

# Prolonged Allergen Exposure Is Associated With Increased Thymic Stromal Lymphopoietin Expression and Th2-Skewing in Mouse Models of Chronic Rhinosinusitis

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**Objectives/Hypothesis:** Chronic rhinosinusitis (CRS) is characterized by a dysfunctional host-environment interaction at the nasal mucosa. Contributions of host susceptibility factors such as atopy and aspirin sensitivity to CRS pathophysiology are well established. However, clinical studies on the effects of environmental factors are limited. This study investigates the histological and immunological effects of allergen exposure duration in animal models.

**Study Design:** Animal study.

**Methods:** A murine model for CRS with nasal polypoid lesions was induced by instilling ovalbumin/staphylococcal enterotoxin B (SEB) into murine nasal cavities for 12 (short term) or 24 weeks (long term). Histopathological changes were observed. Interleukin (IL)-4, IL-17A, IL-10, and interferon (INF)- $\gamma$  levels from nasal lavage fluid were measured using enzyme-linked immunosorbent assay. Gene expressions of IL-25, thymic stromal lymphopoietin (TSLP), IL-4, IL-5, INF- $\gamma$ , C-C motif chemokine ligand (CCL)-11, CCL-24, C-X-C motif chemokine ligand (CXCL)-1, CXCL-2, intercellular adhesion molecule 1, vascular cell adhesion molecule 1, matrix metalloproteinase (MMP)-7, and tissue inhibitor of metalloproteinase (TIMP)-3 were analyzed from the nasal mucosa.

**Results:** Long-term CRS models exhibited increased polypoid lesions, edematous mucosal thickness, and eosinophil infiltration compared with short-term models and showed a higher IL-10 level but lower INF- $\gamma$  and IL-17A protein levels. Moreover, CCL-24 and MMP-7 gene expressions increased whereas TIMP-3 expression decreased in long-term models compared to controls and short-term models. IL-25 and TSLP expressions were upregulated at mRNA and protein levels in short-term and long-term CRS models, respectively. Furthermore, TSLP mRNA expression was positively associated with IL-5 ( $r = 0.8754$ ) and inversely correlated to INF- $\gamma$  ( $r = -0.7212$ ) in CRS models.

**Conclusions:** Prolonged allergen exposure in ovalbumin/SEB-induced CRS models maintains Th2 inflammation and reduces Th1 inflammation, which was associated with upregulation of TSLP.

**Key Words:** Animal model, eosinophil, nasal polyps, rhinosinusitis, thymic stromal lymphopoietin.

**Level of Evidence:** NA

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## INTRODUCTION

Chronic rhinosinusitis (CRS) with nasal polyp (CRSwNP) greatly impacts quality of life and is charac-

terized by accumulation of inflammatory cells and marked tissue remodeling. CRS is typically described as a dysfunctional host-environment interaction at the nasal mucosa.<sup>1</sup> The combination of variable host susceptibility and differential exposure to external stimuli increases the heterogeneity of CRS. Most nasal polyps (NPs) in Western populations show prominent eosinophil infiltration and prevalent asthma,<sup>2,3</sup> whereas Asian CRSwNP exhibits considerable noneosinophilic infiltration in NP tissues and less asthma association, which is attributed to differential host susceptibility.<sup>4,5</sup> In addition, several studies demonstrated different immunohistologic characteristics of CRSwNP endotypes such as the presence of differentiated T-effector cells (Th1, Th2, and Th17) and regulatory T (Treg) cells and characterized of remodeling processes such as fibrosis or edema formation.<sup>4–10</sup> Differences in external stimuli or exposure duration may further explain this heterogeneity. However, studies of the mechanism by which environmental factors influence subtypes or endotypes of CRS are limited in clinical settings.

Recently, we developed the murine allergic CRS with polypoid lesion model using ovalbumin (OVA)/

Additional Supporting Information may be found in the online version of this article.

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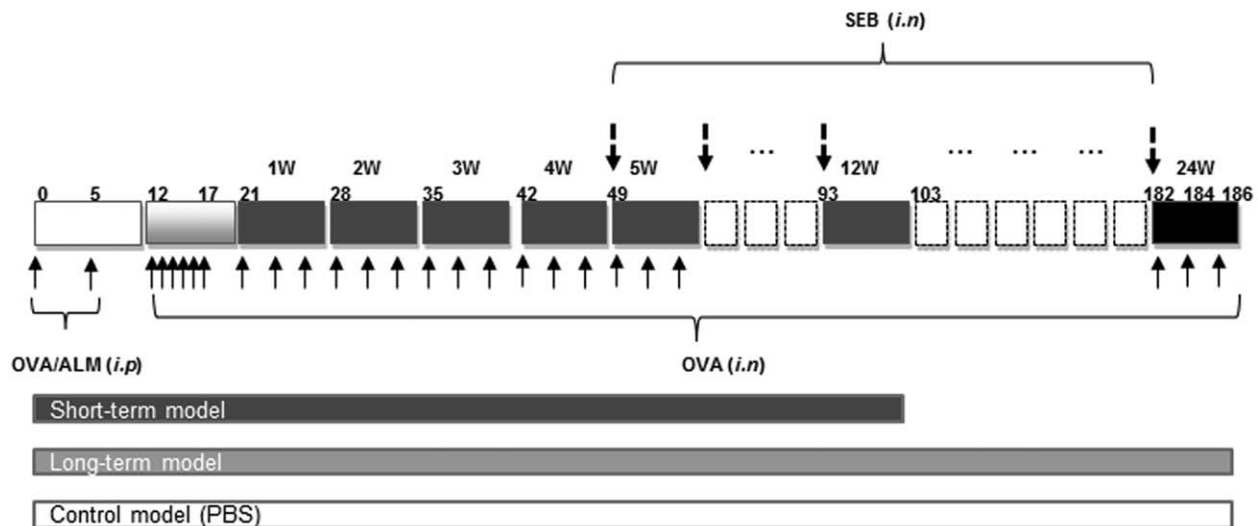


Fig. 1. Protocol for generating the murine model of allergic chronic rhinosinusitis with nasal polypoid lesions. Ovalbumin (OVA) and staphylococcal enterotoxin B (SEB) were administered into the nasal cavity to induce nasal polypoid lesions. ALM = aluminum hydroxide gel; i.n = intranasal instillation; i.p = intraperitoneal injection; PBS = phosphate-buffered saline.

staphylococcal enterotoxin B (SEB),<sup>11</sup> which has been utilized and validated by multiple studies.<sup>12–15</sup> This murine model shows reliable inflammation in the nasal mucosa. Immunohistologically, this model shows higher Th2 cytokine levels, elevated multiple mucosal lesions with prominent eosinophilic infiltration, and combined neutrophilic infiltration. Moreover, similar to human NPs, our murine model exhibits microcavity formation, epithelial ingrowths, within mucosal bulges. Finally, this model shares similar host susceptibility, specifically for the BALB/c genetic background, and is driven by external stimuli such as OVA and SEB and thus is antigen dependent.

Based on these strengths, we employed the murine CRS model herein to enhance our understanding of the role of environmental factors in NP pathophysiology. We hypothesized that the duration of allergen exposure would affect the immunologic characteristics of the nasal mucosa tissues and the expression of epithelial-derived cytokines induced by allergen exposure.

## MATERIALS AND METHODS

### Experimental Protocols

All animal experiments were approved by the IACUC of Boramae Medical Center (No. 2013-0001) and were performed under strict governmental and international guidelines on animal experimentation. Nasal polypoid lesions in mice were induced according to a previously established protocol.<sup>11</sup> Twenty-nine female BALB/c mice (4 weeks old, 20–25 g) were divided into three groups: phosphate-buffered saline (PBS)-treated (control group, n = 10), allergic CRS model (short-term group, n = 11), and long-term allergic CRS model (long-term group, n = 8) (Fig. 1). Briefly, in the control group, PBS was injected intraperitoneally and subsequently instilled intranasally. In the allergic CRS models, mice were injected intraperitoneally with 25  $\mu$ g OVA (Sigma, St. Louis, MO) in 2 mg aluminum hydroxide gel on days 0 and 5, followed by daily intranasal instillation with 3% OVA diluted in 40  $\mu$ L PBS from

day 12 to day 17. Thereafter, the same amount of 3% OVA was instilled three times per week from day 21 to day 102 (12 weeks, short-term group) or day 186 (24 weeks, long-term group). Intranasal instillation was performed in the head-down position, which was maintained for 30 seconds after instillation to prevent pulmonary provocation. In addition, mice were weekly challenged with 10 ng SEB (List Biological Laboratories Inc., Campbell, CA) from day 49 through day 102 (8 weeks, short-term group) or day 186 (20 weeks, long-term group) after OVA instillation. Short-term mice were sacrificed on day 103. And then, long-term and control mice were sacrificed on day 187. Mice were sacrificed 24 hours after the last OVA or PBS challenge. The heads of mice were removed en bloc and fixed in 4% paraformaldehyde for histopathologic analysis. After exposing the nasal cavities of the other mice, the nasal mucosae were meticulously excised using a small curette and microforceps under microscopic view.

### Histopathologic Analysis of Animal Tissues

For evaluation of nasal histopathology, nasal tissues were decalcified, embedded in paraffin, and sectioned coronally (4  $\mu$ m thickness) approximately 5 mm from the nasal vestibule. Several stains were conducted to compare characteristics for polypoid lesions, Masson's trichrome staining for collagen fibers in the subepithelial layer, Sirius red staining for eosinophils, anti-neutrophil antibody ([NIMP-R14] ab2557; 1:50; Abcam, Cambridge, UK) staining for neutrophils, anti-IL-25 antibody (1:300; Millipore, Darmstadt, Germany) staining for IL-25, and anti-thymic stromal lymphopoietin (TSLP) antibody (1:400; Abcam) staining for TSLP. In this study, we used an atlas of normal murine sinonasal anatomy to standardize the anatomic locations being examined according to the previous reports. Briefly, to evaluate the nasal respiratory mucosa without the olfactory one (see Supporting Figure 1 in the online version of this article), we first identified superior and inferior maxillary turbinates. Second, the true maxillary sinus was identified at the regions posterior to the two maxillary turbinates. At this time, three coronal sections that were similar to the sinus cavity were chosen for evaluation to rule out processing errors and confirm mucosal lesion. Finally, 10 areas of the nasal mucosal

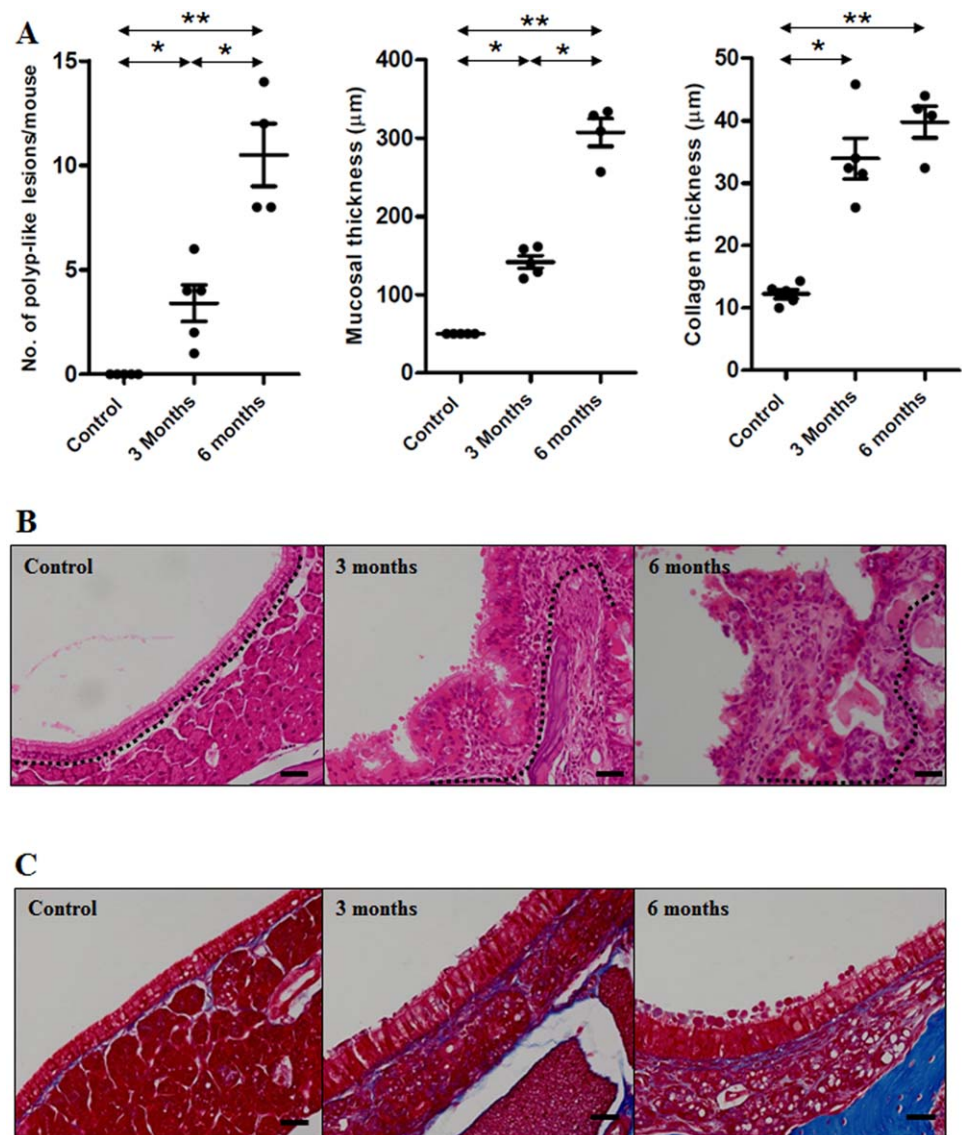


Fig. 2. (A) The number of nasal polypoid lesions, edematous mucosal thickness, and subepithelial collagen deposition in 10 different high-power fields (400 $\times$ ) were compared among the groups. Representative photographs for (B) polypoid lesion, edematous mucosal thickness, and (C) subepithelial collagen deposition on maxillary sinus mucosa from each group of mice (black scale bar = 20  $\mu$ m). Dotted lines represent the border between glandular structure and maxillary sinus mucosa.

sections in the maxillary sinus or lateral wall of the nasal cavity were chosen randomly for evaluation under high-power fields (HPFs) (400 $\times$ ) and measured by two examiners blinded to the groups. Polypoid lesions were defined as distinct mucosal elevations with eosinophilic infiltration and microcavity formation. The mucosal thickness was measured as the distance between the apex of the epithelial cells and the upper border of the subepithelial glands zone using an image analysis system (DP2-BSW software; Olympus, Tokyo, Japan). To assess the mucosal thickness, at least three measurements were made at random points with a minimum distance of 20  $\mu$ m between the points in the appropriate area of each HPF, and the mean of four HPFs was recorded for comparison.

#### Assessment of Cytokines From Mouse Nasal Lavage Fluid

Nasal lavage was performed according to the method previously described.<sup>16</sup> After partial tracheal resection under deep anesthesia, a micropipette was inserted into the posterior choana through the tracheal opening in the direction of the upper airway. Each nasal cavity was gently perfused with

200  $\mu$ L PBS, and the fluid from the nostril was collected and centrifuged. Supernatants were stored at  $-80^{\circ}\text{C}$ . Levels of interferon (IFN)- $\gamma$ , interleukin (IL)-4, IL-17A, and IL-10 in the nasal lavage fluids were measured using enzyme-linked immunosorbent assay (ELISA) kits (BioLegend, San Diego, CA). The lower detection limits of these ELISA kits were 8 pg/mL for INF- $\gamma$ , 0.5 pg/mL for IL-4, 2.7 pg/mL for IL-17A, and 2.7 pg/mL for IL-10.

#### Quantitative Real-Time Polymerase Chain Reaction From Mouse Nasal Tissue

Total RNA was extracted from tissue samples using the TRI reagent (Invitrogen, Carlsbad, CA). One microgram total RNA was reverse transcribed to cDNA using the cDNA Synthesis Kit (*amfiRivert Platinum* cDNA Synthesis Master Mix; GenDEPOT, Katy, TX). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the LightCycler 480 SYBR Green I Master (Roche, Mannheim, Germany). Primers for IL-4 (Mm01275139\_m1), IL-5 (Mm00434284\_m1), C-C motif chemokine ligand (CCL)-11 (Mm00441238\_m1), CCL-24 (Mm00444701\_m1), C-X-C motif chemokine ligand (CXCL)-1

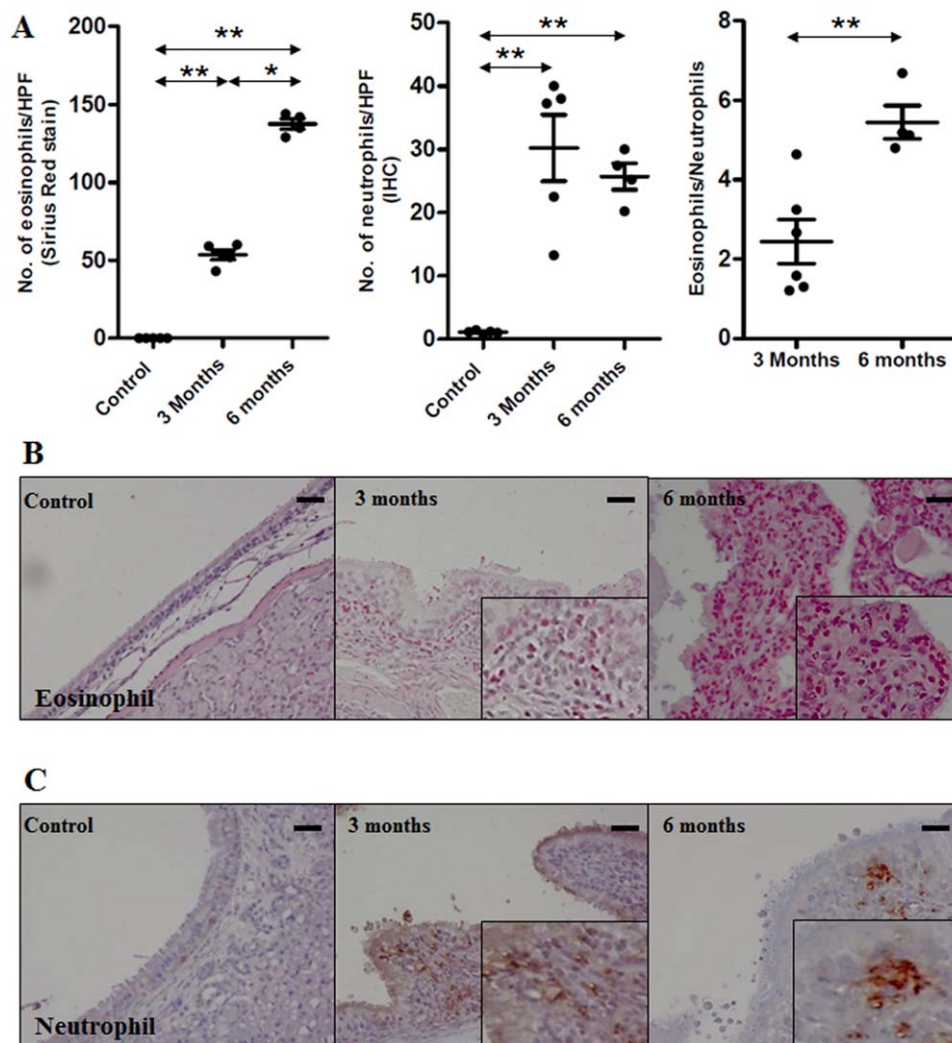


Fig. 3. (A) The numbers of infiltrated eosinophils, infiltrated neutrophils, and the ratio of eosinophils/neutrophils in 10 different high-power fields (HPF) (400 $\times$ ) were compared among the groups. Representative photographs (B) eosinophil and (C) neutrophil on maxillary sinus mucosa from each group of mice (black scale bar = 100  $\mu$ m). IHC = Immunohistochemistry.

(Mm04207460\_m1), CXCL-2 (Mm00436450\_m1), intercellular adhesion molecule (ICAM)-1 (Mm00516023\_m1), vascular cell adhesion molecule (VCAM)-1 (Mm01320970\_m1), matrix metalloproteinase (MMP)-7 (Mm00487724\_m1), tissue inhibitor of metalloproteinase (TIMP)-3; (Mm00441826\_m1), IL-25 (Mm00499822\_m1), TSLP (Mm01157588\_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mm99999915\_g1) were purchased from TaqMan Gene Expression Assay kits (Life Technologies Korea, Seoul, Korea). Predeveloped assay reagent kits containing primers and probes were purchased from Applied Biosystems (Foster City, CA). Expression of GAPDH was used as an internal control for normalization. Cycling conditions were as follows: 95 $^{\circ}$ C for 5 minutes followed by 60 cycles of 95 $^{\circ}$ C for 15 seconds, 60 $^{\circ}$ C for 20 seconds, and 72 $^{\circ}$ C for 20 seconds. To analyze the data, we used Sequence Detection Software version 1.9.1 (Applied Biosystems). Relative gene expression was calculated using the comparative  $2^{-\Delta\Delta CT}$  method.

### Statistical Analysis

Unpaired comparisons were made using the Kruskal-Wallis test and the Mann-Whitney  $U$  two-tailed test and were performed with GraphPad Prism software 6.0 (GraphPad Software Inc., La Jolla, CA). For comparisons between groups, the Kruskal-Wallis test was used to establish the significance of

intergroup variability. The Mann-Whitney  $U$  test was then used for between-group comparisons. The Spearman correlation test was used to determine variable relationships. A  $P$  value  $< .05$  was considered statistically significant.

## RESULTS

### Histologic Evaluation

To examine the expression of nasal polypoid lesions and collagen fiber deposits in the subepithelial layer for short-term and long-term CRS models, we performed H&E and Masson's trichrome staining. We found several polypoid lesions in the maxillary sinus and the junction between olfactory and respiratory epithelia for each CRS model. The number of polypoid lesions and the mucosal thickness significantly increased from the control group to the short-term CRS model to the long-term CRS model (Fig. 2A,B). The subepithelial layer was also considerably thicker in the short-term and long-term CRS models than in the control group (Fig. 2A,B). However, there was no difference between short-term and long-term CRS models. Furthermore, the number of eosinophils was highest in the long-term CRS model, whereas

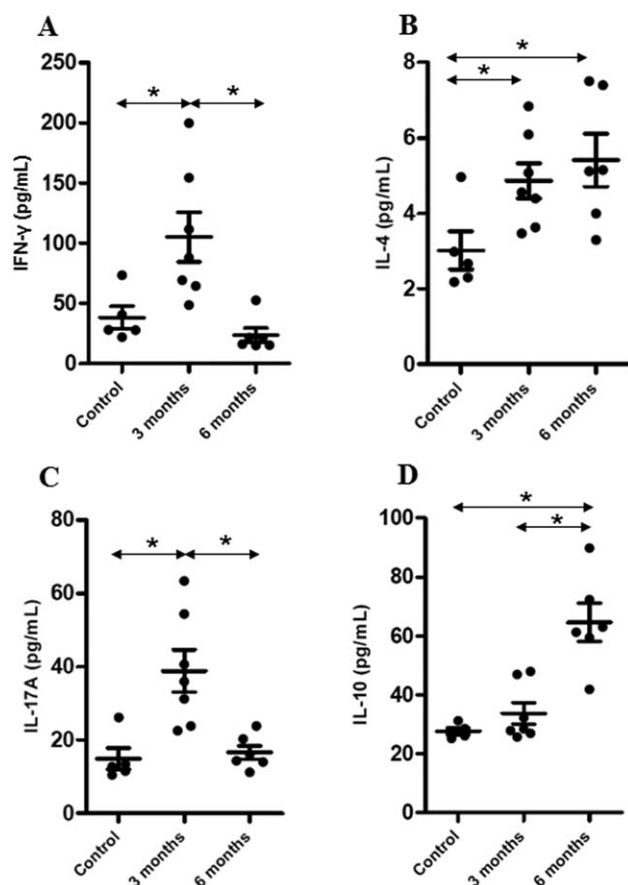


Fig. 4. Cytokine profile from nasal lavage fluids for each group. (A) interferon (IFN)- $\gamma$ , (B) interleukin (IL)-4, (C) IL-17A, (D) IL-10.

the neutrophil infiltration was higher in both short-term and long-term CRS models than in the control group (Fig. 3A–C). However, the eosinophil-to-neutrophil ratio was higher in the long-term CRS model than in the short-term CRS model (Fig. 3A).

#### Assessment of Cytokine Profile in Nasal Lavage Fluid by ELISA

To verify the immunopathologic features of each CRS model, the protein levels of major cytokines IFN- $\gamma$ , IL-4, IL-10, and IL-17A were evaluated in the murine model (Fig. 4). In the short-term CRS model, we observed increased expressions of IFN- $\gamma$ , IL-4, and IL-17A compared with the control group. Additionally, the protein levels of IL-4 and IL-10 were significantly increased in the long-term CRS group compared with the control group. The titers of IFN- $\gamma$  and IL-17A were lower in the long-term CRS model than in the short-term group.

#### Assessment of Nasal Tissues by RT-PCR

To validate the change in histopathology and major cytokine profiles between short-term and long-term CRS models, we evaluated the expression levels of eosinophil chemotactic cytokines (CCL-11 and CCL-24), neutrophil-

recruiting chemokines (CXCL-1 and CXCL-2), adhesion molecules (ICAM-1 and VCAM-1), and tissue remodeling markers (MMP-7 and TIMP-3) (Fig. 5). CCL-11, CCL-24, CXCL-1, CXCL-2, VCAM-1, and MMP-7 were upregulated in both short-term and long-term CRS models compared with control mice, but the long-term CRS model showed greater expression of CCL-24 than the short-term model and downregulation of TIMP-3.

#### Changes of IL-25 and TSLP Gene Expression in Each CRS Model

To further investigate the mechanism of Th2 skewing after prolonged allergen exposure, which is presumed to primarily stimulate the epithelium, the gene expression and protein levels of epithelial-derived cytokines, including IL-25 and TSLP, were measured using quantitative RT-PCR and immunohistochemistry. On the epithelial layer, TSLP expression was significantly upregulated in the long-term CRS model, whereas expression of IL-25 was higher in the short-term CRS model than in the control and long-term models (Fig. 6A–E). Interestingly, the expression of TSLP mRNA showed a positive correlation with that of IL-5 mRNA ( $r = 0.8754$  and  $P = .001$ ) and a negative association with that of IFN- $\gamma$  mRNA ( $r = -0.7212$ ,  $P = .023$ ) (Fig. 6F). Furthermore, association between IL-25 mRNA expression and TSLP was nearly significant in the short-term CRS model ( $r = 0.8286$ ,  $P = 0.058$ ) (Fig. 6G).

#### DISCUSSION

Animal models for human diseases can enhance our understanding of the pathophysiology of diseases and are useful for testing therapeutic targets in vivo. Since we first reported an animal model of allergic CRS,<sup>11</sup> multiple studies have employed this model to investigate nasal polypogenesis.<sup>12–15</sup> In the present study, we also utilized the murine model for allergic CRS with nasal polypoid lesions and extended it with two different protocols designed to vary the duration of allergen exposure (short term vs. long term).

In both animal models, we observed infiltration of mixed inflammatory cells (eosinophils and neutrophils); however, eosinophil infiltration and the eosinophil-to-neutrophil ratio were higher in the long-term CRS model than in the short-term model, which may resemble the inflammatory characteristics of eosinophilic CRSwNP in humans.<sup>4,5,17</sup> Our animal models showed minimal increase of IL-4 concentration compared to control, though this finding has statistical significance. To make this question clear, we investigated the mRNA expression of IL-4 in the mucosa of animal models, because previous study demonstrated that cytokine levels in nasal lavage may not be reliably representative of local inflammatory sites.<sup>18</sup> Interestingly, we could prove that IL-4 gene expression was upregulated more than 100-fold in the mucosa of CRS models compared with controls (see Supporting Figure 2 in the online version of this article). Moreover, we demonstrated downregulation of Th1/Th17 cytokines (IFN- $\gamma$  and IL-17A) and upregulation of CCL-24, an eosinophil chemotactic chemokine, in

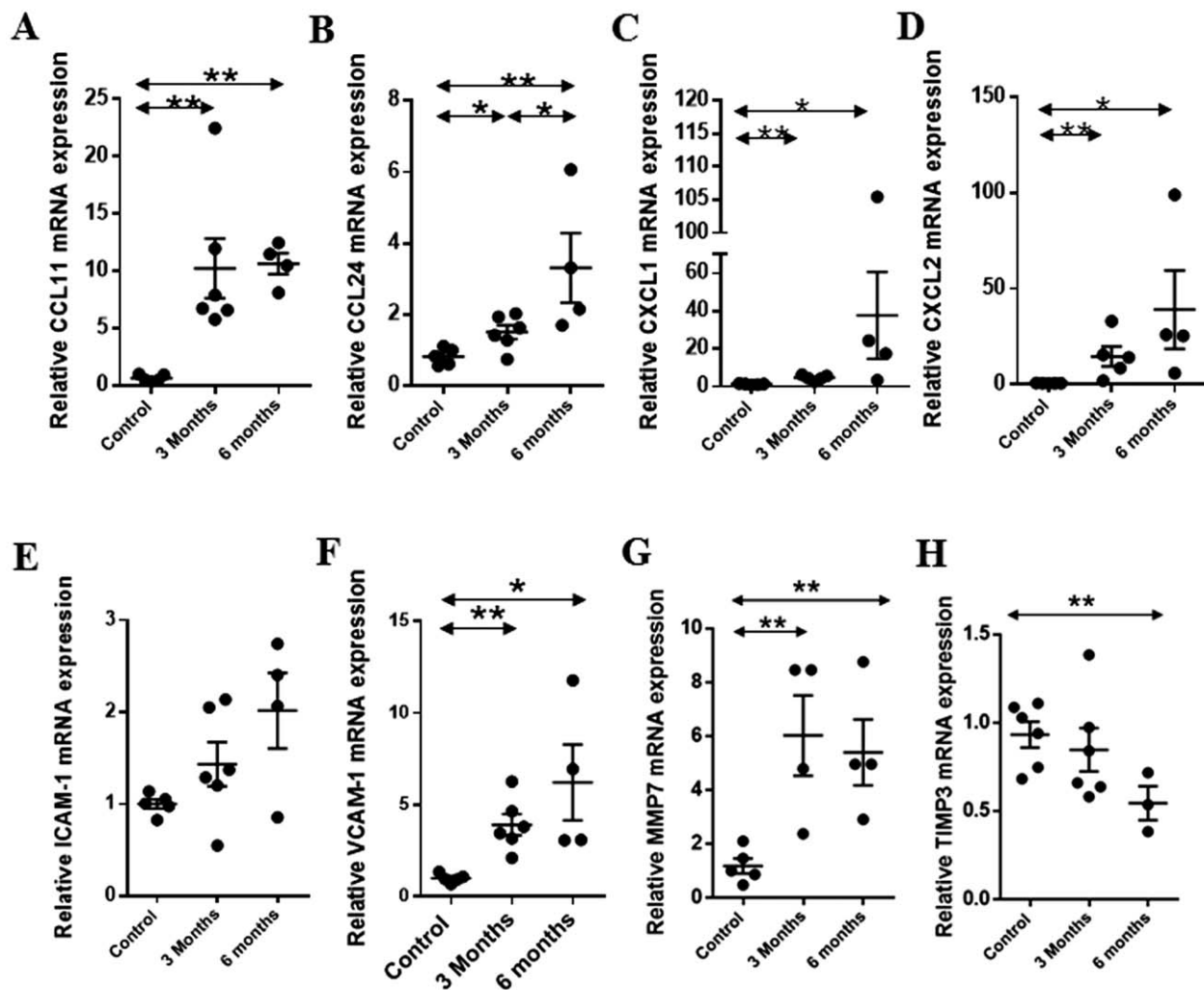


Fig. 5. Analysis of gene expression using quantitative real-time polymerase chain reaction from mouse nasal tissue for each group. (A) C-C motif chemokine ligand (CCL)-11, (B) CCL-24, (C) C-X-C motif chemokine ligand (CXCL)-1, (D) CXCL-2, (E) intercellular adhesion molecule (ICAM)-1, (F) vascular cell adhesion molecule (VCAM)-1, (G) matrix metalloproteinase (MMP)-7, (H) tissue inhibitor of metalloproteinase (TIMP)-3.

the long-term CRS model compared to that in the short-term model. Several studies previously reported that these molecules act as potent mediators by decreasing neutrophil recruitment and increasing eosinophil recruitment in human NPs.<sup>7-9</sup> However, the downregulation of Th1/Th17 cytokines (IFN- $\gamma$  and IL-17A) did not cause the decline of neutrophil recruitment in this study. These findings may be explained by upregulation of the neutrophil-recruiting chemokines (CXCL-1 and CXCL-2) and adhesion molecules (VCAM-1) in the long-term CRS model. On the other hand, increased IL-10 was observed in the long-term CRS model compared with the other groups. We thought this finding might be related to immune tolerance due to external stimuli. However, in this model, the mechanism of immune tolerance seems to be incomplete, because the number of inflammatory cells was still higher in the long-term CRS model than the short-term model. Considering IL-10 is upregulated with time, there is the possibility of immune tolerance in more long-term models.

Furthermore, previous studies reported that the balance between proteases and protease inhibitors is important for degradation of the extracellular matrix (ECM).<sup>19-21</sup> MMPs are ECM degrading enzymes that contribute to edema formation in CRSwNP, and TIMPs are tissue inhibitors of MMPs. Generally, decreased TIMP levels and increased MMP levels are observed in eosinophilic CRSwNP but not in noneosinophilic CRSwNP.<sup>17,19,22,23</sup> In the present study, we found that expression of MMP-7 was increased in both short-term and long-term CRS models, whereas TIMP-3 expression was decreased only in the long-term CRS model compared with the control group. These findings indicate that the long-term CRS model is closer to the remodeling pattern of eosinophilic CRSwNP, which has more extensive edematous formation compared to the noneosinophilic NPs.<sup>17,22</sup>

Innate cytokines (IL-25, IL-33, and TSLP) produced by human nasal epithelial cells in response to external stimuli such as allergens are currently thought to induce

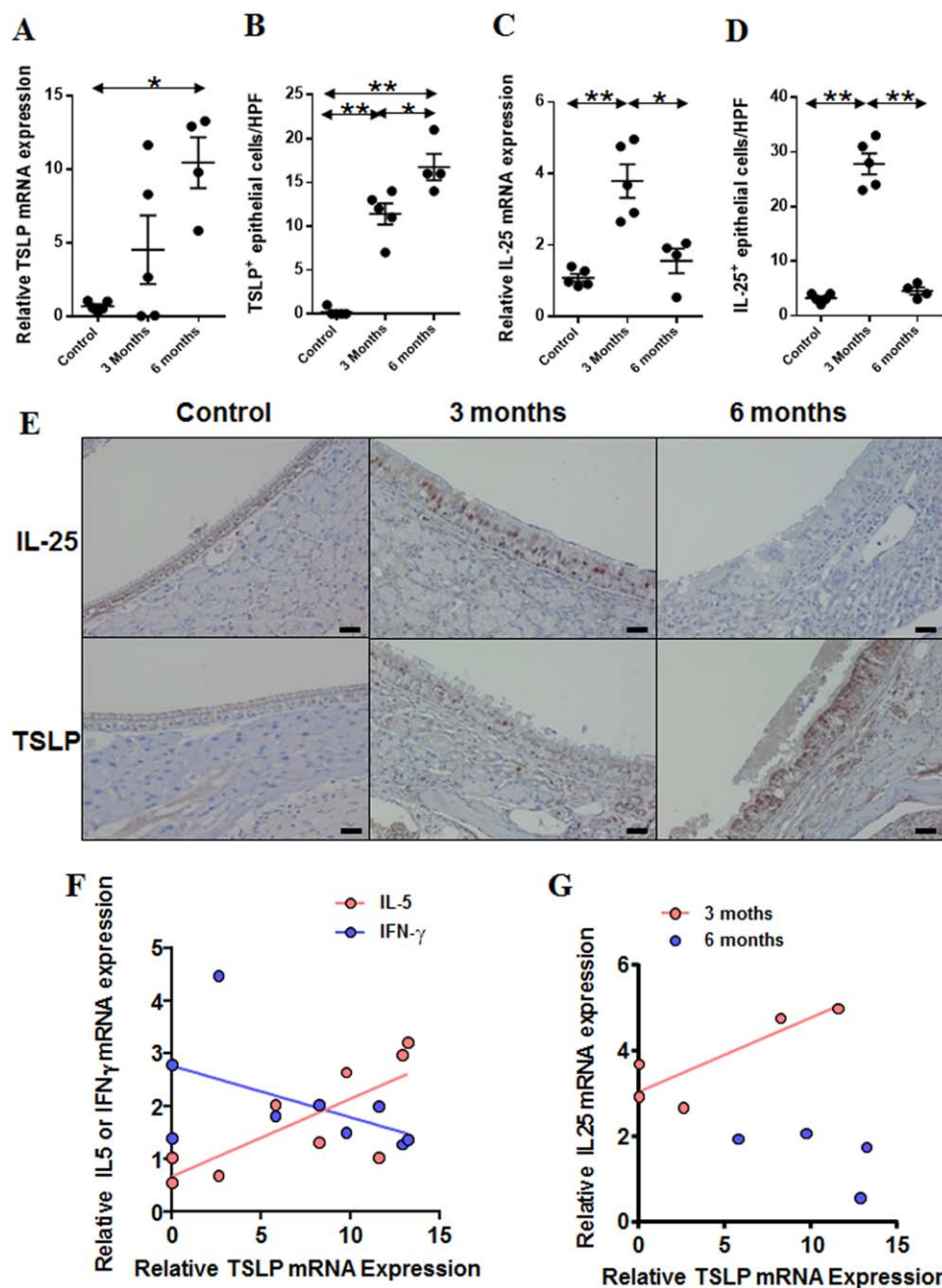


Fig. 6. Expression and correlation of thymic stromal lymphopoietin (TSLP) and interleukin (IL)-25 for each group. (A) TSLP mRNA expression, (B) TSLP<sup>+</sup> epithelial cells, (C) IL-25 mRNA expression, (D) IL-25<sup>+</sup> epithelial cells, (E) representative pictures of TSLP<sup>+</sup> or IL-25<sup>+</sup> epithelial cells, (F) correlation between mRNA expression of TSLP and IL-5/interferon (INF)- $\gamma$ , (G) correlation between mRNA expression of TSLP and IL-25. HPF = high-power fields.

Th2-type immune responses in patients with CRSwNP.<sup>14,24</sup> In this study, we evaluated the profiles of innate cytokines to investigate the mechanism by which prolonged allergen exposure changes from mixed to Th2 inflammation in murine models (Fig. 6) (see Supporting Figure 2 in the online version of this article). Interestingly, the expression of IL-25 was significantly increased in the short-term CRS model compared with the control, but there was no significant change in TSLP expression in this model. However, in the long-term CRS model showing more Th2 inflammatory pattern, we observed increased expression of TSLP with no change in IL-25 expression. Several studies have demonstrated that TSLP plays an important role in promoting the Th2

immune response cytokines and inhibiting IFN- $\gamma$  production.<sup>25–27</sup> Consistent with these studies, our CRS model exhibited positive correlation between TSLP mRNA expression and IL-5 levels and inverse correlation with IFN- $\gamma$  levels.

Expression of epithelial-derived cytokines may be associated with underlying inflammatory types in allergic airway diseases. Recent human studies revealed that IL-25 expression correlated with multiple inflammatory markers, including Th1/Th2/Th17 inflammation.<sup>14,28,29</sup> On the other hand, some reports demonstrated that eosinophilic NPs characterized by Th2 immune response exhibited upregulated TSLP expression and downregulated IL-25 expression.<sup>24,30,31</sup> In agreement with these

reports, our data showed increased IL-25 expression in the short-term CRS model characterized by mixed inflammation and increased TSLP expression in the long-term CRS model typified by Th2 inflammation. Correlation between IL-25 and TSLP in short-term models implies that IL-25 plays a role in inducing TSLP, leading to Th2 inflammation. Interestingly, a previous study reported that IL-25 enhanced dsRNA-induced TSLP production in primary human nasal epithelial cells.<sup>32</sup>

However, this animal model study has some limitations. First, the mouse has a relatively small area of pneumatized sinus structure. Second, olfactory epithelium mostly occupy mouse nasal mucosa, compared with respiratory epithelium. Third, although similar to the immunohistologic characteristics, gross appearance of mouse polypoid lesion is quite different in human nasal polypoid appearance. Lastly, this CRS model is allergen-driven, so it cannot explain all the CRS subtypes because CRS is often not associated with atopy. Therefore, translating animal data into human disease should be carefully considered. Correlation between allergen exposure and inflammatory pattern using human tissues is also required to validate our animal data. In spite of these limitations, our results suggest that allergen exposure upregulates IL-25 expression at a relatively early stage, which may induce levels of TSLP. Persistent allergen exposure may lead to upregulation of TSLP expression. We hypothesize that this mechanism contributes to strong Th2-skewed inflammation at the advanced stage of upper airway inflammation. Dynamic innate cytokine expression based on the duration of allergen exposure may influence inflammatory patterns.

## CONCLUSION

In the present study, we found that the histologic and immunologic characteristics of a murine model of allergic CRS were altered depending on the duration of allergen exposure. Moreover, we demonstrated that prolonged allergen exposure induced dominant eosinophilic inflammation with Th2 skewed inflammation with increased TSLP expression.

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