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Original Article

CD11c(+) dendritic cells coexpressing thymic stromal lymphopoietin receptor in animal model of eosinophilic otitis media

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Conflict of Interest
The authors have no financial conflicts of interest.

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Conceptualization: Atsushi Matsubara. Data curation: Naomi Kudo, Ryutaro Hara, Tomoya

ABSTRACT

Background: Eosinophilic otitis media (EOM) is an intractable middle ear disease often associated with eosinophilic inflammatory airway conditions. Recently, dendritic cells (DCs) have been indicated as an essential component of Th2 allergic inflammation, such as bronchial asthma. DCs are activated by thymic stromal lymphopoietin (TSLP), an epithelial cell-derived cytokine. However, the relationship between TSLP and DCs in EOM remains unknown.
Objective: This study aimed to investigate the relationship between DCs and TSLP and to determine the involvement of DCs in EOM in an animal model.
Methods: Hartley guinea pigs were used as the animal model. Daily ovalbumin (OVA) stimulation of the middle ear was performed for 7 or 14 days. The temporal bone was dissected on the last day of stimulation, and paraffin-embedded sections were prepared. Immunostaining and immunofluorostaining for TSLP receptor (TSLPR) and CD11c, a surface marker of DCs, were performed.
Results: We found CD11c-immunopositive cells in the submucosal area of the middle ear epithelium, particularly around the eustachian tube. TSLPR-immunopositive cells exhibited a similar distribution as CD11c-positive cells.
Conclusion: CD11c positive DCs coexpressing TSLPR were recruited after OVA challenge which might activate Th2 allergic reaction.

Keywords: Otitis media; Eustachian tube; Dendritic cell; Inflammation

INTRODUCTION

Intractable otitis media associated with bronchial asthma was first reported by Tomioka et al. in 1993, and the condition was later termed as eosinophilic otitis media (EOM) because of its characteristic eosinophil-enriched, highly viscous middle ear effusion [1, 2]. Because diagnostic criteria were established in 2011, substantive cases of EOM have been reported, and both the clinical features and pathophysiology of EOM have been gradually uncovered, leading to the establishment of its disease concept and the development of effective treatment [3]. EOM is frequently associated with eosinophilic inflammatory airway conditions, such as bronchial asthma and eosinophilic chronic rhinosinusitis. It has been reported that EOM was improved by optimal treatment and the control of comorbid bronchial asthma, which appears to reflect the concept of "one airway, one disease" [4].

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Previous studies reported various factors involved in the inflammation of EOM, including interleukin (IL)-5, IL-13, eotaxin, ecalectin, and eosinophil cationic protein [5-7]. Our group developed an animal model of EOM and performed experimental studies to clarify the pathophysiology of EOM, revealing the morphological changes of the middle and inner ear and the involvement of factors constituting the EOM pathophysiology [8-10]. Although the mechanism of EOM has been gradually revealed, several aspects of detailed cell-to-cell interactions constituting the disease remain unknown.

Recent studies in the field of allergic diseases have revealed the role of epithelial cell-derived cytokines and inflammatory cells inducing Th2 type allergic inflammation. Among them, dendritic cells (DCs) are activated by thymic stromal lymphopoietin (TSLP), an epithelial cell-derived cytokine, and they in turn stimulate lymphocytes, thereby activating Th2 allergic reactions. TSLP is known to be produced by pulmonary epithelial cells stimulated by protease contained in allergens such as pollen, house dust mites, or fungi [11]. We recently reported the expression of TSLP in patients as well as animal models of EOM, indicating that TSLP is a key trigger in the pathogenesis of EOM [12]. However, the relationship between TSLP and DCs in EOM has not been explored.

We hypothesized that the relationship between TSLP and DCs was also true in EOM. In the present study, we investigated the distribution of DCs and TSLP receptor (TSLPR) positive cells in the middle ear mucosa in an animal model of EOM.

MATERIALS AND METHODS

Tissue preparation

An animal model of EOM using male Hartley guinea pigs (weighing 250–350 g) was constructed as described by Nishizawa et al. [9]. Guinea pigs were kept in normal condition after we purchased them. Briefly, general sensitization with ovalbumin (OVA 014-17074, purity 100%, purchased from FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) was conducted. Specifically, an intraperitoneal injection of 2,000 μg of OVA and 100 mg of aluminum hydroxide (alum) was performed on day 0, followed by 100 μg of OVA and 100 mg of alum on days 7 and 14. Starting on day 21, daily local stimulation with OVA was performed using 1 mL of OVA solution (100 μg/mL) administered via right nasal drip and 0.1 mL of OVA solution (1000 μg/mL) via intratympanic injection through the tympanic membrane of the right ear. The daily application of topical antigen stimulation was continued for 7 or 14 days. Saline was injected to the left nostril and ear as a control. Because eustachian tube functions as "gate keeper" and blocks antigens penetrating into middle ear cavity from nasal cavity, OVA was injected through the tympanic membrane [13]. All procedures were conducted under anesthesia using a mixture of medetomidine hydrochloride, midazolam, and butorphanol tartrate (0.23 mg, 3.0 mg, and 3.75 mg/kg, respectively). Twenty-four hours after the final injection, the temporal bone was dissected, fixed in 10% formaldehyde, and decalcified using EDTA-2Na in 0.1 M Tris (pH, 7.2). The dissected temporal bone was prepared as paraffin-embedded sections. We prepared 10 Hartley guinea pigs, 5 each in the 7- and 14-day stimulation groups. For each animal, the left ear was used as control and the right ear was used for OVA stimulation, which sums up 20 ears. Penetration of eosinophils was observed in the right ear sections and larger number of eosinophils were observed in 14-day stimulation model than in 7-day stimulation model. On the other hand, few eosinophils were found in the left ear section even in 14-day stimulation model [9].

Immunohistochemistry

We utilized the Envision/HRP system (DAKO Cytomation, Biocompare, South San Francisco, CA, USA) to observe immunoreactivity for CD11c, a surface marker of DCs, and TSLPR. To analyze TSLPR expression, we performed immunostaining for cytokine receptor-like factor 2 (CRLF2), which constitutes the TSLPR heterodimer together with IL-7α.

In brief, deparaffinized sections were rehydrated through a graded series of ethanol to phosphate-buffered saline (PBS). Then, the sections were incubated in 0.3% H_2O_2 for 30 min to block endogenous peroxidase activity, followed by Protein Block Serum-Free (Code X0909, DAKO, Carpinteria, CA, USA) for 30 min. Next, the sections were incubated with anti-CD11c antibody (1:200 dilution of integrin alpha X polyclonal antibody, No. 17342-1-AP, Proteintech, Rosemont, IL, USA) and anti-CRLF2 antibody (1:400 dilution of rabbit anti-CRLF2 polyclonal antibody, SAB2900760, Sigma-Aldrich, St. Louis, MO, USA) at 4°C overnight. After incubation in the reagents of the Envision/HRP kit (rabbit) for 30 minutes, the sections were stained with diaminobenzene/H_2O_2 for 5 minutes. The sections were counterstained with hematoxylin and examined under a light microscope. We performed the substitutional control test using normal rabbit serum IgG (I5006-10 mg, Sigma-Aldrich) instead of primary antibody, and no specific staining was observed.

Double immunofluorescent staining

For double immunofluorescent staining, the same sections for immunostaining were prepared and stained as follows. First, we stained nuclei using 4′,6-diamidino-2-phenylindole dihydrochloride. After that, the sections were rinsed well with PBS and incubated with primary antibody (anti-CD11c antibody [1:400 dilution, No. 17342-1-AP], anti-TSLPR antibody [goat anti-mouse TSLPR polyclonal antibody, AF546, R&D Systems, Minneapolis, MN, USA], or anticytokeratin antibody (undiluted solution of monoclonal mouse anti-human cytokeratin clone AE1/AE3 ready-to-use, IS053, DAKO) at 4°C overnight. Third, the sections were incubated with secondary antibody (1:500 dilution of donkey anti-goat IgG, Alexa Fluor 488 [A32814, Thermo Fisher Scientific, Tokyo, Japan], 1:500 dilution of donkey anti-rabbit IgG, Alexa Fluor 647 [A32733, Thermo Fisher Scientific], or 1:500 dilution of donkey anti-mouse IgG, Alexa Fluor 594 [A32744, Thermo Fisher Scientific]) at room temperature for 60 min, and the coverslip was mounted. Images of the sections were taken using a fluorescence microscope (Confocal Quantitative Image Cytometer, CQ1 Yokogawa, Tokyo, Japan).

Cell count and statistical analysis

The sections were observed using an Olympus microscope (BX51, Olympus Corp., Tokyo, Japan) and imaging software (DP2-BSW, Olympus Corp.). Each section was assessed with digital images using a digital camera (DP72, Olympus Corp.). The number of CD11c-immunopositive cells was counted per unit area (0.01 mm^2) in the submucosal area featuring mucosa epithelial cells near the eustachian tube. The average cell count of 5 areas for each model was calculated. The data of 5 models were collected, and the result was analyzed by the unpaired *t* test using Stat Mate ver. 4.01 (ATMS, Tokyo, Japan) to compare the OVA stimulation and control groups. A probability value (p) less than 0.05 was considered statistically significant.

This study was approved by Institutional Animal Care and Use Committee of Hirosaki University (approval number: M10004). All animal experiments were performed in accordance with the Guidelines for Animal Experimentation of Hirosaki University.

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RESULTS

Immunohistology for CD11c and CRLF2

CD11c-immunopositive cells were not found in the submucosal area of the middle ear epithelium in the control group of the 7-day stimulation model (**Fig. 1A**). Conversely, CD11c-immunopositive cells were presented in the submucosal area in the OVA stimulation group of the 7-day stimulation model (**Fig. 1B**). Regarding the 14-day stimulation model, increased numbers of CD11c-immunopositive cells were observed in the submucosal area of the middle ear epithelium compared with the finding in the 7-day stimulation model (**Fig. 1C**).

CRLF2-immunopositive cells appeared to colocalize with CD11c-immunopositive cells in serial sections, although its immunoreactivity was weaker (**Fig. 1D**).

Distribution of CD11c-positive cells

In the OVA stimulation group of the 14-day stimulation model, CD11c-immunopositive cells were abundantly distributed in the area around the eustachian tube (**Fig. 2A**). It should be noted that a relatively large number of CD11c-immunopositive cells were observed around the cartilage of the eustachian tube (**Fig. 2C**). Conversely, the number of CD11c-positive cells was lower in the submucosal area away from the eustachian tube (**Fig. 2B, D**).

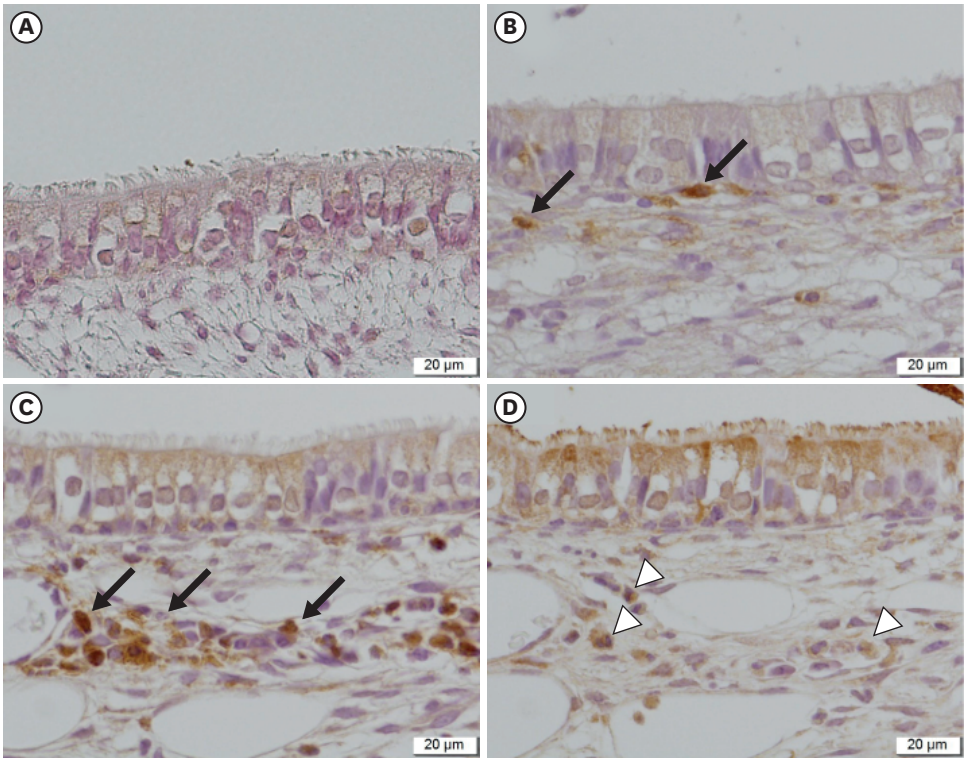

Fig. 1. (A) Immunohistological staining for CD11c and CRLF2. Specific immunopositive cells were not found in the control group. (B) CD11c-immunopositive cells (arrows) were found in the submucosal area of the middle ear epithelium after 7 days of ovalbumin (OVA) stimulation. (C) The number of CD11c-positive cells (arrows) increased by 14 days of OVA stimulation. (D) CRLF2-positive cells (arrowheads) exhibited a similar distribution as CD11c-positive cells after 14 days of OVA stimulation.

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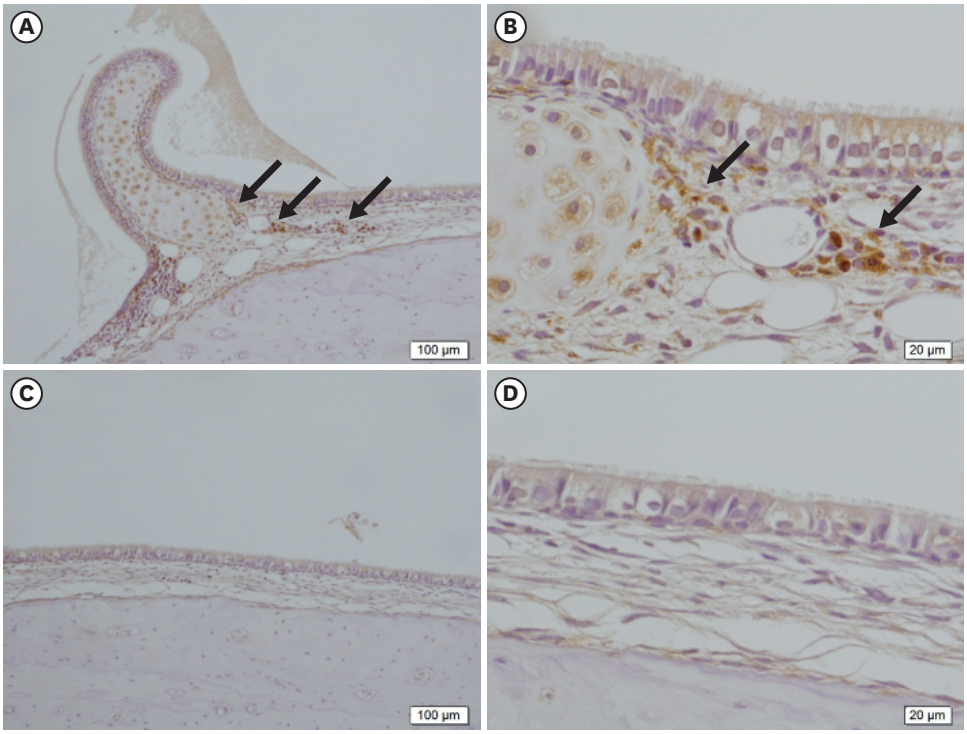

Fig. 2. (A) Distribution of CD11c-positive cells. Abundant CD11c-positive cells (arrows) were found around the eustachian tube. (B) The number of CD11c-positive cells (arrows) tended to decrease with increasing distance from the eustachian tube. Panels B and D represent high-power fields of panels A and C, respectively.

Number of CD11c-positive cells

The number of CD11c-immunopositive cells in the submucosal area of the middle ear epithelium near the eustachian tube is shown in **Fig. 3**. In the 7-day stimulation model, a significantly larger number of CD11c-immunopositive cells were observed in the OVA

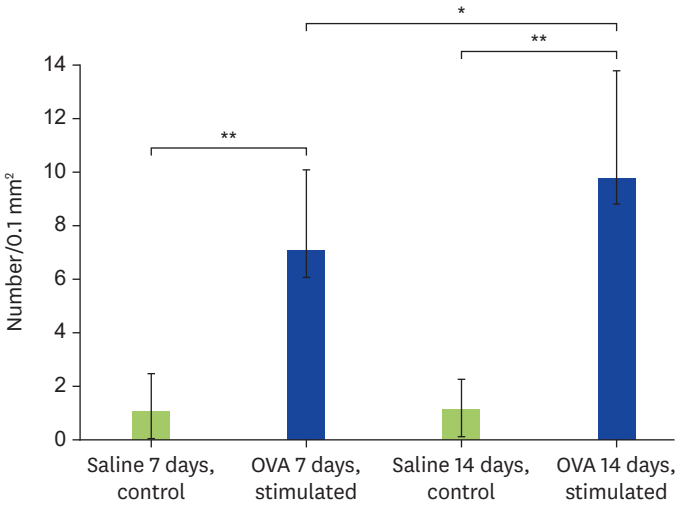

Fig. 3. The number of CD11c-immunopositive cells. The number of CD11c-immunopositive cells was significantly increased following 7 or 14 days of ovalbumin (OVA) stimulation compared with the control group findings. Meanwhile, the number of CD11c-immunopositive cells was significantly higher after 14 days of OVA stimulation than after 7 days of stimulation (n = 20, $^{*}p < 0.05$, $^{**}p < 0.001$).

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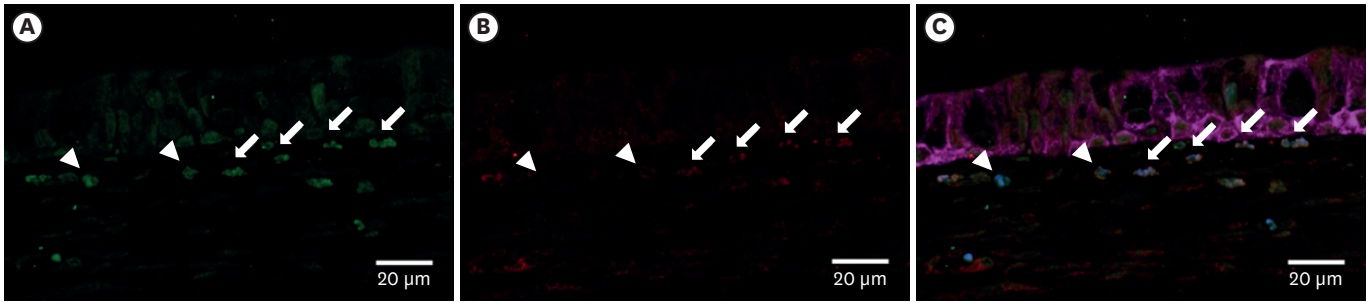

Fig. 4. Double immunofluorescent staining. Double immunofluorescent staining for thymic stromal lymphopoietin receptor (TSLPR) (green, A) and CD11c (red, B). (C) CD11c- and TSLPR-positive cells colocalized (arrows) as white-colored cells. Arrowheads indicate TSLPR-positive CD11c-negative cells. The nuclei were stained blue by 4′,6-diamidino-2-phenylindole dihydrochloride, and mucosa epithelial cells were stained pink by cytokeratin.

stimulation group than in the control group (7.07 ± 3.01 per unit area vs. 1.07 ± 1.38 per unit area, $p < 0.001$). The number of CD11c-immunopositive cells was significantly larger in the OVA stimulation group than in the control group in the 14-day stimulation model (9.8 ± 4.00 per unit area vs. 1.13 ± 1.12 per unit area, $p < 0.001$). In addition, a significantly greater number of CD11c-immunopositive cells were observed in the OVA stimulation group in the 14-day model than in the 7-day model ($p < 0.05$).

Immunofluorescence staining

We performed double immunofluorescence staining for CD11c and TSLPR to investigate the colocalization of CD11c- and TSLPR- positive cells. TSLPR-positive cells were stained green (**Fig. 4A**), and CD11c-positive cells were stained red (**Fig. 4B**). The cells were distributed in the submucosal area of the middle ear epithelium. Note that CD11c- and TSLPR-positive cells partly colocalized, as indicated by white-colored cells in **Fig. 4C**.

DISCUSSION

Since the first case report, pathophysiological nature of EOM has been gradually elucidated. However, most studies exploring the pathophysiology of EOM assessed clinical cases of EOM. Although those studies largely improved our understanding of the detailed mechanism of EOM, full observation of the middle and inner ear is difficult, and experimental studies such as immunostaining cannot be performed.

Our group has continually explored the pathogenesis of EOM utilizing animal models, which made it possible to observe the whole temporal bone construction including the middle ear, eustachian tube, and inner ear [9]. We also performed immunohistological studies for periostin, eotaxin, RANTES, and TSLP [8-10, 12]. Meanwhile, recent studies revealed that TSLP activates CD11c-positive DCs, thereby inducing Th2 type allergic reactions. Yamauchi et al. [14] performed double immunofluorescence staining for CD11c and TSLPR, demonstrating that CD11c and TSLPR double-positive myeloid DCs (mDCs) promote the migration of Th2 cells to the oral lichen planus. Other groups also reported that TSLP induced the production of Th2-related chemokines through mDCs, exacerbating bronchial asthma and atopic dermatitis [15]. We detected CD11c-positive cells in the submucosal area of the middle ear epithelium in our animal model of EOM. Immunostaining for CRLF2 was also performed, revealing that CRLF2-immunopositive cells colocalized with CD11c-positive cells. The results of double immunofluorescent staining also revealed the codistribution of

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CD11c- and TSLPR-positive cells. We previously reported that TSLP-producing cell counts were increased in the middle ear epithelium of an animal model of EOM [12]. Taken together with the results of the current study, we speculate that TSLP activates mDCs, inducing the subsequent production of Th2-related chemokines, in the pathogenesis of EOM.

DCs are divided into 2 functional subsets: conventional DCs (cDCs) and plasmacytoid DCs. cDCs are further categorized by the surface markers CD8α and CD4. The distinction of mDCs and lymphoid DCs is determined by the expression of CD11b, CD123, and CD205. Thus, DCs are classified into at least 6 subsets [16]. In the literature, mDCs were considered to activate Th2 allergic reactions via Th2-related cytokines such as TARC and MDC [15]. Although we speculate that cells immunopositive for both CD11c and TSLPR are mDCs, it is necessary to examine CD8α and CD123 expression to confirm the subset to which the observed cells belong.

Insights from basic and clinical studies led to the development of several effective therapies. Iino et al. [17] reported the beneficial effects of anti-IgE therapy on the symptoms of EOM and progression of bone conduction hearing loss. Mepolizumab, a humanized anti-IL-5 monoclonal antibody, is used for patients with moderate-to-severe bronchial asthma. In a retrospective study of patients with EOM associated with bronchial asthma who were treated with mepolizumab, anti-IL-5 therapy was highly effective in relieving EOM symptoms [18]. Our group demonstrated the expression of TSLP in an animal model of EOM [12]. AMG 157, a human anti-TSLP monoclonal immunoglobulin G2λ that binds human TSLP, is known to reduce allergen-induced bronchoconstriction and indices of airway inflammation before and after allergen challenge in allergic asthma [19]. The results of the present study suggest that CD11c-positive DCs were activated by TSLP through TSLPR. Therefore, an agent blocking TSLPR expressed on DCs would be promising for the treatment of allergic diseases in the future.

Penetration of inflammatory substances via the eustachian tube is considered an important aspect of the pathogenesis of EOM. Iino et al. [20] reported that patients with EOM tended to have patulous eustachian tube function, which allows inflammatory substances in the upper airway to penetrate the middle ear cavity more easily than observed in patients with normal eustachian tube function. Kanazawa et al. [21] reported that fungus-specific IgE and *Staphylococcus aureus* enterotoxin-specific IgE were found in the middle ear effusion of patients with EOM. Because antigens such as fungus and *S. aureus* are also found in upper airway like nasal cavity, it seems reasonable to consider those antigens penetrate middle ear cavity via eustachian tube.

In the present study, we found that CD11c- and TSLPR-positive cells tend to exist mainly around the eustachian tube. Our previous study also detected TSLP in the epithelial cells of the eustachian tube and around the tympanic ostium of the eustachian tube [12]. Morphologically, epithelial cells around eustachian tube have the features of airway epithelium with cilia, and middle ear epithelium distant from eustachian tube lacks such features. Those results seem to indicate that EOM is one of airway allergy similar with bronchial asthma and eosinophilic sinusitis.

The limitation of the present study is that we could not directly show that TSLP activates DCs. We found CD11c positive DCs coexpressed CRLF2 in mucosal area around eustachian tube and we would speculate TSLP stimulates CD11c positive DCs through binding CRLF2. However, we could not perform comparative experiments such as TSLP inhibition test. Further investigation is needed to reveal the accurate relationship between TSLP and DCs in EOM.

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