of the experiment. They were housed in a temperature-controlled facility and maintained on a 12h light/dark cycle and were given with free access to diet and water. All procedures were approved by the University of Tokyo Animal Care Committee and conducted in accordance with the University of Tokyo guidelines for the handling and care of laboratory animals.

1.2 | Experimental protocol

The mice were divided into three groups, namely control, OVA, and ECRS. The procedure is depicted in Figure 1A. Briefly, the ECRS group mice was topically treated once daily with 2 nmol of MC903 (Tocris Bioscience) in 20 μ l of 100% ethanol along with 100 μ g of OVA in 20 μ l of phosphate-buffered saline (PBS) after tape-tripping on the right ear for 14 days. For tape-stripping, the ventral side of the right ear was tape-stripped six times with scotch sealing tape. Next, daily intranasal administration of 2400 μ g of OVA in 40 μ l of PBS was performed for 5 days. The OVA group received 100 μ g of OVA in 20 μ l of PBS together with 20 μ l of 100% ethanol without MC903 after tape-tripping. This group was then challenged intranasally with OVA in a similar fashion to the ECRS group. The control group was given 20 μ l of PBS and 100% ethanol, followed by intranasal administration only with 40 μ l of PBS. All the mice were sacrificed at day 19.

1.3 | Fixation and tissue preparation

The mice were deeply anesthetized using a combination of ketamine and xylazine, fixed by cardiac perfusion with 10 % neutral buffered formalin, and decapitated. After lower jaws were discarded, the remaining parts were further fixed in 10 % neutral buffered formalin for 1 week at room temperature (RT). Then, the specimens were decalcified in Kalkitox (Wako) for 4 hours at RT, washed, dehydrated in a graded ethanol series, and embedded in paraffin. Serial coronal sections (4 μ m thick) were cut at two levels: (1) anterior third of the level of naso-ocular distance, i.e., one-third of the distance from the nose tip to the anterior border of the eyes (Figure 1B); and (2) anterior border of the eyes (Figure 1C). The prepared sections were mounted on MAS-coated slides (Matsunami Glass) and subsequently subjected to Sirius red staining (Muto Pure Chemicals) or immunostaining as described below.

1.4 | Sirius red staining

To investigate the infiltration of eosinophils, Sirius red staining was performed¹. After deparaffinization and rehydration, the sections were incubated with a 0.5% Sirius Red solution dissolved in 100% ethanol for 1 hour at RT, washed in acidified water (0.5% hydrogen chloride), counterstained with hematoxylin, dehydrated and mounted.

1.5 | Immunohistochemistry

To identify basophils, macrophages, and matured olfactory sensory neurons (OSNs) in the specimens, the following primary antibodies were used, respectively: anti-mcpt8 (rat monoclonal, clone TUG8; Biolegend), anti-F4/80 (rat monoclonal, clone CI:A3-1; BIO-RAD), and anti-OMP (goat polyclonal, Wako). Rat IgG isotype-matched control antibody (Invitrogen) and goat IgG isotype-matched control antibody (Invitrogen) were used as negative controls. Mcpt8 and F4/80 are murine basophil-specific marker² and murine macrophage-specific marker³, respectively.

After deparaffinization and rehydration, antigen retrieval was performed. Sections for mcpt8 and OMP immunostaining were subjected to heat-induced antigen retrieval using a citrate buffer solution pH 6.0 (Dako). Sections for F4/80 immunostaining were incubated with Proteinase K working solution (Dako) for 5 minutes at RT. Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxidase (nacalai tesque) in methanol for 10 minutes at RT. Next, the sections were incubated for 30 minutes in Serum-free protein block (Dako) at RT to block nonspecific antibody binding and then incubated with the respective primary antibodies described

above overnight at 4°C. After several washes in PBS, sections were incubated for 30 minutes at RT with horseradish peroxidase conjugated with anti-rat, anti-rabbit, or anti-goat IgG antibodies (Simplestain MAX-PO [Rat], [R], or [G] Nichirei). Following three more washes in PBS, color development was achieved with diaminobenzidine (Simplestain DAB, Nichirei). After washing with distilled water, the sections were counterstained with hematoxylin, dehydrated, and mounted. Histological images of specimens were obtained by fluorescence microscopy BZ9000 (Keyence).

1.6 | Immunofluorescence

To evaluate M2 (F4/80⁺ CD163⁺)⁵ subtype of macrophages, immunofluorescence staining was performed. The following primary antibodies were used, respectively: anti-F4/80 (rat monoclonal, clone CI:A3-1; BIO-RAD) and anti-CD163 (rabbit monoclonal, clone EPR19518; abcam). Rat IgG isotype-matched control antibody (Invitrogen) and Rabbit IgG isotype-matched antibody (Invitrogen) were used as negative controls. After deparaffinization and rehydration, antigen retrieval was performed. For F4/80 immunostaining, Proteinase K working solution (Dako) was used in the same way as mentioned above. For CD163 immunostaining, heat-induced antigen retrieval using a citrate buffer solution pH 9.0 (Dako) was performed. Primary-stained sections were incubated with appropriate Alexa Fluor-conjugated secondary antibodies for 30 minutes at RT. After washing with distilled water, sections were mounted in Vectashield mounting medium with DAPI (Vector Laboratories). Images of specimens were obtained by fluorescence microscopy BZ9000 (Keyence).

1.7 | Histological analyses

To determine the degree of immune cell infiltration into the nasal mucosa, the number of infiltrated cells was manually counted, in the sections cut at the level of the anterior third of the naso-ocular distance. The number of each type of immune cells per 200-μm length of the nasal epithelium was counted in the following four areas: nasal septum, dorsal nasal concha, ventral nasal concha, and maxillary sinus⁶, which is shown in Figure 1B. Then, the mean cell count for each immune cell was calculated.

To analyze any changes in the olfactory epithelium (OE), we used the sections at the level of the anterior boarder of the eyes. The OE thickness and the number of OMP-positive cells per 100-μm length were determined in three areas of the nasal septum: superior, middle, and inferior. Each area is shown in Figure 1C.

1.8 | RNA extraction and quantitative real-time PCR analysis

For RNA extraction, blood removal was performed by cardiac perfusion with PBS. Then, the nasal respiratory mucosa was resected microscopically, minced, and lysed in TRIzol reagent (Invitrogen). Afterward, total RNA was extracted in accordance with the manufacturer's instructions and reverse-transcribed by using a PrimeScript RT Master Mix (Takara Biotechnology). Quantitative real-time PCR was carried out on an Applied Biosystems 7500 Fast Real-Time PCR System. The following primers and probes were used:

IL4 (sense 5'-acggagatggatgtgccaaac, antisense 5'-agcaccttggaagccctacaga),
IL5 (sense 5'-tgaggcttctgtccctactcataa, antisense 5'-ttggaatagcatttccacagtacc),
IL13 (sense 5'-cggcagcatggtatggagt, antisense 5'-attgcaattggagatgttggtcag),
TSLP (sense 5'-aatgaccactgcccaggcta, antisense 5'-ttgtgaggttgattcagacagatg),
IL25 (sense 5'-ctcaacagcagggccatctc, antisense 5'-gtctgtaggctgacgcagtgtg),
IL33 (sense 5'-gatgagatgtctcggtgcttg, antisense 5'-agccgttacggatatggtggtc),
CCL11 (sense 5'-cagatgcaccctgaaagccata, antisense 5'-tgctttgtggcatcctggac),
CCL24 (sense 5'-ccctcatcttgctgcacgtc, antisense 5'-cctctgaaccacagcagctt), and
GAPDH (sense 5'-tgtgtccgtcgtggatctga, antisense 5'-ttgtgttgaaagtcgcaggag).

Gene expression levels were calculated as the difference between the threshold (Ct) value of the target gene and that of GAPDH (Δ Ct) using the comparative threshold cycle ($2^{-\Delta\Delta$ CT) method (n=5 per group).

1.9 | Protein extraction and enzyme-linked immunosorbent assay (ELISA)

Blood samples (60 μ l / mouse) were collected from the tail vein at day 10 and day 19, and allowed to clot at room temperature for 30 min. Serum supernatant was obtained after centrifugation at 10,000 $\times$ g for 5 minutes at room temperature. The nasal mucosa (~8mg) and the right ear tissue (~20mg) were resected and homogenized in 500 μ l of CellLyticTM MT Cell Lysis Reagent (Sigma-Aldrich) with 5 μ l of protease inhibitor cocktail (Sigma-Aldrich) and 120U/mL of benzonase endonuclease (Sigma-Aldrich). Supernatants of the homogenates were obtained after centrifugation at 10,000 $\times$ g for 5 minutes at room temperature. The samples were stored frozen at -80°C before analysis.

The levels of IL-4, IL-5, IL-13, TSLP, IL-25, IL-33, CCL11, and CCL24 in the samples were measured using the Mouse ELISA Kit (Invitrogen) according to the manufacturer's instructions. The absorbance at 450 nm was determined using Microplate Reader ChroMate (Awareness Technology).

1.10 | Statistical analyses

Statistical analyses were conducted by SPSS statistical software (SPSS Inc, Chicago, Illinois). Kruskal–Wallis ANOVA followed by Dunn–Bonferroni test was used to assess the difference between the control, OVA, and ECRS group. Mann–Whitney U-test was used for comparison between right side and left side. Data were plotted as means $\pm$ standard error. A *P* value of <0.05 was considered significant.

2 | Legends of supplementary figures

Figure S1

- (A) Sirius red-stained sections, showing eosinophil infiltration into—from top to bottom—the nasal septum, ventral nasal concha, and maxillary sinus of mice in—from left to right—the control, OVA, and ECRS groups (Scale bar = 50 mm).
- (B) Immunostained sections, showing basophil infiltration into—from top to bottom—the nasal septum, ventral nasal concha, and maxillary sinus of mice in—from left to right—the control, OVA, and ECRS groups (Scale bar = 50 mm).
- (C) Immunostained sections, showing macrophage infiltration into—from top to bottom—the nasal septum, ventral nasal concha, and maxillary sinus of mice in—from left to right—the control, OVA, and ECRS groups (Scale bar = 50 mm).

Figure S2

- (A–C) Immunostained sections, showing M2 macrophage infiltration into the nasal septum (A), dorsal nasal concha (B), and ventral nasal concha (C) of mice in—from left to right—the control, OVA, and ECRS groups (Scale bar = 25 mm).

Figure S3

- (A) Sirius red-stained sections, showing OE thickness in the middle and inferior areas of the nasal septum (Scale bar = 100 mm). (B) Immunostained sections, showing mature OSNs in the middle and inferior areas of the nasal septum (Scale bar = 50 mm). Ctrl, control; OVA, ovalbumin; ECRS, eosinophilic chronic rhinosinusitis.

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