

Anti-IL-33 antibody has a therapeutic effect in a murine model of allergic rhinitis

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Abstract

Background: Interleukin (IL)-33 is involved in the Th2 immune response and could play an essential role in nasal allergy. Therefore, we aimed to investigate the therapeutic potential of anti-IL-33 for allergic rhinitis (AR).

Methods: Twenty-four BALB/c mice were used. In group A (control group, $n = 6$), mice were sensitized and challenged with saline. Group B [ovalbumin (OVA) group, $n = 6$] mice received intraperitoneal and intranasal OVA challenge. In group C (control IgG group, $n = 6$), mice were injected intraperitoneally with rabbit control IgG before OVA challenge. In group D (anti-IL-33 group, $n = 6$), anti-IL-33 was injected before challenge. We evaluated the number of nose-scratching events and external morphology; serum total and OVA-specific IgE; number of eosinophils, neutrophils, and lymphocytes in bronchoalveolar lavage (BAL) fluid; histopathologic examination of nasal cavity; and IL-4, IL-5, and IL-13 in BAL fluid.

Results: Anti-IL-33 treatment significantly reduced the nose-scratching events and ameliorated skin denudation. Serum total and OVA-specific IgE was significantly decreased in group D. The number of eosinophils in BAL fluid was also significantly decreased. Eosinophilic infiltration in the nasal cavity was significantly decreased in group D. IL-4, IL-5, and IL-13 in BAL fluid were also significantly decreased after treatment.

Conclusions: Anti-IL-33 antibody has a therapeutic potential for experimental AR.

Interleukin (IL)-33 is a member of the IL-1 superfamily, and its molecular properties are similar to a number of closely related cytokines including IL-1 α / β , IL-1Ra, and IL-18 (1). IL-33 and its receptor ST2 (formerly known as orphan receptor) promote various activities related to the up-regulation of systemic Th2 response of different cell types such as mast cells, Th2 lymphocytes, eosinophils, basophils, and structural cells such as airway epithelium (1, 2). IL-33 is released by damaged cells undergoing necrotic cell death, suggesting that IL-33 functions as an endogenous danger signal or 'alarmin' particularly in epithelial or endothelial cells that are directly exposed to environmental challenge (3, 4).

In experimental models of asthma, blocking of the IL-33/ST2 signaling cascade reduces the severity of disease (5–9). Anti-IL-33 antibody significantly reduces Th2 cytokine production *in vivo*, eosinophilic inflammation, and airway hyper-reactivity (10). Anti-IL-33 antibody also impedes eosinophilic infiltration and reduces serum IgE titer. Conversely, intranasal IL-33 induces allergic pathology (11). These observations imply that IL-33 could play an essential role in clinical asthma and allergy. However, the precise role of IL-33 in models of asthma still remains to be elucidated (2).

Allergic rhinitis (AR) is a type of nasal allergy, and its pathogenesis is thought to be related to the up-regulation of

Th2 cytokines (12–14). According to the recently proposed ‘united airway disease’ concept, bronchial asthma and AR are not different disease entities, but represent a different spectrum of same disease. Because the nasal cavity is also covered with epithelial cells, IL-33 release from the nasal epithelial cells could act as an ‘alarmin’ and initiate the allergic reaction cascade along with other cytokines. However, nothing is known of the effect of blockade of IL-33 function on the clinical course of AR.

This study was designed to investigate the therapeutic potential of IL-33 blockade in AR in experimental murine model of AR. The following were determined: number of nose-scratching events and change of external morphology because of increased scratching; change of serum total IgE and ovalbumin (OVA)-specific IgE; changes in numbers of eosinophils, neutrophils, and lymphocytes in bronchoalveolar lavage (BAL) fluid; and change of the cytokines IL-4, IL-5, and IL-13 in BAL fluid. As well, the nasal cavity was histopathologically examined.

Materials and methods

Animals

Twenty-four female BALB/c mice, 8–10 weeks old and free of murine-specific pathogens, were obtained from Orient Bio (Seongnam, Korea). The mice were housed in a controlled environment with a 12-h light/dark cycle with free access to food and water. They were maintained on an OVA-free diet. All experimental animals used in this study were handled using a protocol approved by the Institutional Animal Care and Use Committee of Inha University.

Sensitization and antigen challenge

The sensitization and antigen challenge for the murine model of AR was performed as previously described with slight modification (15). Under pathogen-free conditions, mice were sensitized using OVA (Sigma-Aldrich, St. Louis, MO, USA) as follows. Ovalbumin (40 µg/kg) OVA diluted in sterile normal saline was administered along with aluminum hydroxide gel (alum adjuvant, 40 mg/kg) to unanesthetized animals four times by intraperitoneal injection on days 1, 5, 14, and 21. This was followed by daily intranasal challenge with OVA diluted with sterile normal saline intranasally (20 µl of 25 mg/ml OVA per mouse) from days 22 to 35. Twenty-four hours after the last OVA challenge, blood, BAL fluid, lung tissues, and nasal cavity tissues were collected from each mouse.

For blocking experiments, mice also received anti-mouse IL-33 antibody (R&D Systems, Minneapolis, MN, USA), which was produced in goats immunized with purified, *Escherichia coli*-derived recombinant mature mouse interleukin 33 (rmIL-33). Mouse IL-33-specific IgG was purified by mouse IL-33 affinity chromatography. Anti-IL-33 antibody (3.6 µg/mouse) was given intraperitoneally at 30 min before intraperitoneal OVA sensitization and intranasal OVA antigen challenge. As the control, OVA-challenged mice were

given the same amount of rabbit control IgG (purified normal rabbit IgG; R&D Systems) by intraperitoneal injection. In group A ($n = 6$), mice were sensitized and challenged with intraperitoneal and intranasal normal saline as control group. Mice in group B ($n = 6$) received the similar intraperitoneal and intranasal OVA challenge. In group C ($n = 6$; control IgG group), rabbit control IgG was injected intraperitoneally before any OVA challenge. In group D ($n = 6$; anti-IL-33 group), the pretreatment with anti-IL-33 antibody intraperitoneal injection was performed before every OVA challenge.

Evaluation of scratching events and external cell morphology

Twenty-four hours after the last intranasal saline or OVA challenge, each mouse was challenged with intranasal OVA instillation. Immediately after OVA challenge, the number of nose-scratching events over the next 10 min was independently recorded by two observers. For the objective documentation of external morphological change because of increased scratching, photographs were taken in each group.

Serum collection and cell count in BAL fluid

After the kill, mouse serum was collected by aortic puncture. Bronchoalveolar lavage was obtained immediately after the last measurement of airway responsiveness. The trachea was cannulated with a polyethylene tube through which the lungs were lavaged four times with 1.0 ml of saline. The BAL fluid was then filtered through wet gauze. The trypan blue exclusion test was carried out to determine cell viability, and total cell count was performed. The BAL fluid was centrifuged for 10 min at 150 *g*. The pellet was suspended immediately in 4 ml of saline, and total cell numbers in BAL fluid were counted in duplicate with hemocytometer. Then, a 100-µl aliquot was centrifuged in a Model 2 Cytospin cytocentrifuge (Shandon Scientific, Pittsburgh, PA, USA). Differential cell counts were obtained from centrifuged preparations stained with Diff-quick, counting 500 or more cells in each animal at a magnification $\times 1000$ (oil immersion).

Histopathology

The nasal cavity tissues were fixed in EDTA solution for 3 weeks. Then, they were embedded in paraffin using standard methods. Four-micrometer-thick sections were stained with hematoxylin and eosin (H&E) to detect cellular infiltration.

Enzyme-linked immunosorbent assay (ELISA)

Serum levels of total and OVA-specific IgE were measured by ELISA as previously described (16). Total IgE was measured and compared with a mouse IgE standard (BD Pharmingen, San Diego, CA, USA). Because, at the time of the study, there was no known mouse OVA-specific IgE standard, the authors used optical density (OD) at 450 nm without calculating the concentration with standard solution. Levels of IL-4, IL-5, and IL-13 were measured using ELISA

kits (Biosource, Camarillo, CA, USA) according to the manufacturer's instructions and compared with known standards.

Statistical analyses

The Kruskal–Wallis test and Mann–Whitney *U*-test were used for the comparison of number of scratching events, serum levels of total and OVA-specific IgE, number of eosinophils, neutrophils and lymphocytes in BAL fluid, change of Penh and levels of IL-4, IL-5, and IL-13 between groups. The linear regression analyses were performed to find any correlation between the number of scratching event and other parameters. A *P*-value < 0.05 was considered to be statistically significant.

Results

Scratching events and external morphology

Compared with group A (control group, challenged with saline only), mice in group B (AR group, sensitized, and challenged with OVA) and group C (control IgG group, immunized with control IgG) showed significantly more scratching events. After anti-IL-33 antibody treatment, mice in group D (anti-IL-33 group immunized with anti-IL-33 antibody) showed a meaningful decrease in scratching events compared with their allergic counterparts (Fig. 1). Concerning the external morphology, mice in groups B and C showed denuded skin around the nose, probably because of the

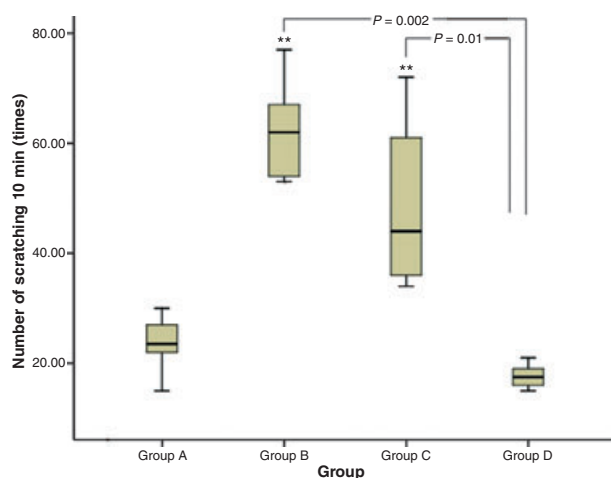


Figure 1 Number of scratching events in 10 min between the treatment groups. Mice in groups B and C scratched their nose significantly more than those in group A. After anti-IL-33 treatment, mice in group D showed significantly less number of scratching events compared with those in group B or C. Group A: control group challenged with saline, group B: allergic rhinitis (AR) group sensitized and challenged with ovalbumin, group C: group with AR and intraperitoneal immunization with control IgG, group D: AR and immunization with anti-IL-33 intraperitoneal immunization. (Kruskal–Wallis test and Mann–Whitney *U*-test, **Significant difference with group A, *P* < 0.01)

increased scratching. However, mice in group D showed intact skin around the nose (Fig. 2).

Serum total and OVA-specific IgE

Serum total and OVA-specific IgE level was significantly elevated in groups B and C compared with group A, which were significantly decreased after treatment with anti-IL-33 antibody (Fig. 3). Linear regression analysis revealed a strong positive linear relationship between the number of scratching events and OVA-specific IgE level ($R = 0.720$, $P < 0.001$).

Analysis of cells in BAL fluid

The number of eosinophils in BAL fluid was significantly increased in groups B and C, and it was significantly decreased by treatment with anti-IL-33 antibody in group D. The number of neutrophils and lymphocytes tended to be decreased in group D compared with those of groups B and C, although without statistical significance in case of lymphocytes (Fig. 4). Linear regression analysis revealed a significant linear correlation between the number of scratching events and the number of eosinophils ($R = 0.650$ and $P < 0.001$), neutrophils ($R = 0.475$ and $P = 0.007$), and lymphocytes ($R = 0.580$ and $P = 0.001$).

Histopathologic examination

Histopathologic examination of the turbinate revealed significantly more infiltration of eosinophils in group B and group C, compared with group A. After the treatment with anti-IL-33 antibody, eosinophilic infiltration was significantly decreased in group D (Fig. 5).

Cytokines in BAL fluid

The titers of IL-4, IL-5, and IL-13 were significantly elevated in group B and group C, compared with those of group A. After treatment with anti-IL-33 antibody, the titers of these cytokines were significantly less than those of group B or group C (Fig. 6). Linear regression analysis revealed significant correlations between the titers of each of these cytokines and number of scratching events (IL-4: $R = 0.665$, $P = 0.001$, IL-5: $R = 0.640$, $P = 0.002$, IL-13: $R = 0.665$, $P = 0.001$).

Discussion

Anti-IL-33 treatment was effective in alleviating allergic symptoms (nose-scratching behavior). Furthermore, the skin denudation because of the excessive scratching was significantly decreased after the treatment. Considering that AR is accompanied with several symptoms and these symptoms significantly impair the quality of life, anti-IL-33 antibody treatment has the potential to improve the quality of lives of the patients with AR.

Presently, the titer of total IgE and OVA-specific IgE was significantly decreased after the anti-IL-33 antibody treat-

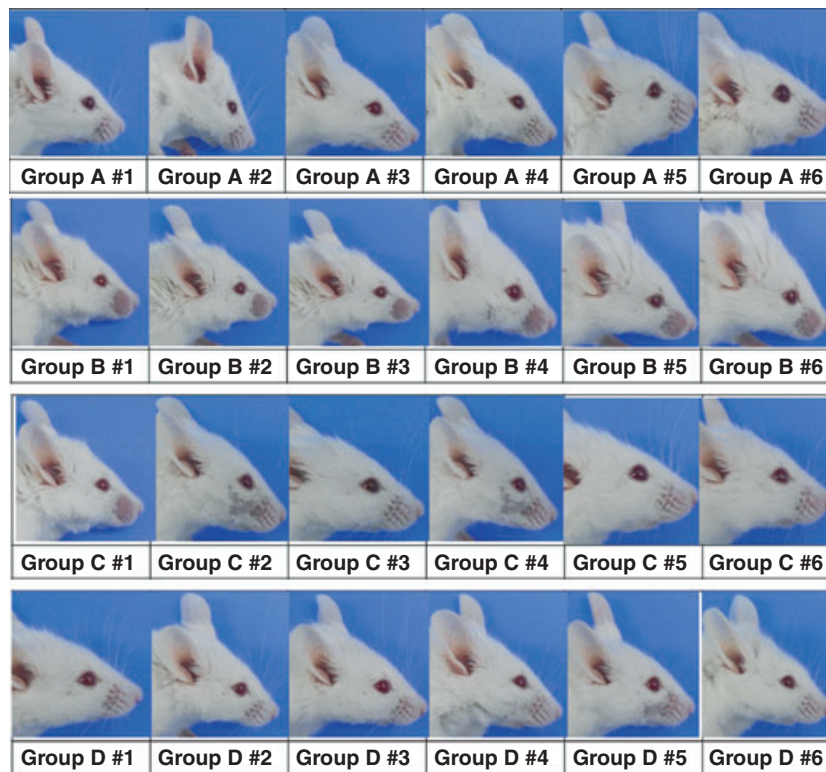


Figure 2 Anti-IL-33 antibody treatment significantly ameliorated the skin lesion induced by nose scratching. Compared with those in group A, mice in groups B and C had denuded skin lesion around their nose possibly because of excessive nose scratching. After

anti-IL-33 treatment, mice in group D showed significantly less skin lesion, which was well correlated with their less number of nose-scratching events.

ment, which correlated well with the results of a previous study (7). This decrease in IgE is thought to be caused by the inhibitory effects on B cell activation and reduced release of Th2 cytokines. Reduced IgE, in turn, causes less degranulation of mast cells and cytokine release. Therefore, through the decrease in IgE and inhibitory effects on the activation of

mast cells, anti-IL-33 antibody treatment is thought to inhibit the systemic allergic response. Because there was a significant correlation between the IgE level and the number of scratching events, we postulate that this inhibition of systemic allergy also contributes to the clinical alleviation of experimental AR.

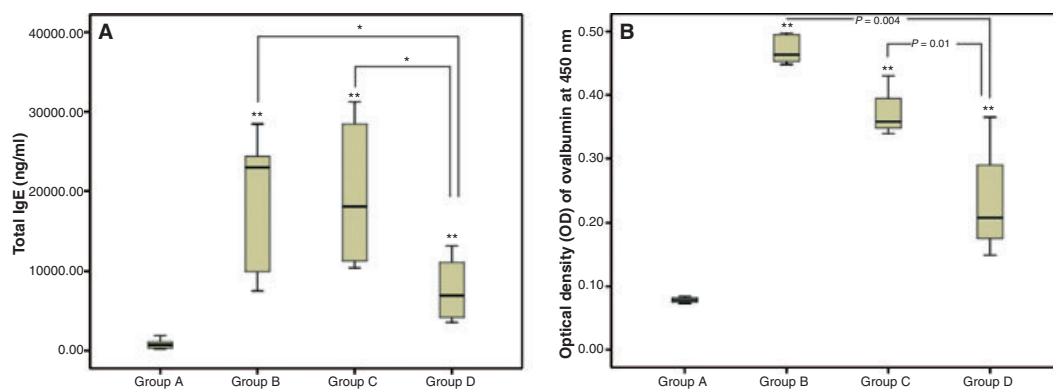


Figure 3 Serum (A) total IgE and (B) ovalbumin (OVA)-specific IgE. Mice in groups B and C had significantly elevated serum level of total and OVA-specific IgE compared with those in group A, which were significantly decreased in group D after anti-IL-33 treatment.

Level of OVA-specific IgE was expressed as optical density (OD) at 450 nm because of the absence of standard OVA-specific IgE kit. (Kruskal–Wallis test and Mann–Whitney *U*-test, * $P < 0.05$, **Significant difference with group A, $P < 0.01$).

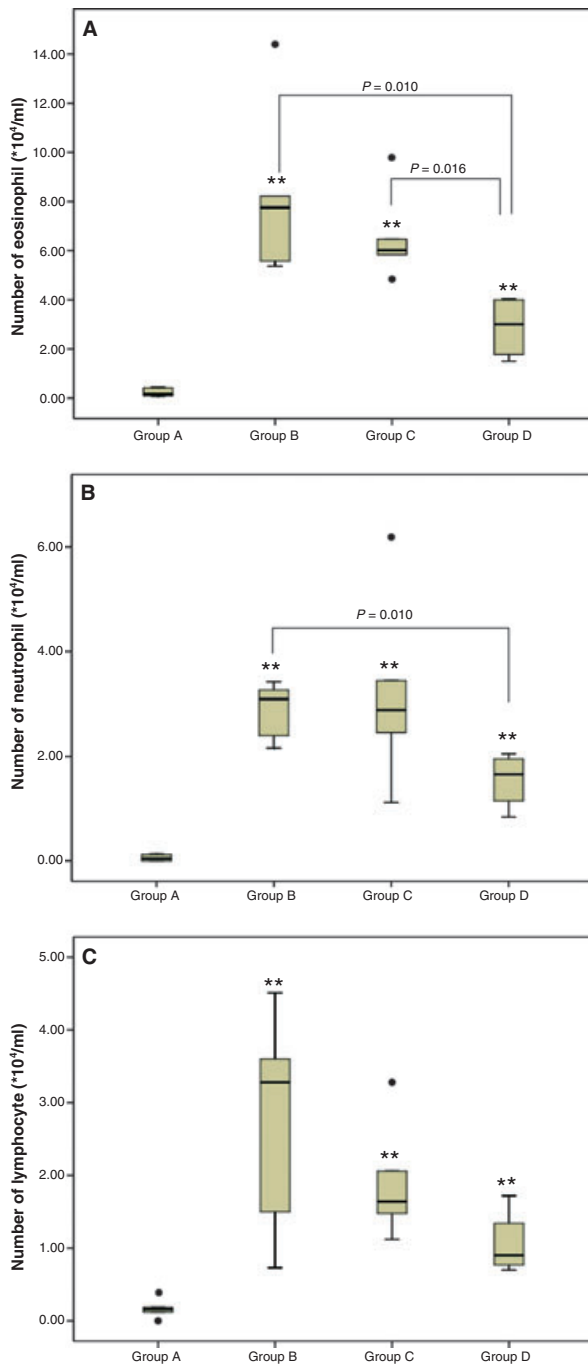


Figure 4 The number of (A) eosinophils, (B) neutrophils, and (C) lymphocytes in BAL fluid. The number of eosinophils in group D was significantly less than those of groups B and C. Although the number of neutrophils and lymphocytes also tended to be decreased in group D compared with group B or C, there were no statistical significances. (Kruskal–Wallis test and Mann–Whitney *U*-test, **Significant difference with group A, $P < 0.01$).

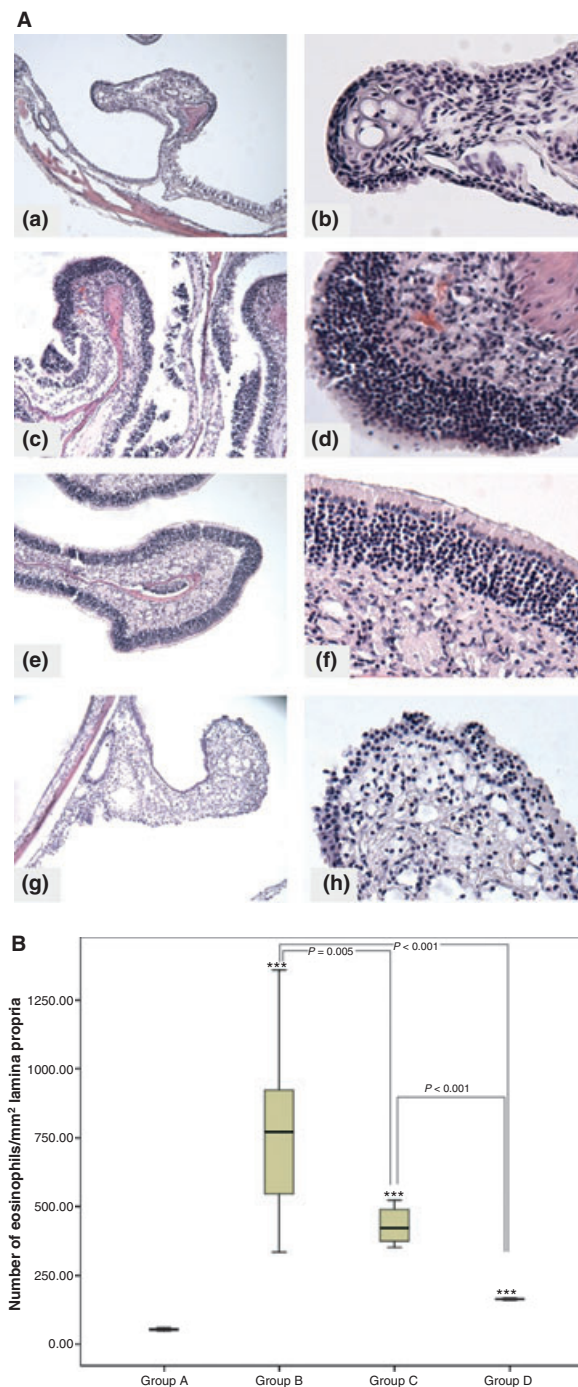


Figure 5 (A) Histopathologic examination of the nasal cavity (turbinates) and (B) comparison of the number of eosinophils in the nasal mucosa between groups. After IL-33 treatment, eosinophilic infiltration of group D was significantly decreased compared with that of group B or C. (Fig. 5A, a, b: group A, c, d: group B, e, f: group C, g, h: group D. Hematoxylin & Eosin stain, $\times 200$ and $\times 400$ each, Kruskal–Wallis test and Mann–Whitney *U*-test, ***Significant difference with group A, $P < 0.001$).

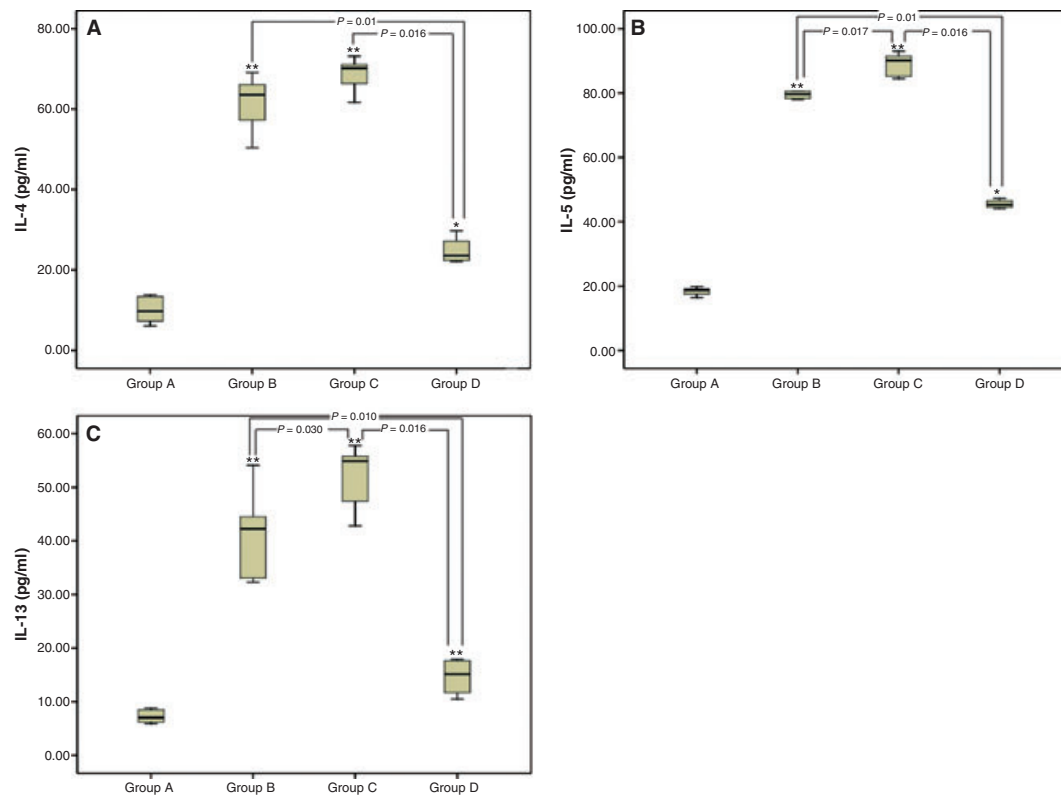


Figure 6 The titers of (A) IL-4, (B) IL-5, and (C) IL-13 in BAL fluid in each group. The titers of IL-4, IL-5, and IL-13 were significantly elevated in mice in groups B and C, which were all significantly

decreased after anti-IL-33 treatment in group D. (Kruskal–Wallis test and Mann–Whitney *U*-test, *: significant difference with group A, $P < 0.05$, **: Significant difference with group A, $P < 0.01$).

We found that the number of eosinophils in BAL fluid was significantly decreased after the anti-IL-33 antibody treatment, which was consistent with previous observations (7). In fact, eosinophils are a direct leukocyte target of IL-33 (17). IL-33 injection in experimental animals can cause profound mucosal eosinophilia and related pathologic changes (18). IL-33 enhances eosinophil adhesion and expression of CD11b, prolongs eosinophil survival, and promotes eosinophil degranulation and release of superoxide anion (19, 20). These actions are thought to be blocked by anti-IL-33 antibody treatment.

There is still controversy about the role of IL-33 on neutrophils. Cherry et al. (19) argued that neutrophils do not express the ST2 receptor and so do not respond to IL-33. On the other hand, Liu et al. (7) reported that the number of neutrophils was slightly decreased after the treatment, although without significance. In our study, the number of neutrophils was significantly decreased after anti-IL-33 treatment, which had statistical significance compared with OVA challenge group. Therefore, further studies designed to elucidate the effect of IL-33 on neutrophils are needed to clarify the issue.

Although without statistical significance, the number of lymphocytes also tended to decrease after anti-IL-33 antibody treatment, which was also in accordance with a previous study (7). This decrease in lymphocytes could have an effect

on the AR through the decreased induction of IL-5 and IL-13. The present results concerning the titers of IL-5 and IL-13 in BAL fluid further support this possibility. The number of eosinophils, neutrophils, and lymphocytes all correlated significantly with the number of scratching events. These findings imply that these cells play a central role in the allergic inflammation of nasal cavity also, and anti-IL-33 antibody treatment exerts therapeutic effect by inhibiting the action of these cells.

To confirm the treatment effect of anti-IL-33 antibody on the experimental AR, the histological examination of the target organ itself, the nasal cavity, is mandatory. This is the first study that documents the decreased eosinophilic infiltration into the nasal mucosa. We conclude that anti-IL-33 antibody has a therapeutic effect on experimental AR.

The titers of IL-4, IL-5, and IL-13 were all significantly decreased after anti-IL-33 antibody treatment in BAL fluid. These cytokines play important roles in the initiation and maintenance of allergic inflammation (21). The development of airway eosinophilia and airway hyper-reactivity is also associated with these cytokines in BAL fluid (7). IL-4 promotes Th2 cell differentiation, synthesis of IgE and up-regulation of receptors for IgE, and secretion of mucus (22–24). IL-5 increases eosinophilic inflammation and airway infiltration (25). IL-13 induces differentiation of B cells and isotype

change to IgE (26). Through the inhibition of these actions, anti-IL-33 antibody exerts its therapeutic effects on allergic inflammation and AR.

One advantage of our study is that the minimum quantity of anti-IL-33 antibody with significant effect and without toxicity to experimental animals was used (3.6 µg per mouse each time). In other studies utilizing anti-IL-33 antibody, amounts as large as 150 µg per mouse were used (7), which could cause significant toxicity and even lethality to the experimental animals. By trial and error, we determined the minimum and optimum quantities of antibody. The result was the absence of mouse death following injection of anti-IL-33 antibody.

Another strength of our study is that we tried to relate the airway findings with those of nasal cavity, uniting the upper and lower airway, consistent with the 'united airway disease' concept. Using this tact, we demonstrated a significant correlation between several parameters of airway inflammation and the number of scratching events. This result supports the 'united airway disease' concept.

Although not included in our study, mast cell is another important cell for the allergic inflammation. Further study to identify the changes in the number of mast cells or their mediators could yield another meaningful result.

In conclusion, we postulate that anti-IL-33 antibody has a therapeutic potential for the alleviation of AR in a murine model. Further studies are needed to identify the exact mechanism of anti-IL-33 antibody to treat AR.

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Author contributions

Young Hyo Kim is the main writer. Tae Yong Yang and Sung-Hye Ahn performed the main animal experiment and gained the preliminary data. Dae Hyun Lim performed English interpretation and correction of the script. Chang-Shin Park performed the statistical analysis. Byong Kwan Son and Jeong Hee Kim performed the statistical analysis and the production of image. Tae Young Jang designed the whole study and is responsible for the study.

Conflicts of interest

There is no conflict of interest related to this study.

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