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Intralymphatic treatment of flagellin-ovalbumin mixture reduced allergic inflammation in murine model of allergic rhinitis.

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Running title: Intralymphatic treatment of flagellin in mouse model of allergic rhinitis

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Authors Contribution

Substantial contributions to conception and design of this study: JHM, YJC; Acquisition of data: EHK, JHK, RS, , JSB, YJC, PSC, JHM; Analysis and interpretation of data: EHK, JHM; Drafting the article: EHK, JHM; Revising it critically for important intellectual content: PSC, JHM; Final approval of the version to be published: PSC, JHM

Abstract

Background: Bacterial flagellin, a Toll-like receptor 5 agonist, is used as an adjuvant for immunomodulation. In this study, we aimed to evaluate the effect and its mechanism following intralymphatic administration of OVA-flagellin (FlaB) mixture in the mouse model of allergic rhinitis.

Materials and Methods: BALB/c mice were sensitized with OVA and treated with an OVA-FlaB mixture via intranasal, sublingual and intralymphatic routes to evaluate the effect of each treatment. Several parameters for allergic inflammation and its underlying mechanisms were then evaluated.

Results: Intralymphatic injection of the OVA-FlaB mixture reduced symptom scores, eosinophil infiltration in the nasal mucosa and total and OVA-specific IgE levels more significantly than intranasal and sublingual administration. Systemic cytokine (IL-4, IL-5, IL-6, IL-17 and IFN- γ) and local cytokine (IL-4 and IL-5) production were also reduced significantly after intralymphatic injection with OVA-FlaB. Double intralymphatic injection of the mixture was more effective than single injection. Moreover, the expression of innate cytokines such as IL-25 and IL-33 in nasal epithelial cells were reduced and the expression of chemokines such as CCL24 (eotaxin-2), CXCL1 and CXCL2 was decreased in the nasal mucosa, suggesting the underlying mechanism for intralymphatic administration of the OVA-FlaB mixture.

Conclusion: Intralymphatic administration of an OVA-FlaB mixture was more effective in alleviating allergic inflammation than intranasal and sublingual administration in a mouse model of allergic rhinitis. This effect may be attributed to the reduced expression of innate cytokines and chemokines. This treatment modality can be considered as a new therapeutic method and agent.

Keywords: Allergic rhinitis; flagellin; intralymphatic injection; mouse model; innate cytokine; chemokine

Toll-like receptors (TLRs) are type 1 transmembrane signaling molecules that recognize pathogen associated molecular patterns or microorganism associated molecular patterns and are usually expressed in innate immune cells(1). Among several TLRs, TLR5 recognizes bacterial flagellin, which is a major protein in bacterial flagellum. *Vibrio vulnificus* is a bacterium that causes severe septicemia when it invades the blood stream through the gastrointestinal mucosa. It usually enters the human body when eaten with contaminated raw fish or shells and causes fever, chills, malaise, nausea or vomiting. *Vibrio vulnificus* has 6 flagellin genes named FlaA to FlaF and FlaB is one of the most important genes responsible for its immunomodulatory effect (2).

The effect of flagellin on airway inflammation has been reported several times, however, its effect on allergic inflammation is still controversial.(3, 4). While Lee *et al.* reported an anti-allergic effect in the mice model of allergic asthma with intranasal administration of a flagellin-OVA mixture (3), Wilson *et al.* reported that flagellin promotes asthma by priming allergic responses to indoor allergens (4), showing conflicting results and necessitating the verification of the immunomodulatory effect of flagellin.

In addition, several administration routes such as intranasal, sublingual, subcutaneous and intralymphatic are used for allergen administration these days. Among them, the intralymphatic route is used for direct administration into the regional lymph node and has recently drawn more attention. This method was reported to induce a stronger cytotoxic T cell response and greater immunogenicity than other routes even with smaller amounts of allergen(5). However, this method has been rarely studied and the effects of intralymphatic administration need to be verified.

Hence, we aimed to evaluate the effect of intralymphatic administration of an OVA-flagellin mixture compared to other routes and to reveal its mechanism in the mouse model of allergic rhinitis.

METHODS

Animals and flagellin preparation

Four-week-old female BALB/c mice were obtained from Nara biotechnology Co. (Seoul, Korea). All animal experiments conducted in this study followed the guidelines and ethics of Institutional Animal Care at the Clinical Research Institute of Dankook University (DK-13-040). Recombinant flagellin B of *vibrio vulnificus* (FlaB) was purified and endotoxin was removed as described previously (2).

Murine allergic rhinitis model and treatment

Mice were divided into several groups according to the protocol and each group consisted of ten mice (Fig. 1A and 2A). The procedures for allergen sensitization and treatment are summarized in Fig. 1A. Briefly, mice were sensitized with an intraperitoneal injection of 25 µg of ovalbumin (OVA; grade V; Sigma, St. Louis, MO) and 1 mg of aluminum hydroxide gel on days 0, 7, and 14. After general sensitization, mice were locally challenged with 100 µg of OVA into their nostrils from day 21 to day 27. Along with sensitization and challenge, selected groups of mice were treated with an administration of the mixture of OVA and FlaB via intranasal, sublingual or intralymphatic route. For intranasal or sublingual administration, 10 µg of FlaB and 10 µg of OVA was used and 1 µg of FlaB and 10 µg of OVA was used for intralymphatic administration. In sublingual administration, OVA-FlaB mixture was sustained in the oral cavity for more than 10 minutes after sedation. For intralymphatic injection, mice were sedated with subcutaneous injection of 2 mg of

zoletil[®] and 0.2 mg of Rompun[®], and cervical lymph node was located after skin incision around neck. Mixture of OVA-FlaB was then injected into the lymph node and skin was sutured (supplementary Fig. 1). For optimization of intralymphatic administration, two different intralymphatic injections of the FlaB-OVA mixture, single and double injection, were performed after general sensitization as shown in protocol (Fig. 2A).

Symptom score and tissue preparation

Fifteen minutes after final OVA challenge on day 27, a blinded observer recorded the frequencies of sneezing and nasal rubbing over a 15-minute interval. Mice were then euthanized 24 hours after the last OVA challenge. After perfusion with 4% paraformaldehyde, the heads of five mice from each group were removed *en bloc* and then fixed in 4% paraformaldehyde. Nasal mucosa was obtained from the mice for reverse transcription-polymerase chain reaction (RT-PCR) and Western blot, and was immediately immersed in liquid nitrogen and stored at -70°C until use.

Eosinophils in the nasal septal mucosa

For evaluation of nasal histology, nasal tissues were decalcified, embedded in paraffin, and sectioned coronally (4 μm thick) approximately 5 mm from the nasal vestibule. Each section was stained with hematoxylin and eosin or Sirius Red, and the number of eosinophils on both sides of the septal mucosa was counted. The number of eosinophils in the submucosal area of the whole nasal septum was counted under a light microscope ($\times 400$ magnification).

Real-time RT-PCR for IL-4, IL-10, and IFN- γ in the nasal mucosa

Total RNA was prepared from the nasal mucosa with TriZol reagent (Invitrogen, Carlsbad, CA). Complementary DNA (cDNA) was synthesized using Superscript reverse transcriptase (Invitrogen) and oligo(dT) primers (Fermentas, Burlington, ON, Canada). For analysis of IL-4 (Mm00445258_g1), IL-10 (Mm00439616_m1), IFN- γ (Mm99999071_m1), and GAPDH (Mm03302249_g1), PDAR (Pre-Developed Assay Reagent) kits of primers and probes were purchased from Applied Biosystems (Foster City, CA). Amplification of IL-4, IL-10, IFN- γ , and GAPDH cDNA was carried out in MicroAmp optical 96-well reaction plates (Applied Biosystems). The reaction was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The average transcript levels of genes were then normalized to GAPDH.

Measurement of cytokines (IL-4, IL-5, IL-6, IL-17 and IFN- γ) in the spleen cell culture

Spleen single-cell suspensions were plated in 96-well tissue culture plates at a final concentration of 10^5 cells/mL using AIM-V media (Gibco, Grand Island, NY, USA). The cells were incubated in a CO₂ incubator at 37°C for 72 hours, stimulated with OVA (1 mg/ml) for 72 hours, and stored at -70°C until cytokines were measured. Cytokines were assayed in culture supernatant using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. After measuring the optical density (OD) at 450 nm, the concentrations of IL-4, IL-5, IL-6, IL-17 and IFN- γ were determined by interpolation from a standard curve and all data were expressed as nanograms per milliliter.

Serum levels of total and OVA-specific immunoglobulin (Ig) E

Serum levels of total and OVA-specific IgE were measured by solid-phase ELISA. Serum samples collected from mice at the time of death were serially diluted and added to 96-well plates coated with 2 µg/ml of purified anti-mouse IgE mAb (clone R35–72; BD Pharmingen, San Jose, CA). A purified mouse IgE isotype (27-74; BD Pharmingen) was used as a standard for total IgE. Nonspecific antigen-antibody reactions were blocked with 3% bovine serum albumin in PBS. To detect total IgE, HRP-conjugated anti-mouse IgE (1 µg/ml, 23G3; Southern Biotechnology, Birmingham, AL) was added to the plate. To detect OVA-specific IgE, 100 µg/ml of OVA was coated, followed by biotinylated Anti-Mouse IgE monoclonal antibody (2 µg/ml ,51-26552E; BD Pharmingen, Inc., San Jose, CA) and Streptavidin-horseradish peroxidase conjugate (BD Pharmingen, Inc., San Jose, CA). The reactions were developed using 3,3',5,5'-tetramethylbenzidine (Moss Inc., Belfast, ME) and terminated by adding 2 N H₂SO₄. The OD was recorded by a luminometer (iEMS Reader; Labsystems, Helsinki, Finland) set at 450 nm. The endpoint titers of OVA-specific IgE are expressed as the reciprocal log₂ of the last dilution of a sample that resulted in an OD value that was 0.1 higher than background.

Immunohistochemistry (neutrophil, eotaxin-2, CXCL1, CXCL2, IL-25 and IL-33)

Nasal mucosa sections were immunostained with Eotaxin-2, CXCL1, CXCL2 , IL-25 and IL-33 using the avidin–biotinylated–horseradish peroxidase (HRP) complex kits (Vector Laboratories, Burlingame, CA, USA). The following antibodies were used; neutrophil (NIMP-R14; 1:100, 1 µg/mL, Abcam®, UK), IL-25 (IL-17E, 1:100, 5 µg/mL, Thermo Fisher Scientific Inc., Rockford, USA), IL-33 (1:40, 5 µg/mL, R&D system, Minneapolis, MN) , Eotaxin-2 (Mouse CCL24/Eotaxin-2/MPIF-2 antibody; 1:1000, 0.2 µg/mL, R&D Systems, MN, USA), CXCL-1 (Anti-GRO alpha antibody; 1:1000, 0.5 µg/mL, Abcam®, UK) and

CXCL-2 (Rabbit anti mouse MIP-2; 1:50, 2 µg/mL, Bio-rad Laboratories Inc., CA, USA).

The detailed procedure was described in our previous research study (6). To score the expression levels, we observed 3 separated high-power fields (HPF) per slide. Three different spots were randomly selected in every HPF, and two independent examiners blinded to the experimental group determined whether IL-25 or IL-33 was expressed in each spot or not.

Western blot for CXCL1, CXCL2, and eotaxin-2 in the nasal mucosa

Protein was obtained from the nasal mucosa of each mouse 24 hours after the final nasal challenge using lysing buffer (0.5% Triton X-100, 150 mM NaCl, 15 mM Tris (pH 7.4) 1 mM CaCl₂, 1 mM MgCl₂). Protein concentrations were determined using BCA protein assay reagent (Thermo Fisher Scientific, Waltham, MA). Samples (95 µg protein per lane) were separated on 8% to 16% Tris-Glycine mini gels (NOVEX, San Diego, CA) and transferred onto PVDF membranes (Amersham Biosciences, Piscataway, NJ). Eotaxin-2 (1:1000; 0.2 µg/mL, R&D system, MN), CXCL-1 (1:1000, 0.5 µg/mL, Abcam®, UK), CXCL-2 (1:50; 2 µg/mL, Bio-rad Laboratories Inc., Hercules, CA, USA), β-actin (1:1000; 0.1 µg/mL, Santa Cruz, CA) antibodies were used as primary antibodies. The membrane was then immunoblotted with a secondary anti-chicken IgY-HRP, secondary anti-goat IgG-HRP, or secondary anti-mouse IgG-HRP (Santa Cruz Biotechnology). The blots were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific).

Quantification of Western blots was performed using the ImageJ software from the National Institutes of Health.

Statistical analysis

Results are shown as the mean $\pm$ SEM. A Mann-Whitney U test was used to compare results between groups. A *p*-value less than 0.05 was considered statistically significant.

RESULTS

The effects of FlaB-OVA mixture treatment according to the route of administration

To determine the effects of the FlaB-OVA mixture according to the route of administration, several administration routes such as intranasal, sublingual and intralymphatic administration were employed following the same protocol (Fig. 1A).

-Symptom score

The nasal symptom scores in the positive control group (group B) were significantly elevated compared to those in the negative control group (group A). The nasal symptom score was significantly lower in the intralymphatic treatment groups compared to the positive control group (both $P < .05$). However, the nasal symptom score in the sublingual and intranasal treatment groups did not change significantly compared to the positive control group (Fig. 1B).

- Total and OVA-specific serum IgE levels

We also evaluated the effects of flagellin treatment on humoral immunity. Total IgE levels and OVA-specific IgE levels decreased significantly only in the intralymphatic treatment group compared to the positive control group (both $P < .05$). These results showed that the intralymphatic treatment group had a greater decrease in both total and OVA-specific IgE levels than the intranasal or sublingual treatment groups (Fig. 1C).

- Histology of nasal mucosa and eosinophil and neutrophil infiltration

Eosinophil count in the positive control group was 188.8 ± 47.7 , which was significantly elevated compared to the negative control group (4.0 ± 2.9 , $P < .001$). Eosinophil counts in the intranasal and sublingual treatment group were 159.5 ± 24.09 and 165.3 ± 73.63 , respectively, slightly reduced but without statistical significance ($P = 0.016$) compared to the positive control (group B). In the intralymphatic treatment group, eosinophil count was significantly decreased (94.3 ± 42.8 , $P < 0.05$), showing decreased eosinophil migration in the nasal mucosa. Sirius red staining showed markedly decreased eosinophil infiltration in the intralymphatic treatment group compared to the positive control group (**Fig. 1D**). Neutrophil infiltration was also enumerated and showed a pattern similar to the eosinophil counts. Neutrophil number in the positive control group was significantly elevated compared with the negative control group. Neutrophil count did not change in the intranasal and sublingual treatment group, but was significantly decreased in the intralymphatic treatment group compared to the positive control group ($P < .05$, Fig. 1E)

- Cytokines in splenocytes culture

Expression of the Th2 cytokine, IL-4 and IL-5 significantly decreased in the spleen cell culture of all treatment groups (Fig. 1F). The intralymphatic treatment group showed a greater decrease in IL-4 than the intranasal and sublingual treatment groups, which is consistent with the observed symptom scores and histology. Expression of the Th1 cytokine, IFN- γ , and Th17 cytokine, IL-17, also decreased significantly in all treatment groups (Fig. 1F).

Optimization of intralymphatic treatment protocol

Since we determined that intralymphatic treatment is more efficacious than intranasal and sublingual administration of the FlaB-OVA mixture, the optimal dosage for intralymphatic administration was evaluated. Two different intralymphatic injections of the FlaB-OVA mixture were performed after general sensitization as shown in protocol (Fig. 2A).

The nasal symptom score decreased in both treatment groups ($P<.05$, Fig. 2B). Total and OVA-specific IgE levels were evaluated and both were more significantly reduced in the double administered group, which is consistent with eosinophil infiltration and cytokine levels (Fig. 2C). Eosinophil infiltration also decreased significantly in all treatment groups and the double administered group showed a greater decrease than the single administered group ($P<.01$, Fig. 2D). Systemic cytokine levels also showed results consistent with the histology. Th2 cytokines, IL-4 and IL-5 decreased significantly in the single and double administered groups and the decrease in IL-5 was more significant in the double administered group than in the single administered group. IL-6, IL-17 and IFN- γ showed a similar pattern, showing a decrease in the single and double administered groups compared to the positive control group (Fig. 2E).

Cytokine expression in the nasal mucosa

Since we knew that double treatment was more efficacious in reducing inflammation, double administration of FlaB was used for further experiments to explore the underlying mechanism (Fig. 3A). To evaluate the local cytokines in the nasal mucosa, mRNA expression of IL-4, IL-5, IL-6, IL-17A and IFN- γ was evaluated. IL-4, IL-5 and IL-6 were significantly decreased in the intralymphatic treatment group. IL-17 did not show much change compared with the positive control group whereas IFN- γ was decreased significantly compared with the positive control group (Fig. 3B).

Chemokines

Chemokines and adhesion molecules are necessary for the migration of effector cells into tissues. To evaluate the mechanisms behind decreased eosinophil and neutrophil infiltration (Fig. 1D, 1E and 2D), the mRNA expressions of chemokines and adhesion molecules such as eotaxin-1, eotaxin-2, CXCL1, CXCL2, VCAM1 and ICAM1 were measured in the nasal mucosa of each group using qPCR. The expression of eotaxin-1 and eotaxin-2 was increased significantly in the positive control group and only eotaxin-2 was decreased significantly in the intralymphatic treatment group (Fig. 3C). Neutrophil chemokines, CXCL1 and CXCL2, were not induced in the positive control group and they were decreased significantly in the intralymphatic treatment group. For VCAM1 and ICAM1, there were no significant differences among the 3 groups (Fig. 3C). To confirm the mRNA expression, eotaxin-2, CXCL1 and CXCL2 expressions were investigated using immunohistochemistry and Western blot. Eotaxin-2 was stained mainly in the subepithelial layer and multiple cells were positively stained. Quantitative analysis showed that the number of eotaxin-2 positive cells increased significantly in the positive control group and decreased significantly in the intralymphatic treatment group (Fig. 3D). CXCL1 was mainly stained in the epithelial layer and CXCL1 expression was decreased in the intralymphatic treatment group (Fig. 3D). CXCL2 immunohistochemistry showed decreased expression in the intralymphatic treatment group (Fig. 3D) and Western blot confirmed decreased expression of those chemokines in intralymphatic treatment group (Fig. 3E). Collectively, these results showed decreased expression of eotaxin-2, CXCL1 and CXCL2 in the nasal mucosa, which might be associated with the mechanism of decreased inflammatory cell infiltration in the intralymphatic treatment group.

Expression of IL-25 and IL-33 in the nasal mucosa

Recently, innate cytokines were studied thoroughly and the relationship between allergic diseases and innate cytokines has been reported. Hence, we evaluated innate cytokines such as IL-25 and IL-33 using immunohistochemistry. We found that the expressions of both innate cytokines, IL-25 and IL-33, were increased in the positive control group and were decreased significantly in the intralymphatic treatment group, suggesting that the innate cytokines were down-regulated by intralymphatic FlaB-OVA treatment. Quantitative analysis also confirmed decreased IL-25 and IL-33 positive cells in the intralymphatic treatment group (Fig. 3F).

DISCUSSION

Flagellin is a structural component of bacterial flagella that activates TLR5 and the intracellular pattern recognition receptor Ipaf (1). Several studies have revealed the effectiveness of flagellin in terms of adjuvants as well as its ability for immunomodulation (2, 7-9). Although several flagellin-based vaccines for infectious diseases have already entered clinical trials, the potential uses of flagellin as an adjuvant for immunomodulation needs to be further explored.

The present study clarified the effects of intralymphatic treatment with a flagellin-OVA mixture in an allergic rhinitis mouse model, and demonstrated an important role for intralymphatic administration of flagellin-OVA mixture in Th2 responses in the mouse model of allergic rhinitis. Intralymphatic treatment with the flagellin OVA mixture decreased the allergic symptom score. This anti-allergic effect could be associated with not only the inhibition of Th2 cytokines and eosinophil infiltration, but also with decreased innate cytokines and chemokines in the nasal mucosa.

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First, we compared the effects of the OVA-flagellin mixture applied via several administration routes such as intranasally, sublingually and intralymphatically. We used more flagellin (10 µg) for intranasal and sublingual administrations than for intralymphatic administration (1 µg), since more exposure to flagellin and antigen can occur with intralymphatic administration than with the other routes. As lymph nodes contain a high number of professional antigen presenting cells such as dendritic cells, direct administration of the antigen into the lymph node should be the most efficient route (10). Johansen *et al.* also showed that intralymphatic injection of MHC class I-binding peptide enhanced immunogenicity by as much as 10^6 times compared to subcutaneous and intradermal vaccination, showing implications for clinical immunotherapy (5). Among the three routes we compared, the intralymphatic route showed the most consistent anti-allergic effect in the mouse model for allergic rhinitis. Although intranasal and sublingual administrations also reduced cytokines such as IL-4, IL-5 and IL-17 in the spleen cell culture, the allergic symptom score, inflammatory cell infiltration and ovalbumin specific IgE decreased only in the intralymphatic administration group, suggesting higher efficacy for intralymphatic administration in terms of anti-allergic effects.

Although several groups have reported the immunomodulatory effects of flagellin, its immunomodulatory effect on allergic inflammation showed some contradictory results depending on experimental setting. Lee *et al.* reported that intranasal administration of the OVA-flagellin mixture reduced allergic inflammation in an OVA induced allergic asthma model (3). Schulke *et al.* reported that the fusion protein of flagellin and ovalbumin suppresses Th2 response and prevents intestinal murine allergy (8). However, Wilson *et al.* claimed that flagellin promotes asthma by priming allergic responses to indoor allergen, showing proinflammatory results in the animal model (4). An *in vitro* study also showed that flagellin induces the expression of thymic stromal lymphopoietin, which is related to Th2

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inflammation, in human keratinocytes, implying that flagellin may increase Th2 inflammation (11). Previous studies claimed that flagellin could prime Th1 responses (12, 13), Th2 responses (14) or both Th1 and Th17 responses (15), depending on flagellin dosage, preparation and route of administration. However, it is not clear in which conditions flagellin can induce immunomodulatory effects in a specific direction for Th1, Th2, or Th17 and these should be verified.

Chemokines and adhesion molecules, such as eotaxin-1, eotaxin-2, VCAM and ICAM are important for eosinophilic infiltration. In this study, the expression levels of eotaxin-1, ICAM-1 and VCAM-1 did not decrease in the intralymphatic treatment group. Only Eotaxin-2 was markedly induced in the positive control group and was reduced in the treatment group. A recent study showed that eotaxin-2 had a more dominant role with airway eosinophilia than eotaxin-1, which supports our data (16). The observed decreased expression of eotaxin-2, confirmed through immunohistochemistry and Western blot, might suggest that intralymphatic injection of the OVA-FlaB mixture prevents recruitment of eosinophil in the nasal mucosa. In addition, CXCL1 and CXCL2 play important roles in attracting neutrophil infiltration (17). Our study showed that both CXCL1 and CXCL2 were downregulated in the intralymphatic administration group, which is consistent with the decreased neutrophil infiltration in the intralymphatic administration group.

IL-25, also known as IL-17E, is a cytokine that belongs to the IL-17 superfamily and may be the proinflammatory cytokine favoring Th2 immune responses. Th2 cells, mast cells and epithelial cells secrete IL-25. We showed that epithelial and subepithelial expression of IL-25 was increased in the positive control group and that intralymphatic treatment significantly suppressed the expression, suggesting another anti-allergic mechanism for intralymphatic treatment. IL-33 is a dual-function cytokine that functions as an intracellular regulator of gene expression and also as an alarm mediator (18) that initiates sterile

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inflammation in response to cellular destruction. A recent report showed that nuclear IL-33 sequesters nuclear NF- κ B and reduces NF- κ B-triggered gene expression to dampen proinflammatory signaling, suggesting that nuclear expression of IL-33 reduces proinflammatory cytokines (19). Immunohistochemistry of IL-33 showed that it was highly expressed in the nucleus in the negative control group. Its expression was increased not only in the cytosol of epithelial cell but also in the subepithelial layer in the positive control group, which is consistent with the previous report that nuclear IL-33 is decreased in inflamed tissue in the positive control group and slightly increased in the intralymphatic treatment group. A wide variety of cells including mast cells, dendritic cells, macrophages, endothelial cells and epithelial cells can express IL-33 and our study showed that IL-33 was expressed in both epithelial and subepithelial inflammatory cells.

There was a caveat in this study that the surgical procedure performed in intranasal group could be a confounding factor. To address this caveat we performed mouse experiment using intralymphatic PBS treatment (Supplementary Fig. 2). However, intralymphatic PBS treatment group showed almost similar result with positive control group in every respect including symptom score, Total and OVA-specific IgE, eosinophil infiltration in the nasal mucosa and splenic cytokines, suggesting that the surgical procedure of intralymphatic injection is not a confounding factor.

In summary, intralymphatic administration of OVA-FlaB mixture was more effective in inhibiting symptoms and other allergic parameters in a mouse model of allergic rhinitis than intranasal and sublingual administration. This effect can be attributed not only to decreased Th2 cytokine release and to total and OVA-specific IgEs secretion from B cells but also to reduced expression of innate cytokines such as IL-25 and IL-33 as well as chemokines. Thus, this treatment modality can be considered as a new therapeutic method and agent.

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Figure legends

Figure 1 Intralymphatic administration of FlaB-Ovalbumin mixture reduced allergic inflammation in mouse model of allergic rhinitis **A.** Experimental protocol. BALB/c mice were sensitized with ovalbumin (OVA) and 1 mg of aluminum hydroxide gel (Alum) on days 0, 7, and 14 (general sensitization). All groups except for group A received intranasal OVA from day 21 to day 27. In addition to sensitization and challenge, selected groups of mice were treated with a FlaB-OVA mixture via different routes of administration (group C: intranasal, group D: sublingual, group E: intralymphatic) 3 hours before intranasal OVA challenge. **B.** Symptom score of nasal rubbing and sneezing for 15 minutes. Intralymphatic administration of FlaB-OVA mixture suppressed allergic symptoms of rubbing and sneezing. **C.** Intralymphatic administration of the FlaB-OVA mixture suppressed total and OVA-specific Immunoglobulin. **D.** Intralymphatic administration of FlaB-OVA mixture suppressed eosinophil infiltration in the nasal mucosa ($\times 400$ magnification; Scale bar, 25 μm). Eosinophil counts in the nasal mucosa. Eosinophil count was significantly reduced in treatment group E compared to the positive control (group B) and the other administration groups (groups C and D)). **E.** Neutrophil infiltration was also significantly reduced in the intralymphatic administration group ($\times 400$ magnification; Scale bar, 25 μm). **F.** Systemic cytokine production from spleen cell culture. Interleukin IL-4, IL-5, IL-6, IL-17 and IFN- γ were reduced in all treatment groups. IL-4 was reduced more significantly in the intralymphatic treatment group. FlaB; flagellin B, IN; intranasal, SL; sublingual, IL; intralymphatic, (-) con; negative control group, (+) con; positive control group

Figure 2 Double intralymphatic injection of FlaB-ovalbumin mixture was more effective than single injection in reducing allergic inflammation. **A.** Experimental protocol. **B.** Symptom score of nasal rubbing and sneezing for 15 minutes. Intralymphatic administration

of FlaB-OVA mixture suppressed allergic symptoms of rubbing and sneezing. **C.** Total and OVA-specific Immunoglobulin levels. The double injection group had significant reduction of OVA-specific IgE. **D.** Eosinophil counts in the nasal mucosa ($\times 400$ magnification; Scale bar, 25 μm , Sirius red staining). Eosinophil count was significantly reduced in both the single and double injection group compared to the positive control (group B). The double injection group had more reduction than the single injection group ($\times 400$ magnification; Scale bar, 25 μm). **E.** Systemic cytokine production from spleen cell culture. Both single and double injection of the FlaB-ovalbumin mixture significantly reduced Interleukin IL-4, IL-5, IL-6, IL-17 and IFN- γ . IL-5 was more significantly reduced in the double injection group. FlaB; flagellin B, IL; intralymphatic, (-) con; negative control group, (+) con; positive control group

Figure 3 Intralymphatic administration of FlaB-ovalbumin mixture suppressed production of cytokines, chemokines and innate cytokines in the nasal mucosa. A.

Experimental protocol. **B.** Local cytokine production in the nasal mucosa. Intralymphatic administration of FlaB-ovalbumin mixture reduced IL-4, IL-5, IL-6 and IFN- γ in the nasal mucosa. **C.** Local chemokine profiles in the nasal mucosa. Eotaxin-2 and the neutrophil chemokines, CXCL1 and CXCL2, were significantly reduced in the intralymphatic administration group. **D.** Immunohistochemistry of eotaxin-2, CXCL1, and CXCL2. Eotaxin-2 and CXCL1 was significantly reduced and CXCL2 was slightly decreased in the intralymphatic administration group ($\times 400$ magnification; Scale bar, 25 μm). **E.** Western blot of Eotaxin-2, CXCL1 and CXCL2 in the nasal mucosa. **F.** Immunohistochemistry of IL-25 and IL-33. The expression of IL-25 in the epithelial and subepithelial layers was decreased in the intralymphatic treatment group. The expression of IL-33 was increased in the cytosol of the epithelium and subepithelial inflammatory cell in the (+) control group and that was decreased in the intralymphatic treatment group ($\times 400$ magnification; Scale bar, 25 μm).

FlaB; flagellin B, IL; intralymphatic, (-) con; negative control group, (+) con; positive control group

Fig. 1

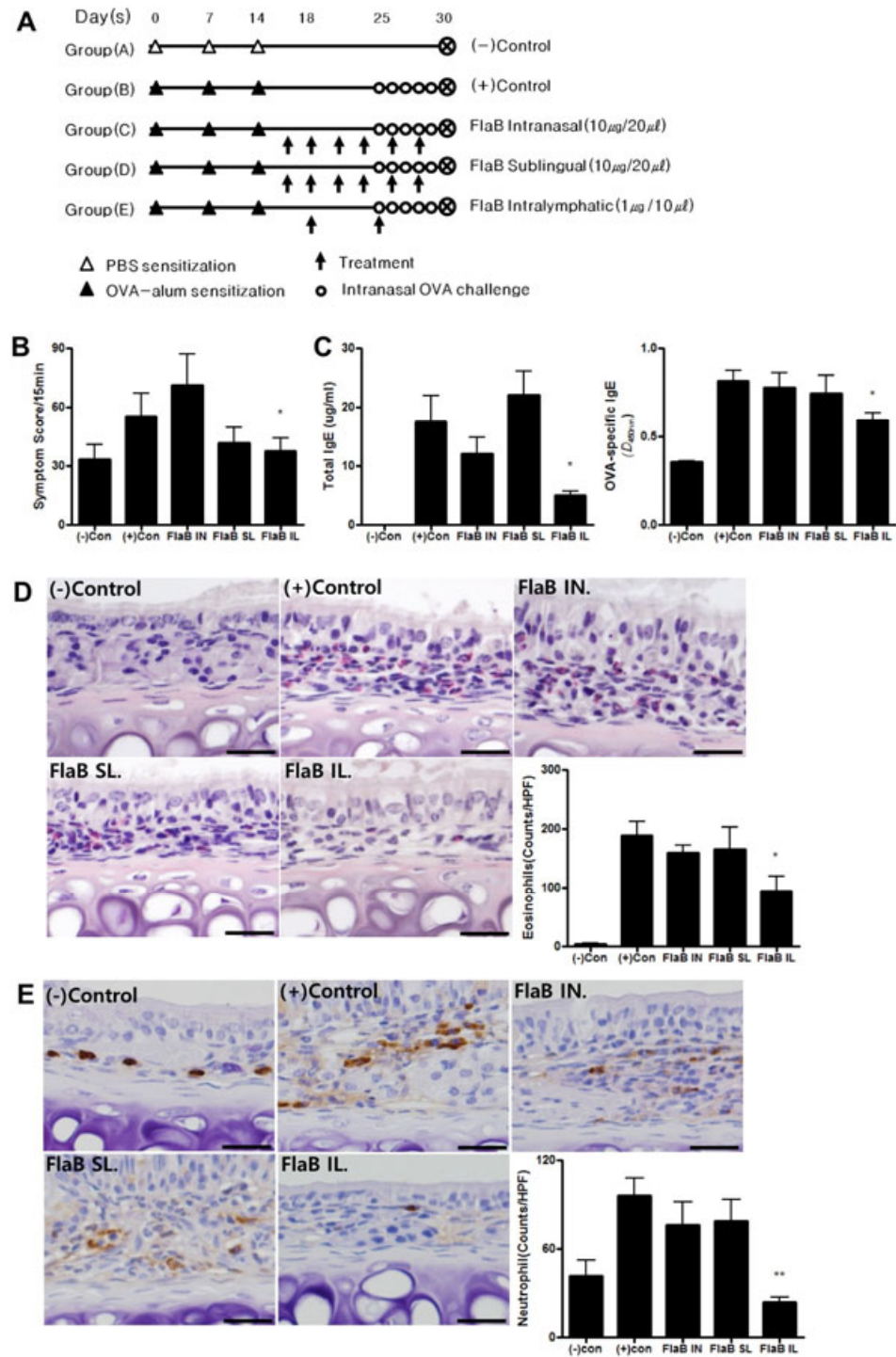


Fig. 1

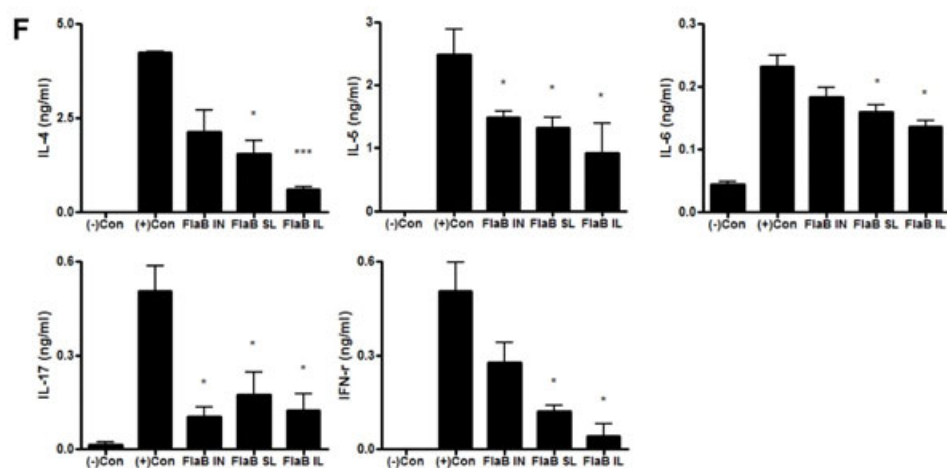


Fig. 3

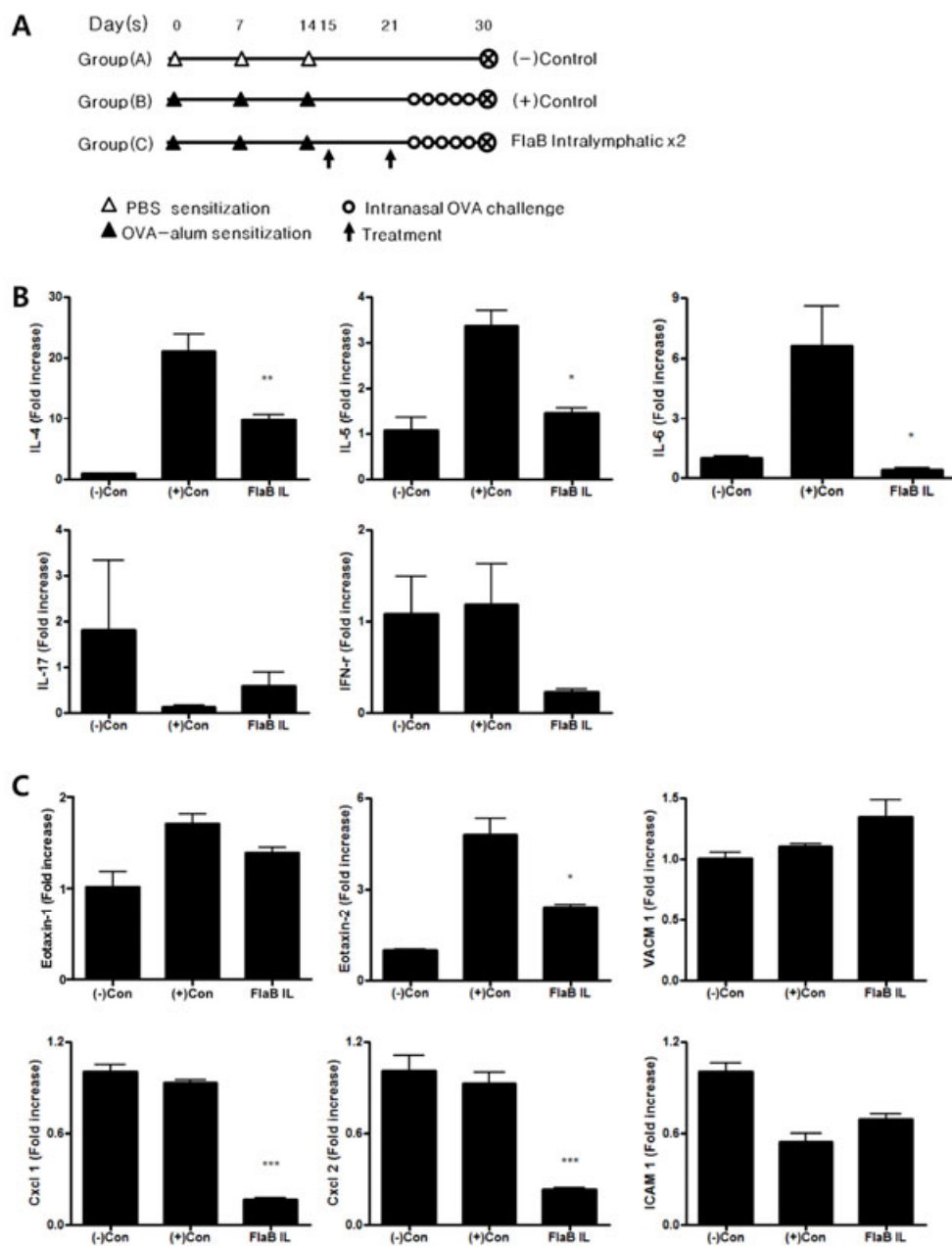
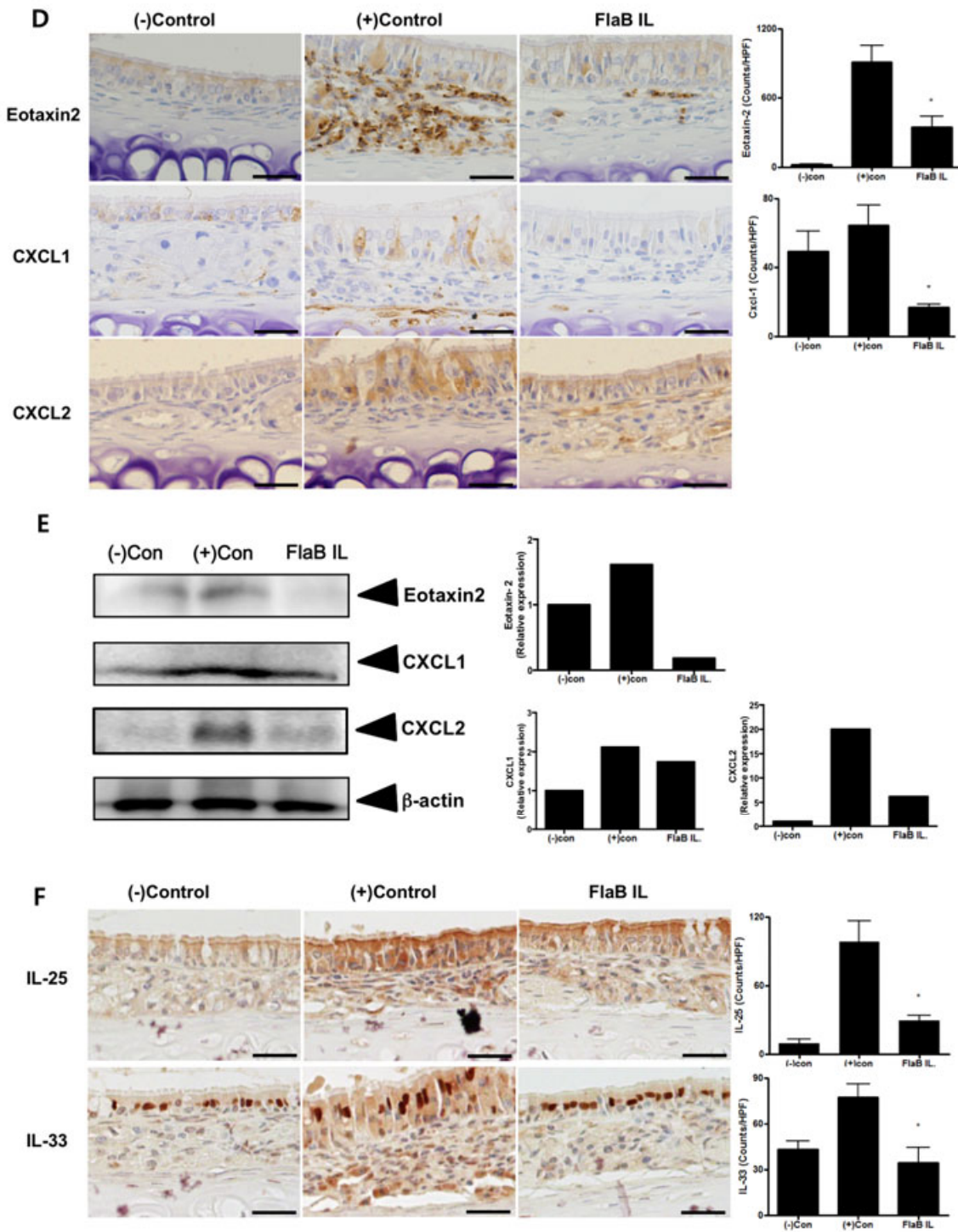
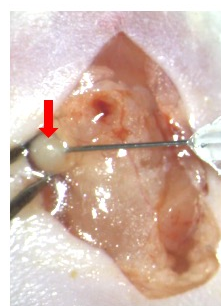
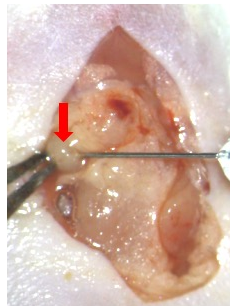
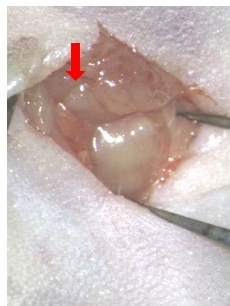
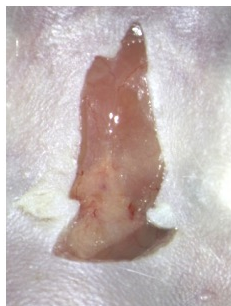


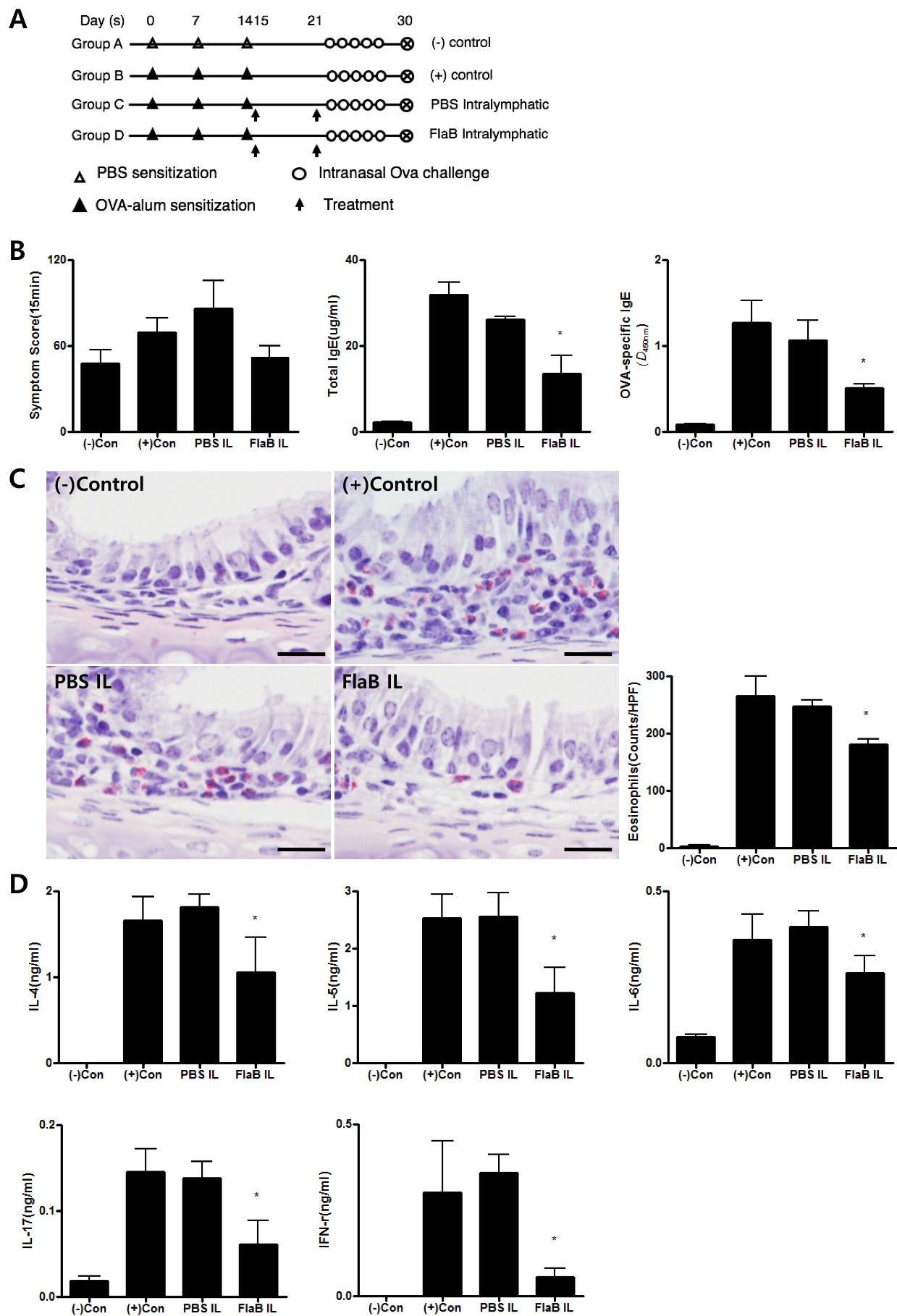
Fig. 3



Supplementary Fig. 1



Supplementary Fig. 2



Supplementary Figure 1

The intralymphatic injection procedure. After skin incision in the neck area, cervical lymph node (red arrow) can be easily identified around sublingual and submandibular gland area. Insulin syringe (31G, BD Ultra-Fine II, NJ, USA) was gently inserted into the lymph node and 10 μ l of mixture was administered gently.

Supplementary Figure 2 Intralymphatic administration of PBS did not reduce allergic parameters and showed similar results with positive control group **A.** Experimental protocol. **B.** Symptom score of nasal rubbing and sneezing for 15 minutes and Total and OVA-specific IgE levels **C.** Eosinophil counts in the nasal mucosa (each bar represents 25 μ m). **D.** Systemic cytokine production from spleen cell culture. Interleukin IL-4, IL-5, IL-6, IL-17 and interferon- γ were measured. FlaB; flagellin B, IL; intralymphatic, (-) con; negative control group, (+) con; positive control group.