**ORIGINAL ARTICLE**
Iran J Allergy Asthma Immunol
October 2017; 16(5): 404-417.

# Interleukin-37 Relieves Allergic Inflammation in a House Dust Mite Allergic Rhinitis Murine Model

**Do Hyun Kim[1], Si Won Kim[1], Soo Whan Kim[2], and Jun-Myung Kang[2]**

*[1] Department of Otolaryngology-Head and Neck Surgery, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul, Korea*
*[2] Department of Otolaryngology-Head and Neck Surgery, Bucheon St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul, Korea*

***Received: 18 November 2016; Received in revised form: 17 March 2017; Accepted: 6 May 2017***

## ABSTRACT

The purpose of this study was to investigate the effects of interleukin-37 (IL-37) on a *Dermatophagoides farinae* (*Der f*)-induced murine model of allergic rhinitis (AR).

BALB/c mice, except the control groups, were sensitized intraperitoneally and challenged intranasally with *Der f* (*Der f* group). The IL-37 and IL-37+anti-CD25 groups were administered IL-37 intranasally. The IL-37+anti-CD25 groups were administered anti-CD25 monoclonal antibody intraperitoneally before challenge. Allergic symptoms and the average eosinophil number were counted. The levels of cytokines and transcription factors in the nasal mucosa were measured by Real-Time polymerase chain reaction (PCR) and western blotting. The levels of *Der f*-specific immunoglobulin E (IgE) were measured. The $CD4^+CD25^+Foxp3^+$T cells among splenic mononuclear cells were analyzed by flow cytometry.

The allergic symptom scores and *Der f*-specific IgE levels were lower in the IL-37 group compared to the *Der f* group. Additionally, the levels of the transcription factor GATA-3 and ROR-γt and those of the cytokines IL-4, IL-5, IL-13, and IL-17, representing both T helper (Th)2 and Th17 responses, were lower in the IL-37 group in comparison with the *Der f* group. However, the Th1 response was not suppressed after administration of IL-37. IL-37 increased the IL-10 level; however, Real-Time PCR, western blotting, and flow cytometry results showed the limited action of IL-37 on $CD4^+CD25^+Foxp3^+$T cells.

This study demonstrates that intranasal IL-37 can suppress Th2 and Th17 responses in an AR murine model. Furthermore, these data suggest that IL-10 is increased, but $CD4^+CD25^+Foxp3^+$T cells are not correlated with the IL-37-induced mechanism.

**Keywords**: Allergy; Dermatophagoides farinae; Mice; Rhinitis; Regulatory; T Lymphocytes

**Corresponding Authors:** Soo Whan Kim, MD, PhD;
Department of Otolaryngology-Head and Neck Surgery, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea. Banpo-daero 222, Seocho-gu, Seoul 137-701, Republic of Korea. Tel: (+82 2)2258 6214, Fax: (+82 2)535 1354, E-mail: kshent@catholic.ac.kr

Jun-Myung Kang, MD, PhD;
Department of Otolaryngology, Head and Neck Surgery, Bucheon St. Mary's Hospital, College of Medicine, The Catholic University of Korea. 2 Sosa-dong, Wonmi-gu, Bucheon, Kyounggi-do 420 –717, Republic of Korea. Tel: (+82 032)3407 051, Fax: (+82 032)340 2674
E-mail: entkjm@catholic.ac.kr



Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

## INTRODUCTION

Allergic rhinitis (AR) is one of the most common chronic illnesses worldwide, and its prevalence has increased rapidly over the last few decades.[1] Despite advances in the understanding of allergic inflammation mechanisms, the disease symptoms usually cannot be controlled completely with current therapies. Therefore, adaptive immunity, as well as innate immunity and immune tolerance, have been the subject of focus in AR treatment. Additionally, innate immunity was revealed to regulate adaptive immune responses and affect the activity and function of dendritic cells (DCs) and epithelial cells.[2-4]

Interleukin 37 (IL-37), formerly termed IL-1 family member 7, has been shown to down-regulate the expression levels of pro-inflammatory cytokines in chronic inflammatory diseases.[5-7] IL-37 expression has been reported in the lung, lymph nodes, spleen, and thymus, as well as in activated peripheral blood mononuclear cells, DCs, plasma cells, and bronchial epithelial cells.[6,8,9] Although the open reading frame of IL-37 has, thus far, not been identified in murine cells, several studies have reported that human IL-37 demonstrates effects on murine as well as human cells.[6,7,10-16] IL-37 has been reported to promote the generation of semi mature tolergenic DCs[13,17] and to suppress the activity of pro-inflammatory cytokines such as IL-1α, IL-1β, TNF-α, IL-6, IL-23, and GM-CSF,[6-9,18,19] but also to play a role in adaptive immunity.[12,13,20]. However, its effects on the role of regulatory T cells (Treg) are controversial.[6,7,13,14,17]

Thus, this study was designed to investigate the therapeutic potential of IL-37 in AR using a *Dermatophagoides farinae* (*Der f*)-induced murine model of AR and to clarify its effect on immune tolerance. To our knowledge, this is the first study to evaluate the effect of topical IL-37 treatment in a murine model of AR.

## MATERIALS AND METHODS

### Experimental Animals

Forty 6-week-old, healthy female BALB/c mice were used in the present study. The mice were housed in a controlled environment under a 12-hour light/dark cycle with free access to food and water. This experiment was performed with the approval of the Institutional Animal Care and Use Committee at the Catholic University of Korea (No.2014008003).

### Reagents

*Der f* crude body extract (Arthropods of Medical Importance Resource Bank, College of Medicine, Yonsei University, Seoul, Korea) was used as the allergen. The *Der f* crude extract was solubilized and stored at -70°C and dissolved before use. The recombinant human IL-37 (Adipogen AG, Liestal, Switzerland) and anti-CD25 (clone PC61; eBioscience, San Diego, CA, USA) monoclonal antibodies (mAb) were prepared in sterile phosphate-buffered saline (PBS).

### Sensitization, Antigen challenge, and Treatment

The 40 mice were randomized into four groups: control (*n*=10), *Der f* (AR, *n*=10), IL-37 (*n*=10), and IL-37+anti-CD25 mAb (*n*=10). Allergen sensitization and challenge for the development of the AR mouse model are summarized in Figure 1. On days 0, 7, and 14, all of the mice, except those in the control group, were immunized by intraperitoneal injection of 100 μg *Der f* absorbed on the 1 mg aluminum hydroxide (Sigma-Aldrich, St. Louis, MO, USA). Mice in the IL-37 and IL-37+anti-CD25 group were treated intranasally with 1 μg IL-37 dissolved in 50 μL sterile PBS as described previously[12,21] on days 26, 27, 28, 29, and 30. Next, 10 days after sensitization, these mice were challenged intranasally with 20 μg *Der f* in 20 μL PBS for 6 consecutive days. Mice in the IL-37+anti-CD25 group were treated intraperitoneally with 250 μg anti-CD25 mAb 1 day before the first intra-nasal challenge with *Der f* (on day 24). The control group received PBS intranasally instead of *Der f.* [22]

### Evaluation of Allergic Symptoms

The numbers of sneezing and nose-rubbing motions during the 15 minutes after the final allergen challenge were recorded and compared between the experimental groups by two observers blinded to the experimental groups.[22,23]

### Nasal Mucosal Tissue Evaluation

Mice were sacrificed and decapitated 24 hours after the last allergen challenge. The heads were fixed in 4% paraformaldehyde for 3 days at 4°C, washed in running water, decalcified for 3 days using Calci-Clear Rapid (National Diagnostics, Atlanta, GA, USA)[24] at room temperature, dehydrated by passage through a graded

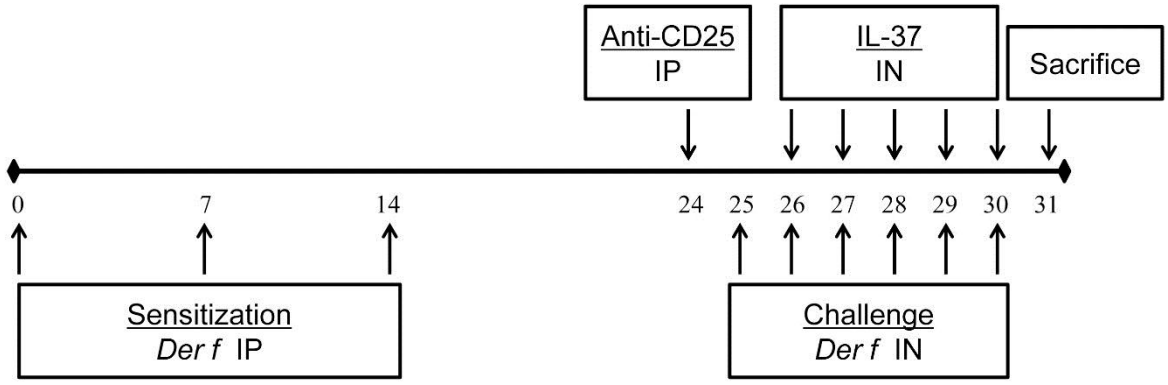


**Figure 1. Schematic representation of the experimental allergic rhinitis model and treatment protocol**
**Der f, Dermatophagoides farinae; IN, intranasal administration; IP, intraperitoneal administration; Anti-CD25, anti-CD25 monoclonal antibody**
**The units are days.**

alcohol series, and embedded in paraffin blocks. These blocks were cut into 4-μm-thick sections and stained with hematoxylin and eosin (H&E) to evaluate the general morphology and number of eosinophils in the lamina propria of the nasal mucosa. The average number of eosinophils was counted in four areas around the nasal septa areas under a light microscope (×400). The individuals who counted the cells were blinded to the animals' group assignments.

**Measurement of the *Der f*-Specific Levels of Immunoglobulin E (IgE) in the Serum**

Samples were collected 24 hours after the last *Der f* challenge. The levels of *Der f*-specific IgE in the serum were measured using an enzyme-linked immunosorbent assay kit (ELISA; Bethyl, Montgomery, TX, USA).

**Cytokines and Transcription Factors in Nasal Mucosal Tissue**

Using cytokines from nasal mucosal tissue, interferon (IFN)-γ for the T helper 1 (Th1) immune reaction, interleukin (IL)-4, 5, 13 for the T helper 2 (Th2) immune reaction, IL-17 for the T helper 17 (Th17) immune reaction, and IL-10 and transforming growth factor (TGF)-β for the Treg immune reaction, were measured by real-time polymerase chain reaction (PCR). Simultaneously, the relative mRNA expression levels of the transcription factors T-bet, GATA-3, ROR-γt, and Foxp3 were measured by real-time PCR.

Total RNA was extracted from nasal mucosal tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and first-strand cDNA was reverse transcribed using random primers (TaKaRa, Otsu, Japan).[25,26] The oligonucleotide primer sequences used in this study was presented in Table 1. The mRNA levels of IFN-γ, IL-4, IL-5, IL-13, IL-17, IL-10, TGF-β, T-bet, GATA-3, ROR-γt, Foxp3, and GAPDH were determined by real-time PCR using the CFX96 Real Time PCR Machine (Bio-Rad, Hercules, CA, USA) and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). The results were normalized relative to GAPDH expression and expressed as the absolute mRNA expression level for the cytokines and as the fold increase relative to the expression of the control group for the transcription factors.

**Western Blotting**

Western blot analysis was used to detect the relative protein expression patterns of transcription factors (T-bet, GATA-3, ROR-γt, and Foxp3) and cytokines (IFN-γ, IL-4, IL-5, IL-13, IL-17, IL-10, and TGF-β) within the nasal mucosa of the control, *Der f*, IL-37, and IL-37+anti-CD25 groups. Nasal mucosal tissues were homogenized and extracted using Precellys Lysing kits (Bertin Technologies, Rockville, MD, USA) and T-PER tissue protein extraction reagent (Pierce, Rockford, IL, USA) with protease inhibitor cocktail (Roche, Mannheim, Germany) according to

manufacturer's protocol. Protein concentrations were determined by bicinchoninic acid (BCA) reagent kit (Pierce, Rockford, IL, USA). Proteins were separated by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Then, the proteins were transferred onto polyvinylidene fluoride membranes (Milipore, Bedford, MA, USA). After being blocked with 5% skim milk (BD BioSciences, San Jose, CA, USA), the membranes were incubated with the primary antibody at 4 °C for 24 h. After washing the membranes three times, the membranes were probed with horseradish peroxidase-labeled secondary antibodies. Finally, immunoreactive bands were visualized using enhanced chemiluminescence (ECL) detection kit (Bio-rad, Richmond, CA, USA).

Results were based on the gray-scale target gene to GAPDH ratios to indicate the relative levels of the proteins encoded by the target genes using anti-GATA-3, -Foxp3, anti-IL-5, -IL-10, -IL-13, -IL-17, -IL-10, -TGF-β, and -ROR-γt antibodies (Santa Cruz Biotechnology, Dallas, TX, USA).

**Flow Cytometry**

For cell-surface staining, aliquots of $10^6$ splenic mononuclear cells were incubated with fluorescein isothiocyanate-conjugated anti-mouse CD4 (GK1.5) antibody (eBioscience, San Diego, CA, USA). For intracellular staining, cells stained with CD4 were incubated with fixation/permeabilization working solution, and Fc receptors were blocked with excess mouse Fc block. Next, the cells were stained with phycoerythrin-Cy5-conjugated anti-mouse Foxp3 (FJK-16s) and anti-allophycocyanin-CD25 antibodies (eBioscience, San Diego, CA, USA). $CD4^+CD25^+Foxp3^+T$ cells were analysed by flow cytometry (FACS Calibur flow cytometer; Becton Dickinson, San Jose, CA, USA).[26,27]

**Table 1. The primer sequences used in the allergic rhinitis murine model groups (control, Der f-induced allergic rhinitis, IL-37 treated Der f-induced allergic rhinitis, and IL-37+anti-CD25 treated Der f-induced allergic rhinitis)**

| Cytokines and transcription factors | | Primer sequences |
|---|---|---|
| IFN-γ | forward primer | 5′-AGAGCCAGATTATCTCTTTCTACCTCAG-3′ |
| | reverse primer | 5′-CCTTTTTCGCCTTGCTGTTG-3′ |
| IL-4 | forward primer | 5′-TCAACCCCCAGCTAGTTGTC-3′ |
| | reverse primer | 5′-AAATATGCGAAGCACCTTGG-3′ |
| IL-5 | forward primer | 5′-GACGAGGCAGTTCCTGGAT-3′ |
| | reverse primer | 5′-GCATATGGTATCCCTTGCATT-3′ |
| IL-13 | forward primer | 5′-CCTCTGACCCTTAAGGAGCTTAT-3′ |
| | reverse primer | 5′-CGTTGCACAGGGGAGTCT-3′ |
| IL-17 | forward primer | 5′-TTTAACTCCCTTGGCGCAAAA-3′ |
| | reverse primer | 5′-CTTTCCCTCCGCATTGACAC-3′ |
| IL-10 | forward primer | 5′-ATGCTGCCTGCTCTTACTGACTG-3′ |
| | reverse primer | 5′-CCCAAGTAACCCTTATCCTGC-3′ |
| TGF-β | forward primer | 5′-CACCATCCATGACATGAACC-3′ |
| | reverse primer | 5′-TCATGTTGGACAACTGCTCC-3′ |
| T-bet | forward primer | 5′-GCCAGGGAACCGCTTATA-3′ |
| | reverse primer | 5′-CCTTGTTGTTGGTGAGCTTTA-3′ |
| GATA-3 | forward primer | 5′-CTGGATGGCGGCAAAGC-3′ |
| | reverse primer | 5′-GTGGGCGGGAAGGTGAA-3′ |
| ROR-γt | forward primer | 5′-ACCTCCACTGCCAGCTGTGTGCTGTC-3′ |
| | reverse primer | 5′-TCATTTCTGCACTTCTGCATGTAGACTGTCCC-3′ |
| Foxp3 | forward primer | 5′ -GAAAGCGGATACCAAATGA-3′ |
| | reverse primer | 5′- CTGTGAGGACTACCGAGCC-3′ |
| GAPDH | forward primer | 5′-GCACAGTCAAGGCCGAGAAT-3′ |
| | reverse primer | 5′-GCCTTCTCCATGGTGGTGAA-3′ |

*GAPDH; glyceraldehyde 3-phosphate dehydrogenase

**Statistical Analysis**

All measured parameters are expressed as means±standard deviation. Differences among the groups were analyzed using the Kruskal-Wallis test. In cases of statistical significance, the ranked parameters were compared by one-way analysis of variance and Bonferroni's multiple comparison tests. A *p* value less than 0.05 was considered to reflect statistical significance. All statistical analyses were conducted using SAS version 9.3 (SAS Institute, Cary, NC, USA).

## RESULTS

**Allergic Symptoms**

The number of episodes of sneezing motion was 1.60±1.58 in the control group, 45.6±11.48 in the *Der f* group, 16.6±5.38 in the IL-37 group, and 19.2±6.51 in the IL-37+anti-CD25 group. The number of episodes of nose-rubbing motion was 8.67±3.13 in the control group, 111.1±18.19 in the *Der f* group, 33±8.47 in the IL-37 group, and 44.7±8.41 in the IL-37+anti-CD25 group. The number of episodes of sneezing and nose-rubbing motions was significantly lower in the control group than in the other groups (all $p<0.001$; Figure 2). The IL-37 and IL-37+anti-CD25 groups showed significantly fewer sneezing and nose-rubbing motions than those of the *Der f* group (all $p<0.001$; Figure 2). The number of episodes of nose-rubbing motion was significantly higher in the IL-37+anti-CD25 group than in the IL-37 group ($p=0.011$; Figure 2B).

**Serum *Der f*-Specific IgE**

The serum *Der f*-specific IgE levels were significantly higher in the *Der f* group (2.54±0.80 ng/mL, $p<0.001$) than in the control (0.3±0.20 ng/mL), IL-37 (2.54±0.80 ng/mL, $p=0.005$), and IL-37+anti-CD25 (1.57±0.85 ng/mL, $p=0.019$) groups. The control group had a significantly lower serum *Der f*-specific IgE level than that of each of the other groups (IL-37, $p=0.005$; all of the others, $p<0.001$). The IL-37 group showed significantly lower serum *Der f*-specific IgE levels than those of the *Der f* group ($p=0.001$; Figure 3).

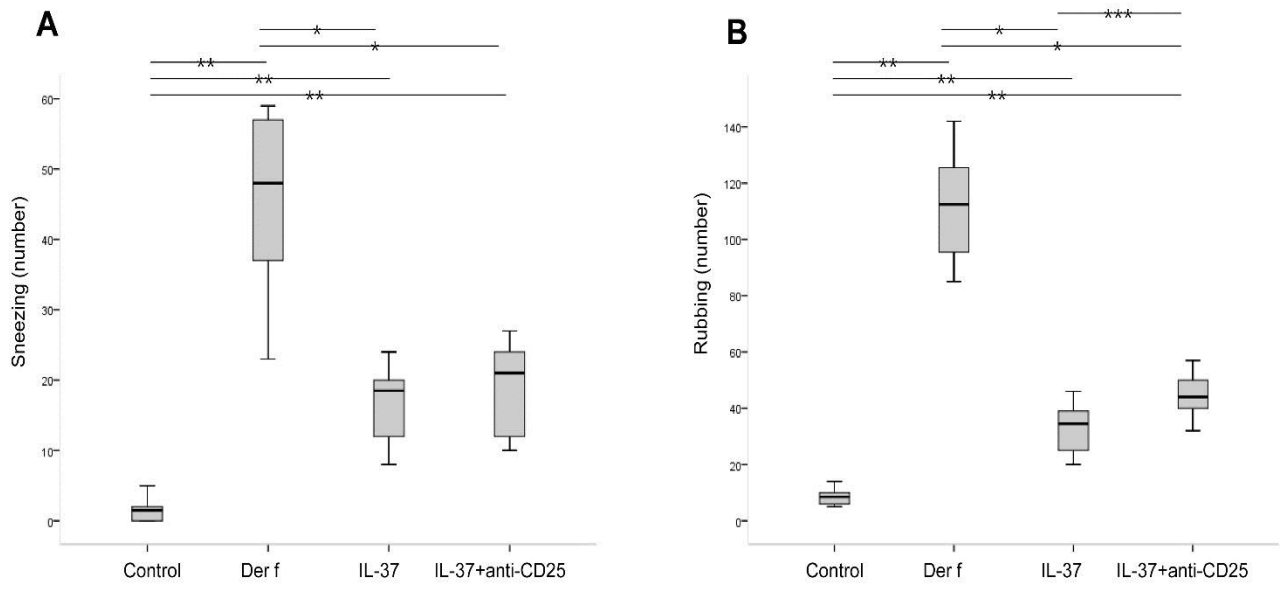


**Figure 2. Nasal symptom scores measured in the allergic rhinitis murine model groups (control, Der f-induced allergic rhinitis, IL-37 treated Der f-induced allergic rhinitis, and IL-37+anti-CD25 treated Der f induced allergic rhinitis). Sneezing (A) and nose-rubbing motions (B) of the control (n=10), Der f (n=10), IL-37 (n=10), and IL-37+anti-CD25 (n=10) groups. The box and whisker plots show the medians and interquartile ranges. The error bars represent standard deviations. *P<0.05, Der f vs. IL-37; **$p$<0.05, control vs. Der f; ***$p$<0.05, Der f vs. IL-37+anti-CD25.**

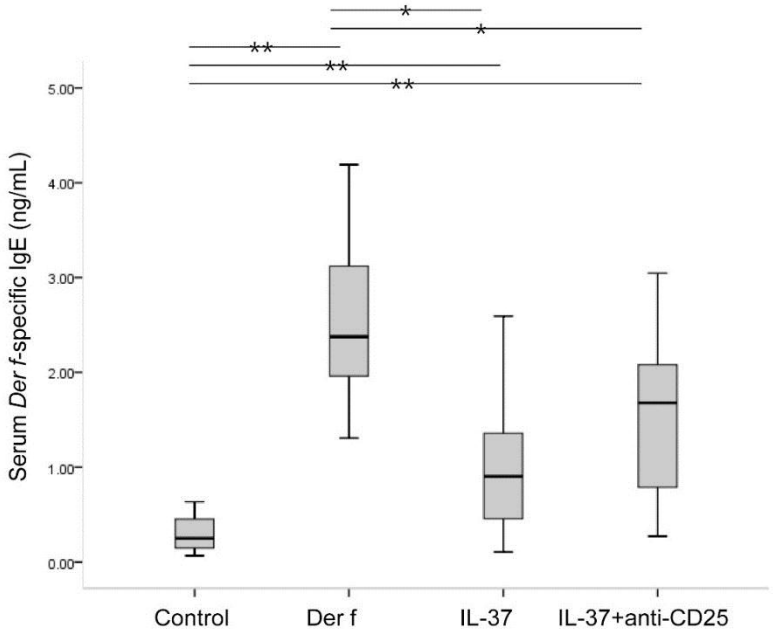


**Figure 3. Serum levels of Dermatophagoides farinae-specific immunoglobulin E measured in the allergic rhinitis murine model groups (control, Der f-induced allergic rhinitis, IL-37 treated Der f-induced allergic rhinitis, and IL-37+anti-CD25 treated Der f-induced allergic rhinitis). The box and whisker plots show the medians and interquartile ranges. The error bars represent standard deviations. *$p$<0.05, Der f vs. IL-37; **$p$<0.05, control vs. Der f.**

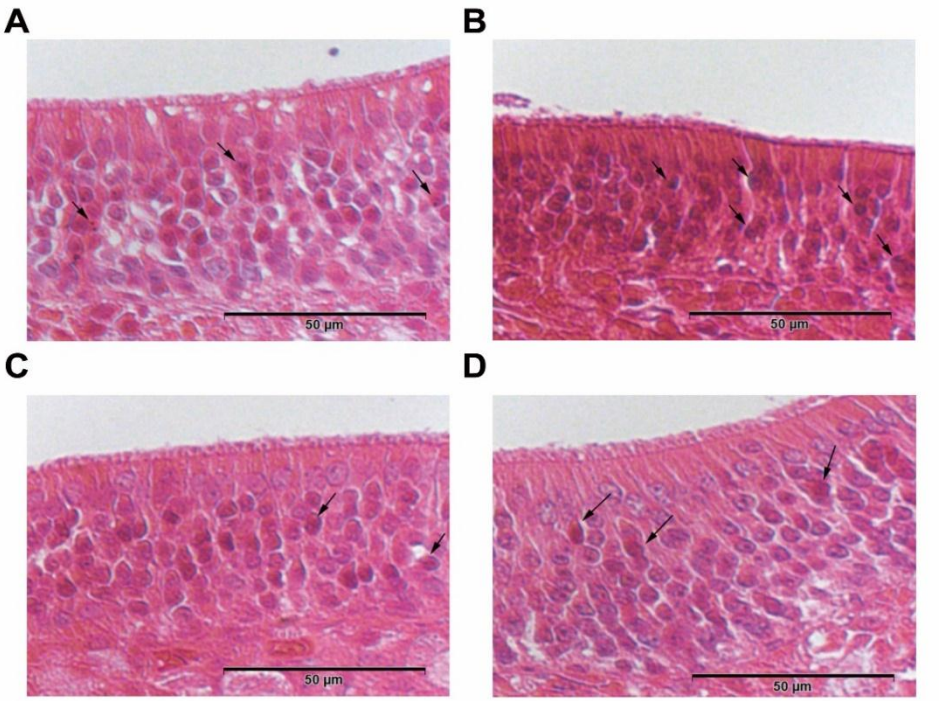


**Figure 4. Histologic results of nasal mucosa in the allergic rhinitis murine model groups (control, Der f-induced allergic rhinitis, IL-37 treated Der f-induced allergic rhinitis, and IL-37+anti-CD25 treated Der f induced allergic rhinitis): (A) control group, (B) Der f group, (C) IL-37 group, and (D) IL-37+anti-CD25 group (hematoxylin and eosin staining; original magnification, ×400). The black arrows indicate the infiltration of eosinophils.**

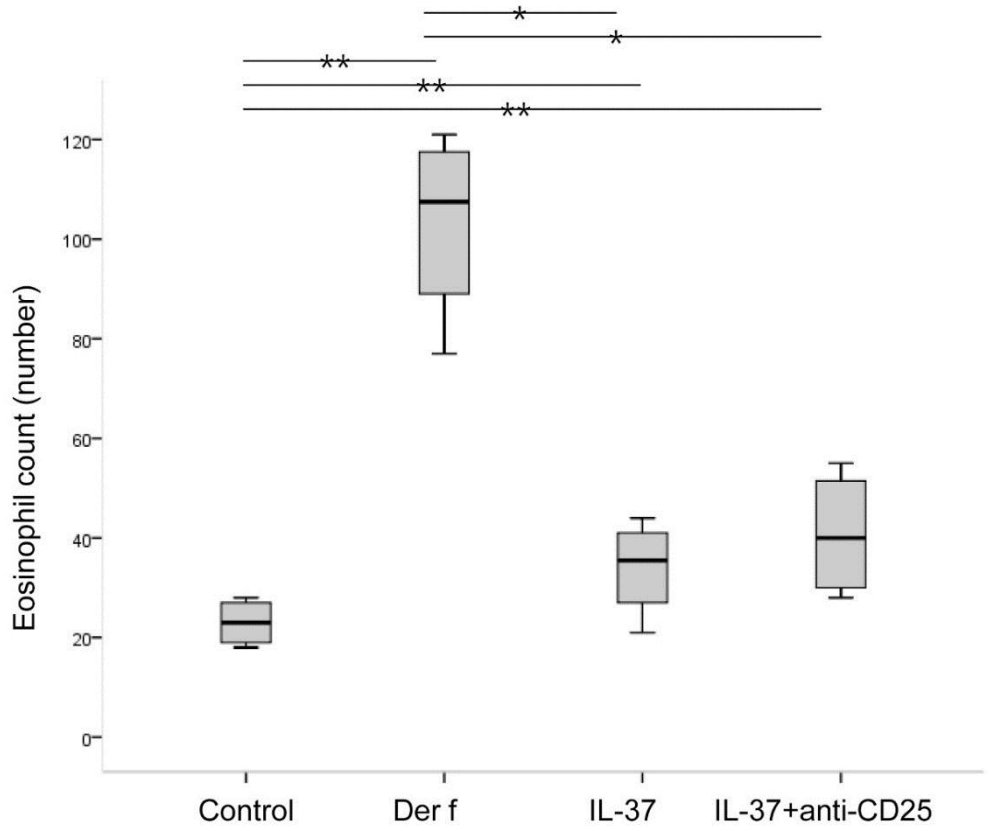


**Figure 5. Eosinophil counts in the nasal mucosa in the allergic rhinitis murine model groups (control, Der f-induced allergic rhinitis, IL-37 treated Der f-induced allergic rhinitis, and IL-37+anti-CD25 treated Der f-induced allergic rhinitis). The box and whisker plots show the medians and interquartile ranges. The error bars represent standard deviations. *$p$<0.05, Der f vs. IL-37; **$p$<0.05, control vs. Der f.**

**Eosinophil Counts in the Lamina Propria**

Figure 4A-D shows eosinophil infiltration in the lamina propria. The eosinophil numbers were 22.6±3.47 in the control group, 103.8±15.13 in the *Der f* group, 33.9±7.29 in the IL-37 group, and 40.4±8.04 in the IL-37+anti-CD25 group. The eosinophil count was significantly higher in the *Der f* group than in the control, IL-37, or IL-37+anti-CD25 groups (all $p$<0.001). The control group had a significantly lower eosinophil count than those in the IL-37 ($p$=0.001) and IL-37+anti-CD25 ($p$<0.001) groups (Figure 5).

**Real-Time PCR and Western Blotting**

The mRNA expression levels of cytokines (IFN-γ, IL-4, IL-5, IL-13, IL-17, IL-10, and TGF-β) and transcription factors (T-bet, GATA-3, ROR-γt, and Foxp3) were measured by real-time PCR. Concurrently, the protein expression patterns of the cytokines and transcription factors were evaluated by western blotting.

In the Th1 response, the relative mRNA level of the transcription factor T-bet showed no significant difference among the groups (Figure 6A). Additionally, the expression level of IFN-γ mRNA showed no difference among the groups, except between the IL-37+anti-CD25 and control groups ($p$=0.003, Figure 6B).

In the Th2 response, the relative level of GATA-3 mRNA was significantly higher in the *Der f* group than in the IL-37 and control groups (both $p$<0.001; Figure 7A). The expression levels of IL-4, -5, and -13 mRNA were all significantly higher in the *Der f* group than in the IL-37 ($p$=0.015, 0.001, and <0.001), IL-37+anti-CD25 ($p$=0.029, 0.002, and 0.001) and control groups ($p$=0.035, <0.001, and <0.001; Figure 7B, C, and D, respectively).

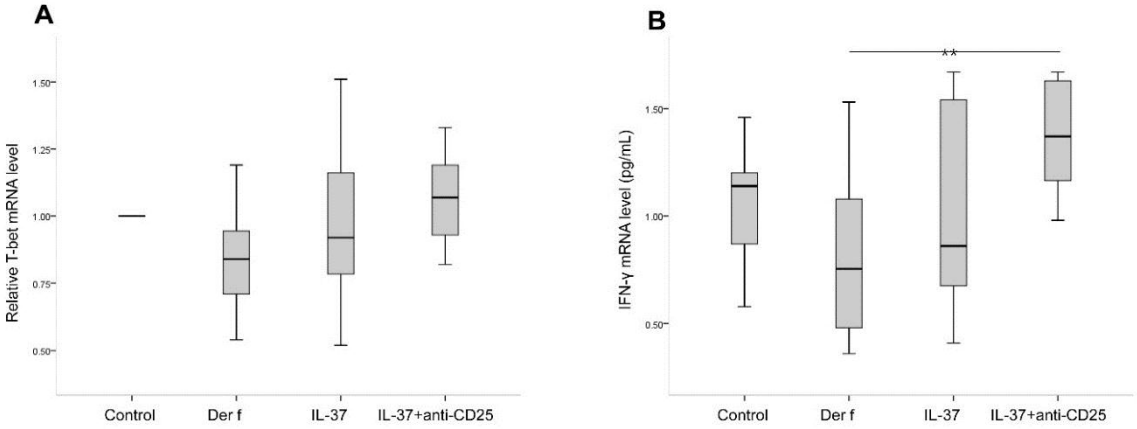


**Figure 6. Quantitative analysis by real-time PCR of cytokine levels in the nasal mucosa of the allergic rhinitis murine model groups (control, Der f induced allergic rhinitis, IL-37 treated Der f-induced allergic rhinitis, and IL-37+anti-CD25 treated Der f-induced allergic rhinitis) in the Th1 response: (A) T-bet and (B) IFN-γ. The results were normalized relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The box and whisker plots show the medians and interquartile ranges. The error bars represent standard deviations. **$p$<0.05, control vs. Der f.**

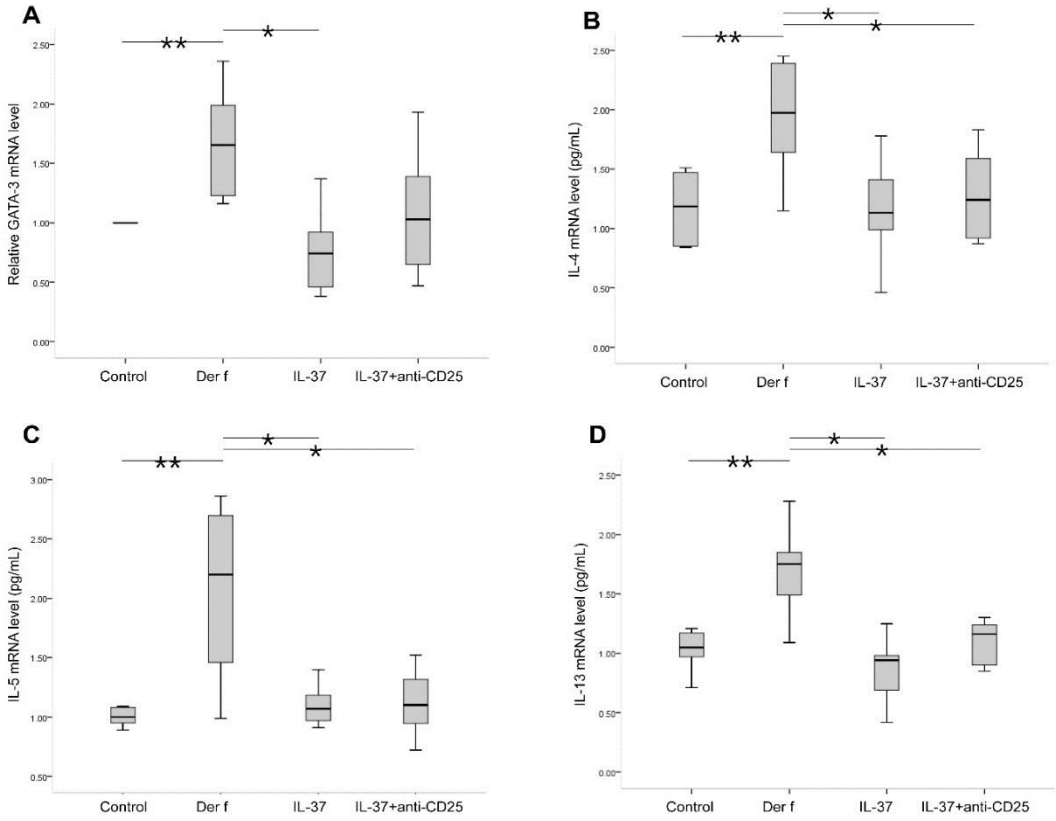


**Figure 7. Quantitative analysis by real-time PCR of the cytokine levels in the nasal mucosa of the allergic rhinitis murine model groups (control, Der f induced allergic rhinitis, IL-37 treated Der f induced allergic rhinitis, and IL-37+anti-CD25 treated Der f induced allergic rhinitis) in the Th2 response: (A) GATA-3, (B) IL-4, (C) IL-5, and (D) IL-13. The results were normalized relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The box and whisker plots show the medians and interquartile ranges. The error bars represent standard deviations. *$p$<0.05, Der f vs. IL-37; **$p$<0.05, control vs. Der f.**

In the Th17 response, the relative level of ROR-γt mRNA was significantly higher in the *Der f* group than in the other groups (all, $p<0.001$) and significantly lower in the IL-37 than control group ($p=0.002$; Figure 8A). Additionally, the level of IL-17 mRNA was significantly higher in the *Der f* group than in the other groups (control, $p<0.001$; IL-37, $p<0.001$; IL-37+anti-CD25, $p=0.002$) and significantly lower in the IL-37 than IL-37+anti-CD25 group ($p=0.023$; Figure 8B).

For the Treg response, we evaluated the mRNA expression levels of Foxp3, IL-10, and TGF-β. The relative expression levels of Foxp3 were significantly lower in the IL-37+anti-CD25 group than in the other groups (all, $p<0.001$). The *Der f* group showed a significantly lower Foxp3 level than the control group ($p<0.001$; Figure 9A). The expression level of IL-10 mRNA was also significantly lower in the *Der f* group than in the IL-37 ($p<0.001$) and IL-37+anti-CD25 groups ($p=0.011$). The IL-37 group showed a significantly higher IL-10 level than the control group ($p=0.004$; Figure 9B). The level of TGF-β was significantly lower in the IL-37+anti-CD25 group than in the other groups (all, $p<0.001$; Figure 9C).

We conducted western blotting to confirm the express patterns of the above cytokines and transcription factors. Because we did not perform a quantitative test, we checked the general gray-scale patterns of each factor (Figure 10).

### Flow Cytometric Analysis of $CD4^+CD25^+Foxp3^+$T Cells from Splenic Mononuclear Cells

We sorted CD4-expressing cells and measured their expression of Foxp3 and CD25 (Figure 11A). $CD4^+CD25^+Foxp3^+$T cells accounted for 2.71±0.79% of all splenic mononuclear cells in the control group, 2.46±0.61% in the *Der f* group, 2.26±0.28% in the IL-37 group, and 2.02±0.13% in the IL-37+anti-CD25 group. The IL-37+anti-CD25 group had a significantly lower percentage of $CD4^+CD25^+Foxp3^+$T cells than those of the other groups (control, $p=0.002$; *Der f*, $p=0.043$; IL-37, $p=0.029$; Figure 11B).

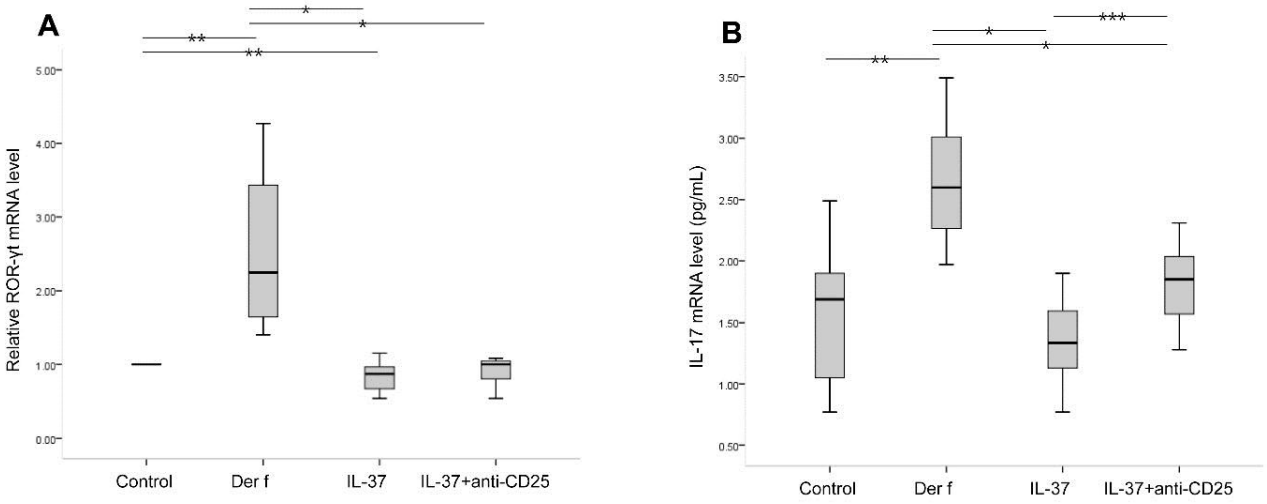


**Figure 8. Quantitative analysis by real-time PCR of the cytokine levels in the nasal mucosa of the allergic rhinitis murine model groups (control, Der f induced allergic rhinitis, IL-37 treated Der f induced allergic rhinitis, and IL-37+anti-CD25 treated Der f induced allergic rhinitis) in the Th17 response: (A) ROR-γt and (B) IL-17. The results were normalized relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The box and whisker plots show the medians and interquartile ranges. The error bars represent standard deviations. *$p<0.05$, Der f vs. IL-37; **$p<0.05$, control vs. Der f; ***$p<0.05$, Der f vs. IL-37+anti-CD25.**

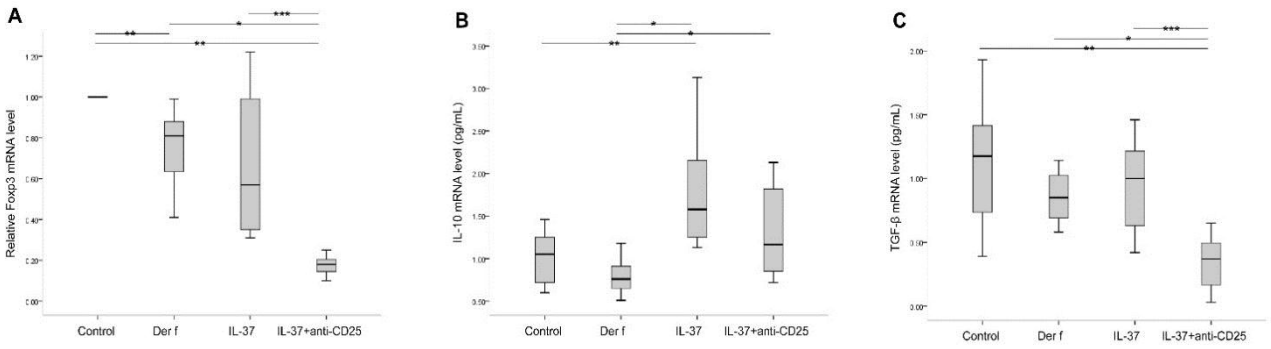


**Figure 9. Quantitative analysis of the cytokine levels by real-time PCR from the nasal mucosa of the allergic rhinitis murine model groups (control, Der f induced allergic rhinitis, IL-37 treated Der f induced allergic rhinitis, and IL-37+anti-CD25 treated Der f induced allergic rhinitis) in the regulatory T response: (A) Foxp3, (B) IL-10, and (C) TGF-β. The results were normalized relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The box and whisker plots show the medians and interquartile ranges. The error bars represent standard deviations. *$p$<0.05, Der f vs. IL-37; **$p$<0.05, control vs. Der f.**

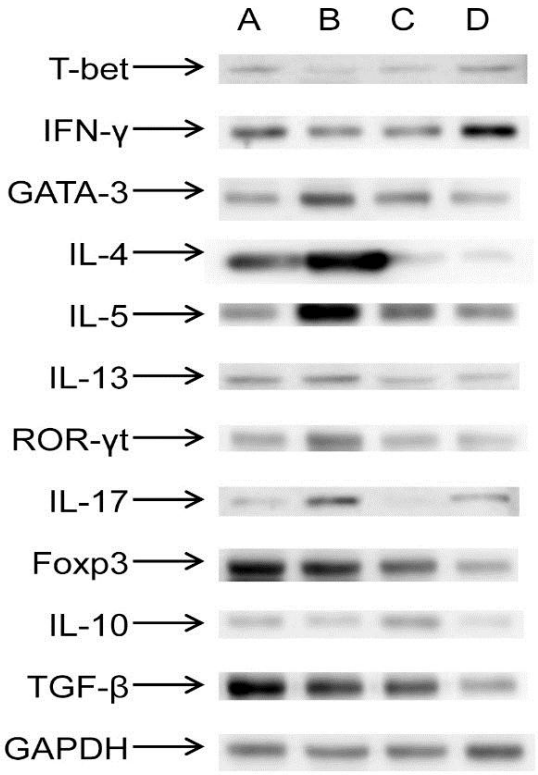


**Figure 10. Cytokines and transcription factors measured by western blotting to confirm protein expression of the cytokines and transcription factors in the allergic rhinitis murine model groups (control, Der f induced allergic rhinitis, IL-37 treated Der f induced allergic rhinitis, and IL-37+anti-CD25 treated Der f induced allergic rhinitis): (A) control group, (B) Der f group, (C) IL-37 group, and (D) IL-37+anti-CD25 group.**

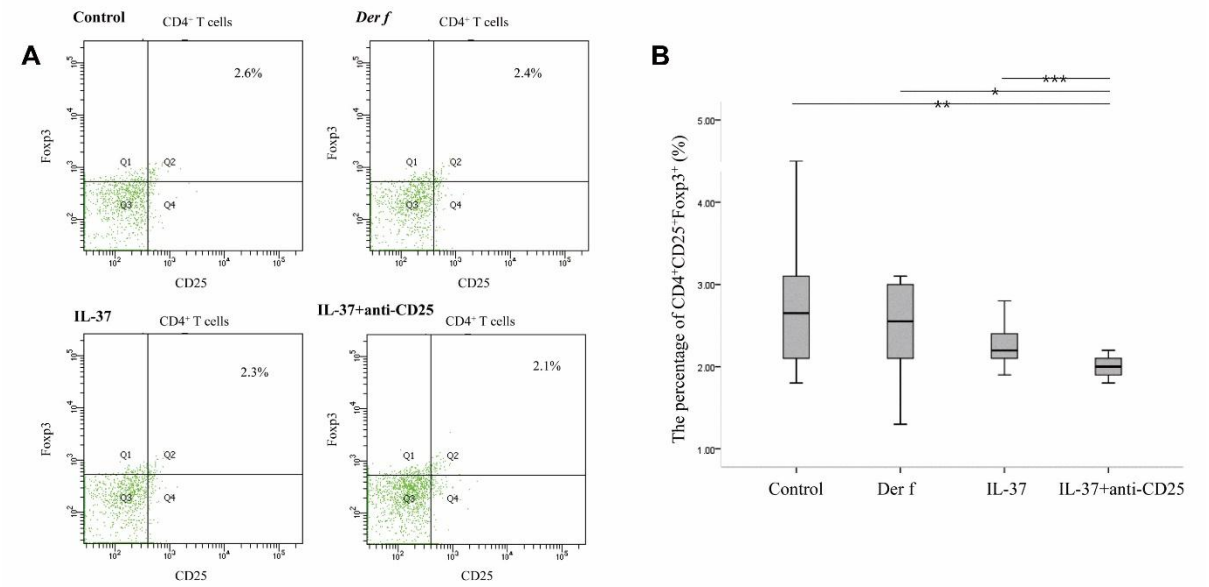


**Figure 11. Flow cytometric analysis of CD4+CD25+Foxp3+T cell subsets in the allergic rhinitis murine model groups (control, Der f induced allergic rhinitis, IL-37 treated Der f induced allergic rhinitis, and IL-37+anti-CD25 treated Der f induced allergic rhinitis). (A) Representative fluorescence-activated cell sorting analysis. The upper right quadrant represents CD4+CD25+Foxp3+T cells. (B) The percentage of splenic mononuclear cells that are CD4+CD25+Foxp3+T cells. The box and whisker plots show the medians and interquartile ranges. The error bars represent standard deviations. *$p$<0.05, Der f vs. IL-37; **$p$<0.05, control vs. Der f; ***$p$<0.05, Der f vs. IL-37+anti-CD25.**

## DISCUSSION

The conventional treatment of AR has limitations because of the side effects and failure of long-term symptomatic treatments or low compliance with allergen avoidance.[28] Thus far, intranasal steroid and specific allergen immunotherapy have been applied clinically, and their long-term effects have been controversial. Consequently, the roles of adaptive immunity as well as innate immunity, such as involvement of Toll-like receptors and immune tolerance, in AR have been investigated. Early studies showed that IL-37 regulates the expression of pro-inflammatory cytokines in chronic inflammatory diseases,[5-7] but its effect on adaptive immunity[12,13] has been reported recently. We demonstrated the anti-inflammatory effect in an AR mouse model. IL-37 treatment was effective in alleviating allergic symptoms, such as sneezing and nose-scratching motions. Additionally, histologic changes were observed. Tissue eosinophil recruitment in the lamina propria was significantly decreased after IL-37 treatment. Additionally, immune suppression after *Der f* induced a Th2 and Th17 cytokine milieu, identified at both the protein and mRNA levels. The IL-37 treatment groups showed a significant reduction in serum IgE levels and GATA-3, IL-4, IL-5, IL-13, ROR-γt and IL-17 mRNA levels, compared with the *Der f* group. IL-4 plays a pivotal role in the allergic response, including induction of isotype switching to IgE, expression of vascular cell adhesion molecule-1, promotion of eosinophil migration across the endothelium, mucus secretion, and differentiation of Th2 lymphocytes. IL-5 is essential for the differentiation, maturation, and survival of eosinophils.[29] Additionally, IL-13 is the most potent inducer of goblet cell differentiation and mucus production.[30] The anti-inflammatory effect of IL-37 via the IL-18Rα and IL-1R8 (SIRR or TIR8) receptor pathways has been reported.[12,31] In addition, IL-17 is related to airway hyperreactivity and mucus hypersecretion in the upper airway, including in AR.[32,33] Th17 suppression has been explained by the inhibition of the IL-1 receptor pathway and mTOR kinase activation.[34,35] As a result, the intranasal administration of IL-37 could suppress these allergic

reactions via Th2 and Th17. Moreover, we found that the local administration of IL-37 did not suppress the Th1 milieu, such as the transcription of IFN-γ or T-bet mRNA. IL-18Rα and IL-1R8 receptors directly regulate Th17 but not Th1 cell development.[35] From this point of view, the intranasal administration of IL-37 could be a novel and potent AR treatment candidate.

It has been suggested that IL-37 promotes the generation of IL-10[13,16,17] and $CD4^+CD25^+Foxp3^+$Treg.[13,17] However, other studies have shown opposite results.[6,7,12,14] Thus, we clarified the effect of IL-37 on Treg via evaluation of cell-surface CD25, a well-known marker of Treg. $CD25^+$Treg in mice were treated with anti-CD25 mAb, which was reported to reverse the effects of Bacillus Calmette-Guérin-stimulated DCs and berberine to reduce allergic inflammation.[36,37] This effect was limited in the anti-CD25 mAb treatment group in our study because of the negligible contribution of Treg. Thus, in our study, elevated IL-10 levels might contribute to semi mature tolergenic DCs[13,17] or affect B cells, monocytes/macrophages and natural killer cells through a mode other than $CD25^+$Treg. Additionally, in our study, $CD4^+CD25^+Foxp3^+$T cells were not significantly increased in the IL-37 group compared with the control and AR groups, according to real-time PCR, western blot analysis in the nasal mucosa, or flow cytometric analysis in splenic mononuclear cells. These results confirmed the limited action of IL-37 on $CD25^+$Treg and can be understood within the same context that TGF-β levels in the IL-37 administrated groups were comparable with those in the control and AR groups.

An important strength of the current study is the use of *Der f*, which is a common allergen associated with AR patients. Additionally, to determine the effect of Treg, we used anti-CD25 mAb treatment and real-time PCR, western blot analysis and flow cytometry.

Our study had several limitations. First, there were relatively small numbers of mice in each group. However, lots of conducted experiments showed significant statistical differences between the groups. Second, although many studies showed human IL-37 effects on murine model, it is important to clarify the exact mechanism of IL-37 on allergic rhinitis.

Taken together, our study demonstrated that IL-37 can reduce Th2 and Th17 cell-related allergic inflammation in an AR mouse model. Additionally, we found that the administration of IL-37 has a minimal effect on Th1. In conclusion, we postulate that IL-37 has a therapeutic potential for the alleviation of AR in a mice. Further studies are needed to identify the exact mechanism underlying IL-37 treatment of AR.

## ACKNOWLEDGEMENTS

This study was supported by a grant from the E.N.T. Fund of the Catholic University of Korea made in the program year of 2016. The sponsors had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## REFERENCES

1. Bousquet J, Khaltaev N, Cruz AA, Denburg J, Fokkens WJ, Togias A, et al. Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). Allergy 2008; (Suppl 86):8-160.
2. Granucci F, Zanoni I, Feau S, Ricciardi-Castagnoli P. Dendritic cell regulation of immune responses: a new role for interleukin 2 at the intersection of innate and adaptive immunity. Embo j 2003; 22(11):2546-51.
3. Hammad H, Lambrecht BN. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. Nat Rev Immunol 2008; 8(3):193-204.
4. Hoebe K, Janssen E, Beutler B. The interface between innate and adaptive immunity. Nat Immunol 2004; 5(10):971-4.
5. Kumar S, McDonnell PC, Lehr R, Tierney L, Tzimas MN, Griswold DE, et al. Identification and initial characterization of four novel members of the interleukin-1 family. J Biol Chem 2000; 275(14):10308-14.
6. Nold MF, Nold-Petry CA, Zepp JA, Palmer BE, Bufler P, Dinarello CA. IL-37 is a fundamental inhibitor of innate immunity. Nat Immunol 2010; 11(11):1014-22.
7. Sharma S, Kulk N, Nold MF, Graf R, Kim SH, Reinhardt D, et al. The IL-1 family member 7b translocates to the nucleus and down-regulates proinflammatory cytokines. J Immunol 2008; 180(8):5477-82.
8. Bufler P, Azam T, Gamboni-Robertson F, Reznikov LL, Kumar S, Dinarello CA, et al. A complex of the IL-1 homologue IL-1F7b and IL-18-binding protein reduces IL-18 activity. Proc Natl Acad Sci U S A 2002; 99(21):13723-8.
9. Pan G, Risser P, Mao W, Baldwin DT, Zhong AW, Filvaroff E, et al. IL-1H, an interleukin 1-related protein that binds IL-18 receptor/IL-1Rrp. Cytokine 2001;

13(1):1-7.
10. Bulau AM, Fink M, Maucksch C, Kappler R, Mayr D, Wagner K, et al. In vivo expression of interleukin-37 reduces local and systemic inflammation in concanavalin A-induced hepatitis. ScientificWorldJournal 2011; 11:2480-90.
11. Bulau AM, Nold MF, Li S, Nold-Petry CA, Fink M, Mansell A, et al. Role of caspase-1 in nuclear translocation of IL-37, release of the cytokine, and IL-37 inhibition of innate immune responses. Proc Natl Acad Sci U S A 2014; 111(7):2650-5.
12. Lunding L, Webering S, Vock C, Schroder A, Raedler D, Schaub B, et al. IL-37 requires IL-18Ralpha and SIGIRR/IL-1R8 to diminish allergic airway inflammation in mice. Allergy 2015; 70(4):366-73.
13. Luo Y, Cai X, Liu S, Wang S, Nold-Petry CA, Nold MF, et al. Suppression of antigen-specific adaptive immunity by IL-37 via induction of tolerogenic dendritic cells. Proc Natl Acad Sci U S A 2014; 111(42):15178-83.
14. McNamee EN, Masterson JC, Jedlicka P, McManus M, Grenz A, Collins CB, et al. Interleukin 37 expression protects mice from colitis. Proc Natl Acad Sci U S A 2011; 108(40):16711-6.
15. Patel FJ, Volkmann DT, Taylor GW, Hansson MA, Anderson JF, Zhou Y, et al. IL-37 reduces inflammatory response after cerebral ischemia and reperfusion injury through down-regulation of pro-inflammatory cytokines. Cytokine 2014; 69(2):234-9.
16. Wu B, Meng K, Ji Q, Cheng M, Yu K, Zhao X, et al. Interleukin-37 ameliorates myocardial ischaemia/reperfusion injury in mice. Clin Exp Immunol 2014; 176(3):438-51.
17. Chen HM, Fujita M. IL-37: a new player in immune tolerance. Cytokine 2015; 72(1):113-4.
18. Bufler P, Gamboni-Robertson F, Azam T, Kim SH, Dinarello CA. Interleukin-1 homologues IL-1F7b and IL-18 contain functional mRNA instability elements within the coding region responsive to lipopolysaccharide. Biochem J 2004; 381(Pt 2):503-10.
19. Smith DE, Renshaw BR, Ketchem RR, Kubin M, Garka KE, Sims JE. Four new members expand the interleukin-1 superfamily. J Biol Chem 2000; 275(2):1169-75.
20. Charrad R, Berraies A, Hamdi B, Ammar J, Hamzaoui K, Hamzaoui A. Anti-inflammatory activity of IL-37 in asthmatic children: Correlation with inflammatory cytokines TNF-alpha, IL-beta, IL-6 and IL-17A. Immunobiology 2016; 221(2):182-7.
21. Mousavi T, Salek Moghadam A, Falak R, Tebyanian M. Co-administration of CpG oligonucleotides and chenopodium album extract reverse IgG2a/IgG1 ratios and increase IFN-gamma and IL-10 productions in a murine model of asthma. Iran J Allergy Asthma Immunol 2008; 7(1):1-6.
22. Kim BY, Park HR, Shin JH, Kim SW, Cho JH, Park YJ, et al. The Serine Protease Inhibitor, 4-(2-aminoethyl) Benzene Sulfonyl Fluoride Hydrochloride, Reduces Allergic Inflammation in a House Dust Mite Allergic Rhinitis Mouse Model. Allergy Asthma Immunol Res 2014; 6(6):558-66.
23. Kim YH, Yang TY, Park CS, Ahn SH, Son BK, Kim JH, et al. Anti-IL-33 antibody has a therapeutic effect in a murine model of allergic rhinitis. Allergy 2012; 67(2):183-90.
24. Minty A, Chalon P, Derocq JM, Dumont X, Guillemot JC, Kaghad M, et al. Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. Nature 1993; 362(6417):248-50.
25. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 2000; 100(6):655-69.
26. Yamashita M, Ukai-Tadenuma M, Miyamoto T, Sugaya K, Hosokawa H, Hasegawa A, et al. Essential role of GATA3 for the maintenance of type 2 helper T (Th2) cytokine production and chromatin remodeling at the Th2 cytokine gene loci. J Biol Chem 2004; 279(26):26983-90.
27. Fields ML, Hondowicz BD, Metzgar MH, Nish SA, Wharton GN, Picca CC, et al. CD4+ CD25+ regulatory T cells inhibit the maturation but not the initiation of an autoantibody response. J Immunol 2005; 175(7):4255-64.
28. Bousquet J, Schunemann HJ, Samolinski B, Demoly P, Baena-Cagnani CE, Bachert C, et al. Allergic Rhinitis and its Impact on Asthma (ARIA): achievements in 10 years and future needs. J Allergy Clin Immunol 2012; 130(5):1049-62.
29. Rosenberg HF, Phipps S, Foster PS. Eosinophil trafficking in allergy and asthma. J Allergy Clin Immunol 2007; 119(6):1303-10.
30. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, et al. Interleukin-13: central mediator of allergic asthma. Science 1998; 282(5397):2258-61.
31. Nold-Petry CA, Lo CY, Rudloff I, Elgass KD, Li S, Gantier MP, et al. IL-37 requires the receptors IL-18Ralpha and IL-1R8 (SIGIRR) to carry out its multifaceted anti-inflammatory program upon innate signal transduction. Nat Immunol 2015; 16(4):354-65.
32. Liu Y, Zeng M, Liu Z. Re: CEA-2015-0096-CR-AJW, Th17 polarization and upper airways: new insights:

Clinical relevance of Th17 response in allergic rhinitis: more evidence. Clin Exp Allergy 2015; 45(12):1875.

33. Xuekun H, Qintai Y, Yulian C, Gehua Z. Correlation of gammadelta-T-cells, Th17 cells and IL-17 in peripheral blood of patients with allergic rhinitis. Asian Pac J Allergy Immunol 2014; 32(3):235-9.
34. Garlanda C, Dinarello CA, Mantovani A. The interleukin-1 family: back to the future. Immunity 2013; 39(6):1003-18.
35. Gulen MF, Kang Z, Bulek K, Youzhong W, Kim TW, Chen Y, et al. The receptor SIGIRR suppresses Th17 cell proliferation via inhibition of the interleukin-1 receptor pathway and mTOR kinase activation. Immunity 2010; 32(1):54-66.
36. Kim BY, Park HR, Jeong HG, Kim SW. Berberine reduce allergic inflammation in a house dust mite allergic rhinitis mouse model. Rhinology 2015; 53(4):353-8.
37. Kim YJ, Kim HJ, Kang MJ, Yu HS, Seo JH, Kim HY, et al. Bacillus Calmette-Guerin Suppresses Asthmatic Responses via CD4(+)CD25(+) Regulatory T Cells and Dendritic Cells. Allergy Asthma Immunol Res 2014; 6(3):201-7.