

Cytokine modulation by IL-35 in mice with allergic rhinitis

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ABSTRACT

Background: Interleukin (IL) 35 was recently identified as an additional anti-inflammatory and immunosuppressive cytokine. However, the role of IL-35 in allergic rhinitis is not well understood. The effect of IL-35 on other cytokines in allergic responses is also unclear.

Objective: To investigate, in mice with allergic rhinitis, the effect of IL-35 on other cytokines associated with allergic rhinitis.

Methods: A murine model of allergic rhinitis was established, and splenic cells were collected. Ovalbumin-specific allergic T-cell response was measured. The production of cytokines (T helper 1 interferon-gamma), Th2 (IL-4, IL-5, IL-13), Th17 (IL-17), IL-12 family (IL-12, IL-23, IL-27), IL-2, tumor necrosis factor-alpha, transforming growth factor-beta, and IL-10) stimulated with antigen was also measured in the presence or absence of IL-35.

Results: IL-35 significantly inhibited the ovalbumin-specific T-cell response. It also significantly reduced the production of IL-4, IL-5, IL-13, IL-17, IL-23, and TNF-alpha, and significantly increased the production of IL-2, IL-10, and IL-27.

Conclusion: This study showed that IL-35 inhibits allergic T-cell response and has the ability to modulate the production of IL-2, IL-4, IL-5, IL-10, IL-13, IL-17, IL-23, IL-27, and TNF-alpha in mice with allergic rhinitis. This study also indicated the possibility of a novel therapy with IL-35 for the control of allergic rhinitis.

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Epstein-Barr virus-induced gene 3 and the p35 subunit of interleukin (IL) 12 have been reported to form a heterodimeric hemopoietin, IL-35, which, in 2007, was identified as an additional anti-inflammatory and immunosuppressive cytokine.¹ IL-35 generates regulatory T (Treg) cells that express IL-35 but do not express Foxp3, transforming growth factor (TGF)- β , or IL-10 (IL-35 producing inducible regulatory T cells [iTreg35 cells]).²

Huang *et al.*³ showed that IL-35 inhibited immunoglobulin E (IgE) production and IL-4, IL-5, and IL-13 in bronchoalveolar lavage fluid (BALF) induced by a dust-mite allergen-specific memory/effector Th2 cell line. Whitehead *et al.*⁴ also showed that IL-35 production by Treg cells suppressed the levels of IL-17 and Th17-mediated allergic airway hyperresponsiveness. However, reported investigations of IL-35 in allergic rhinitis are very limited and few. The effect of IL-35 on other cytokines in allergic responses is also unclear. We, therefore, investigated the effect of IL-35 on Th1 (IFN- γ), Th2 (IL-4, IL-5, IL-13), Th17 (IL-17), the IL-12 family (IL-12, IL-23, IL-27), IL-2, TNF- α , TGF- β , and IL-10 cytokines, which are associated with allergic rhinitis, to elucidate the mechanism of IL-35 effects in allergic rhinitis model mice.

MATERIALS AND METHODS

Immunization

Eight-week-old male BALB/c mice were purchased from Japan SLC Ins (Shizuoka, St Louis, MO) and injected with 2 mg Al(OH)₃ twice on days 0 and 14. The same mice were challenged intranasally on days 21 through 27 with ovalbumin (OVA) (200 μ g). Immediately after the last nasal challenge, the number of sneezing and nasal rubbing movements was counted for 20 minutes according to the previously described method,⁵ and samples were collected on day 28. All the mice were housed in an environmentally controlled animal facility at Nagoya City University. The protocols were approved by

the guidelines for care and use of animals at Nagoya City University. Every effort was made to minimize any discomfort of the animals.

Measurement of OVA-specific IgE

Mouse serum titers of OVA-specific IgE were measured by enzyme-linked immunosorbent assay (ELISA). Briefly, ELISA plates were coated with antimouse IgE monoclonal antibody (Yamasa, Tokyo, Japan). Nonspecific binding was blocked, and sera were added to the plate. After adding biotinylated OVA, the plates were incubated with Avidin-peroxidase (Vector Laboratories, Burlingame, CA). The tetramethylbenzidine microwell peroxidase substrate system (KPL, Gaithersburg, MD), which develops a deep blue color when reacted with peroxidase conjugates in ELISA, was applied according to the manufacturer's instructions after washing. Optical density was measured at 450 nm.

Isolation of Splenic Dendritic Cells and CD4⁺CD25⁻ T cells

Dendritic cells (DC) were isolated from spleens of mice by gradient centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sala, Sweden) and were labeled with monoclonal antimouse CD11c antibody-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD11c⁺ cells were isolated by using a magnetic column.

CD4⁺CD25⁻ T cells were isolated from spleens by using the magnetic-activated cell sorting (MACS) system according to manufacturer's instruction. CD4⁺ T cells were negatively selected with a MACS negative selection CD4 isolation kit (Miltenyi Biotec), and CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were then separated by anti-CD25 MACS beads (Miltenyi Biotec).

OVA-Specific T-Cell Response

CD4⁺CD25⁻ T cells were isolated from spleens in allergic rhinitis model mice. All CD4⁺CD25⁻ T cells (4 $\times$ 10⁵ cells/well) and DCs (4 $\times$ 10⁴ cells/well) were cultured in 96-well plates for 72 hours in the presence of OVA antigen.

To assess the inhibitory capacity of IL-35, the isolated splenic CD4⁺CD25⁻ T cells and DCs of allergic mice were cultured in 96-well plates with 100 μ g/mL OVA antigen in the presence or absence of 10 ng/mL IL-35 fusion protein (IL-35:Fc [the crystallizable fragment], Chimerigen Laboratories, San Diego, CA). IL-35 and OVA were added on the day when T cells were isolated. In the control group, Fc

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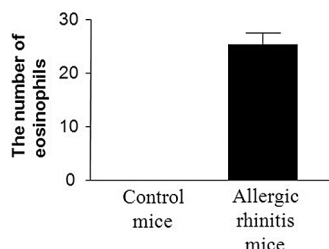


Figure 1. Eosinophilia in allergic rhinitis. Mice were injected with OVA and alum, and were intranasally challenged with OVA (allergic rhinitis mice) or phosphate buffered saline alone (control mice). After sacrificing the mice, nasal sections were fixed and decalcified, and Luna staining was performed. The numbers of eosinophils per high power field (magnification range 10×40) were counted microscopically in the nasal septum.

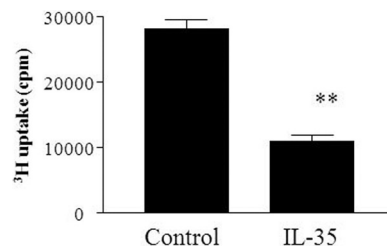


Figure 2. Inhibition of OVA-specific T-cell response by IL-35. Mice were injected with OVA and alum, and intranasally challenged with OVA. Splenic OVA-specific T-cell responses were measured cultured with or without IL-35. ** $p < 0.01$ by Student's *t*-test.

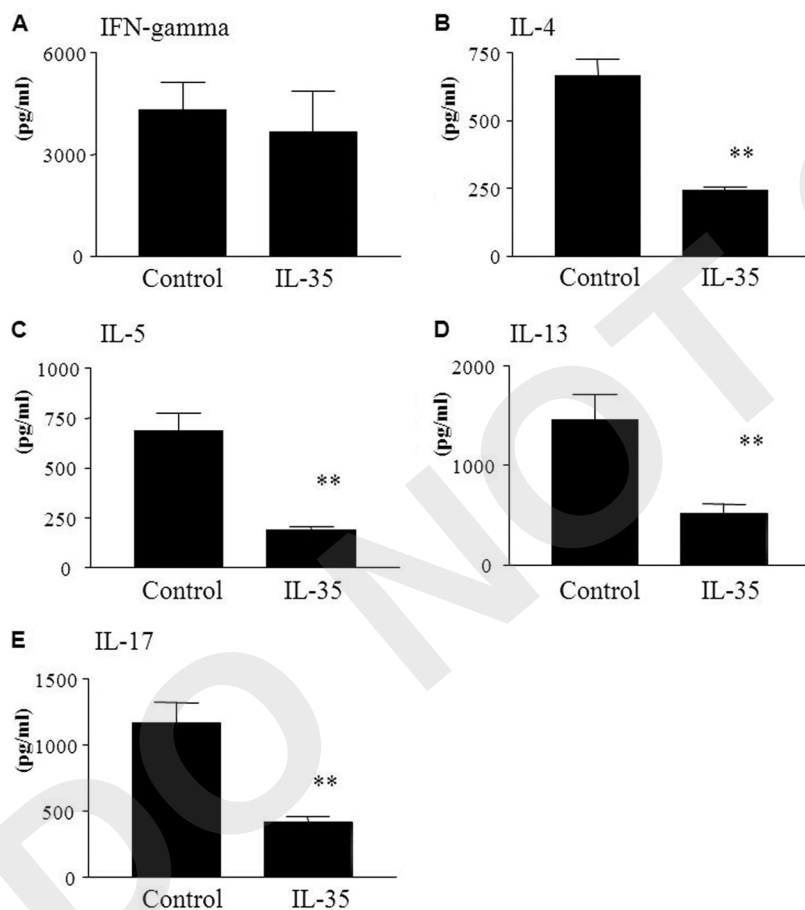


Figure 3. Effect of IL-35 on Th1, Th2, and Th17 cytokines. Mice were injected with OVA and alum, and intranasally challenged with OVA. Splenic $\text{CD4}^+\text{CD25}^-$ T cells and DCs were cultured in the presence of OVA with or without IL-35. Levels of (A) IFN- γ , (B) IL-4, (C) IL-5, (D) IL-13, or (E) IL-17 in the supernatant were measured by using ELISA. ** $p < 0.01$, Student's *t*-test.

IgG1 control, but not IL-35:Fc, was also added. IL-35:Fc and Fc IgG1, which we used, were produced in the same cells, chinese hamster ovary cells. Cells were cultured for 3 days and pulsed with $1 \mu\text{Ci}$ of [^3H] thymidine (Perkin Elmer, Billerica, MA) for the last 16 hours of culture. Cells were harvested onto glass fiber filters, and incorporated radioactivity was quantitated by using liquid scintillation counter. Results were expressed as a mean $\pm$ SE cpm of triplicate cultures.

Measurement of Cytokine Release by *In Vitro* Splenocytes

Spleen $\text{CD4}^+\text{CD25}^-$ T cell (2×10^6 cells/mL) and DC (2×10^5 cells/mL) suspensions were cultured for 72 hours, stimulated with $100 \mu\text{g/mL}$ OVA antigen in the presence or absence of IL-35 (10

ng/mL). IL-35 and OVA were added on the day when T cells were isolated. Quantities of cytokines (IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-17, IL-23, IL-27, TNF- α , IFN- γ , and TGF- β) in the culture supernatants were determined by using a sandwich ELISA with monoclonal antibodies specific for each cytokine. Plates were coated with anti-mouse IL-2 (BioLegend, San Diego, CA), IL-4 (PeproTech, Rocky Hill, NJ), IL-5 (PeproTech), IL-10 (PeproTech), IL-12 (PeproTech), IL-13 (PeproTech), IL-17 (PeproTech), IL-23 (BioLegend), IL-27 (BioLegend), TNF- α (PeproTech), IFN- γ (PeproTech), or TGF- β (R&D System, Minneapolis, MN). The culture supernatant was then added, and the plates were incubated with the second antibody of biotinylated antimouse IL-2 (BioLegend), IL-4 (PeproTech), IL-5 (PeproTech), IL-10 (PeproTech), IL-12 (PeproTech), IL-13 (PeproTech), IL-17 (PeproTech), IL-23 (BioLegend), IL-27 (BioLegend), TNF- α (PeproTech), IFN- γ (PeproTech),

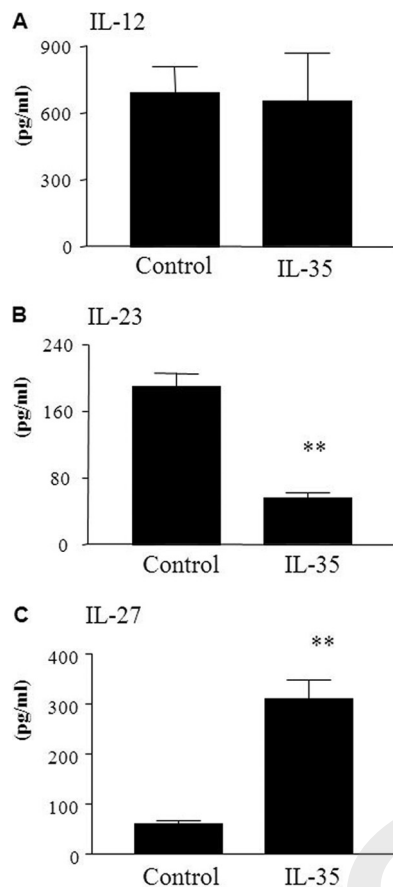


Figure 4. Effect of IL-35 on IL-12 family (IL-12, IL-23, IL-27). Mice were injected with OVA and alum, and intranasally challenged with OVA. Splenic CD4⁺ CD25⁻ T cells and DCs were cultured in the presence of OVA with or without IL-35. Levels of (A) IL-12, (B) IL-23, or (C) IL-27 in the supernatant were measured by using ELISA. ***p* < 0.01, Student's *t*-test.

or TGF- β (R&D System). Standard curves were generated by using recombinant cytokines.

Real-Time Polymerase Chain Reaction

Total RNA was isolated from DCs and T cells after stimulation with 100 μ g/mL OVA antigen in the presence or absence of IL-35 (10 ng/mL) by using Trizol (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. In brief, 20 μ g ribonucleic acid (RNA) was digested with 10 units of deoxyribonuclease I at 37°C for 30 minutes, extracted with phenol:chloroform (3:1), precipitated with ethanol, washed with 70% ethanol, and finally dissolved in 20 μ L of RNase-free water. To generate the first-strand complementary DNA, the SuperScript Preamplification System (Invitrogen Life Technologies).

Real-time polymerase chain reactions (PCR) were performed by using SYBR Green PCR Master mix (Stratagene, La Jolla, CA) and 100 nM of gene-specific primers. The PCR reaction conditions were 95°C for 10 minutes, 95°C for 30 seconds, 58°C for 1 minute, and 72°C for 30 seconds (40 cycles). Mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) messenger RNA was used for normalization to ensure equal amounts of starting RNA.

The primers used in this study were the following:

IL-2, sense 5'-GAGCCTTATGTGTGTAAGC-3' and antisense 5'-GTGCTCCTGTGCAACAGCGC-3';

IL-4, sense 5'-CATGGTGGCTCAGTACTACG-3' and antisense 5'-TCTTTCTCGAATGTACCAGG-3';

IL-5, sense 5'-TCCCTGCTACTCTCCCCAAAC-3'; and antisense 5'-TGGCACAGTCTGATTTCATACATAGG-3';

IL-10, sense 5'-GGACAACATACTGCTAACCGAC-3' and antisense 5'-AAAATCACTCTTCACCTGCTCC-3';

IL-12, sense 5'-CATCGATGAGCTGATGCAGT-3' and antisense 5'-CAGATAGCCCCATCACCCTGT-3';

IL-13, sense 5'-GCTTATTGAGGAGCTGAGCAACA-3' and antisense 5'-GGCCAGGTCCACACTCCATA-3';

IL-17, sense 5'-CTGCTGAGCCTGGCGGTAC-3' and antisense 5'-CATTGCGGTGGAGAGTCCAGGG-3';

IL-23, sense 5'-TGCTGGATTGCAGAGCAGTAA-3' and antisense 5'-CTGGAGGAGTTGGCTGAGTC-3';

IL-27, sense 5'-GGCCAGGYGACAGGAGACC-3' and antisense 5'-CAGCTTGTAACCAAGCAAGGG-3';

IFN- γ , sense 5'-GGACAATCTCTTCCCCACCC-3' and antisense 5'-CATGAAAATCCTGCAGAGCC-3';

TNF- α , sense 5'-AAGGGAGAGTGGTCAGGTTGCC-3' and antisense 5'-CCTCAGGGAAGAGTCTGGAAAGG-3';

TGF- β , sense 5'-TGGAGCAACATGTGGAATC-3' and antisense 5'-GTCAGCAGCCGTTACCA-3';

GAPDH, sense 5'-TGATGACATCAAGAAGGTGGTGA-3' and antisense 5'-TCCTTGGAGGCCATGTAGGCCAT-3'.

Pathology

After being fixed in 10% buffered formalin, the heads were decalcified and sectioned. Three-micrometer-thick cryosections of nasal tissue were stained with Luna staining. The number of eosinophils in the nasal mucosa of the nasal septum was evaluated microscopically by using a high power field (magnification range 10 $\times$ 40).

RESULTS

Establishment of a Murine Model of Allergic Rhinitis

To confirm the establishment of the model of allergic rhinitis, we measured OVA-specific IgE, the number of sneezes and nasal rubbings, and eosinophils at the nasal septum. On day 28, OVA-specific IgE was detected in mice injected with OVA and alum. On day 35, the number of sneezes and nasal rubbings after intranasal challenge with OVA also significantly increased compared with mice that were intranasally challenged with phosphate buffered saline alone after injection with OVA and alum (control mice) (data not shown). Eosinophils at the nasal septum were also seen in these mice but not in the control mice (Fig. 1).

Inhibition of OVA-Specific Allergic T-Cell Response by IL-35

To evaluate the capacity of IL-35 to affect the T-cell response, which is associated with an allergic response, we measured the OVA-specific T-cell response in allergic rhinitis model mice with OVA antigen in the presence and absence of IL-35. The T-cell response stimulated with IL-35 was found to be significantly lower than T-cell response without IL-35 (*p* < 0.01) (Fig. 2).

Effect of IL-35 on Th1, Th2, Th17 cytokines

To investigate the effect of IL-35 on Th1 and Th2 cytokines, we measured the level of Th1 cytokine (IFN- γ) and Th2 cytokines (IL-4, IL-5, and IL-13) cultured with OVA in the presence and absence of IL-35. IL-35 did not significantly change IFN- γ production (Fig. 3A). However, IL-35 significantly reduced the production of IL-4, IL-5, and IL-13 (*p* < 0.01) (Fig. 3B, C, and D). Next, we measured the level of IL-17, which is a Th17 cytokine, produced by OVA stimulation. IL-35 significantly inhibited the production of IL-17 (*p* < 0.01) (Fig. 3E).

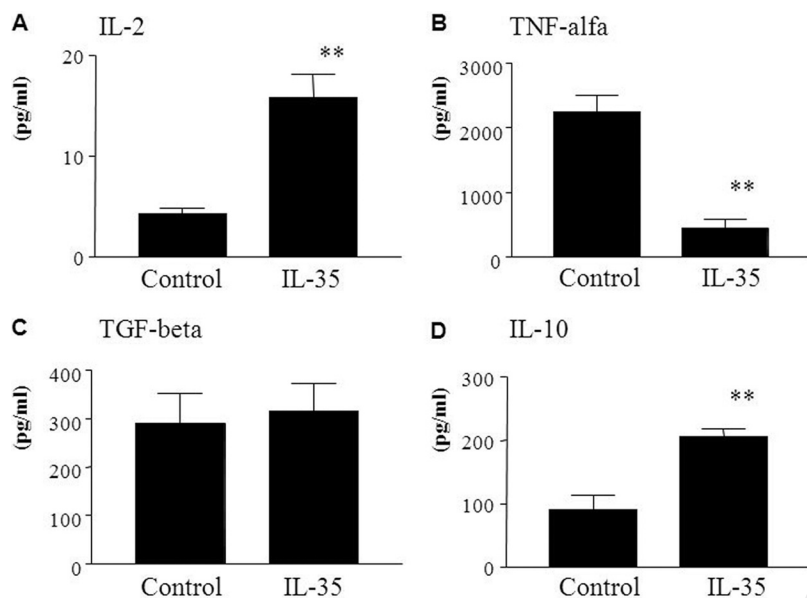


Figure 5. The effect of IL-35 on IL-2, TNF- α , TGF- β , and IL-10. Mice were injected with OVA and alum, and intranasally challenged with OVA. Splenic CD4⁺CD25⁻ T cells and DCs were cultured in the presence of OVA with or without IL-35. Levels of (A) IL-2, (B) TNF- α , (C) TGF- β , or (D) IL-10 in the supernatant were measured by using ELISA. ** $p < 0.01$ by Student's *t*-test.

Effect of IL-35 on IL-12 Family Cytokines (IL-12, IL-23, IL-27)

IL-35 is considered to be a new member of the IL-12 family. Therefore, we examined the effect of IL-35 on other IL-12 family cytokines (IL-12, IL-23, IL-27). Isolated splenic cells were cultured with or without IL-35. IL-12, IL-23, and IL-27 production by these cells was then measured. Although IL-35 did not significantly change IL-12 production (Fig. 4 A), it significantly suppressed IL-23 ($p < 0.01$) (Fig. 4 B). It also significantly increased IL-27 production ($p < 0.01$) (Fig. 4 C).

Effect of IL-35 on IL-2, TNF- α , TGF- β , and IL-10

We then measured the effect of IL-35 on IL-2, TNF- α , TGF- β and IL-10, which are associated with allergic responses. IL-35 significantly increased the level of IL-2 ($p < 0.01$) (Fig. 5A). It also significantly inhibited the production of TNF- α ($p < 0.01$) (Fig. 5 B). Although IL-35 did not significantly change TGF- β (Fig. 5 C), it significantly increased the production of IL-10 ($p < 0.01$) (Fig. 5 D).

Effect of IL-35 on Transcript Levels for Cytokines

Next, we examined the effect of IL-35 on transcript levels of cytokines by using real-time PCR. Cytokine modulation was detected at the messenger RNA level by real-time PCR. IFN- γ , Th1 cytokine, did not significantly change by IL-35 (Fig. 6A). IL-35 significantly reduced IL-4, IL-5, IL-13 (Fig. 6, B–D). IL-35 also significantly reduced transcript levels of IL-17, IL-23, and TNF- α (Fig. 6, E, G, J). Also, IL-35 did not significantly change transcript level of IL-12 and TGF- β (Fig. 6, F and K). Furthermore, IL-35 significantly increased transcript levels of IL-27, IL-2, and IL-10 (Fig. 6, H, I, and L).

DISCUSSION

Allergic rhinitis results from the aberrant expansion of CD4⁺ T cells that produce type 2 cytokines, such as IL-4, IL-5, and IL-13.⁶ This study showed that IL-35 inhibited IL-4, IL-5, and IL-13 production, which indicated that IL-35 may attenuate allergic rhinitis in the mouse model, which is consistent with the previous report of Huang *et al.*³, which showed that IL-35 can inhibit IgE production and IL-4, IL-5, and IL-13 in BALF.

IL-12, IL-23, IL-27, and IL-35 are members of the IL-12 family. Peng *et al.*⁷ showed that IL-23 signaling enhances Th2 polarization. Mice

with transgenic overexpression of IL-23R exhibited increased airway infiltration of eosinophils and Th2 cytokine production, whereas those mice deficient in IL-23 displayed reduced airway inflammation. Li *et al.*⁸ also reported that silencing IL-23 expression with a small hairpin RNA reduced the number of eosinophils and neutrophils in BALF and the levels of serum IgE, IL-23, IL-17, and IL-4. This study showed that IL-35 significantly inhibited IL-23 production, which indicates that IL-35 may inhibit allergic responses through the reduction of IL-23 production. IL-27 has two distinct functions in immune responses: one as an initiator of Th1-type immune responses and the other as an attenuator of immune and/or inflammatory responses.⁹ Intranasal administration of IL-27 inhibits OVA-induced airway hyperresponsiveness and inflammation in OVA-sensitized animals. Thus, IL-27 could provide a novel therapeutic method for treating Th2-associated diseases, *e.g.*, bronchial asthma.¹⁰ This study showed that IL-35 significantly increased IL-27 production, which indicates that IL-35 may attenuate allergic rhinitis through upregulation of IL-27 in the mouse model.

IL-17 plays a crucial role in driving neutrophil influx into the airway.¹¹ Makihara *et al.*¹² showed that expression of IL-17 in the nasal mucosa was associated with nasal eosinophilia and clinical severity in allergic rhinitis. Baumann *et al.*¹³ also showed that IL-17 may aggravate nasal mucosal allergy. Also, Niedbala *et al.*⁴ showed that, compared with wild-type mice, EBI-3 (one of the IL-35 subunits) deficient mice had higher levels of IL-17 in BALF and continued to display airway hyperresponsiveness after multiple antigen challenge. Although this study showed that IL-35 significantly inhibited IL-17 production, this was consistent with the previous report of Whitehead. IL-23 also has the ability to induce naive precursor cells to differentiate into Th-17 cells,¹⁴ and to induce CD4⁺ Th17 cells to produce IL-17.^{15–17} IL-35 may inhibit the Th17 response through IL-23 reduction, which results in inhibition of airway hyperresponsiveness. Also, TNF- α can mediate the recruitment of neutrophils and eosinophils into the airways in antigen-driven airway inflammation in mice, potentially *via* upregulation of epithelial and endothelial adhesion molecules.¹⁸ TNF- α treatment reduced allergic responses in an allergic rhinitis model.¹⁹ Anti-TNF- α inhibited allergic symptoms, eosinophilia in the nasal mucosa, total and specific IgE levels, and Th2 cytokine. This study showed that IL-35 may inhibit TNF- α , which indicates that IL-35 may inhibit the allergic response through reduction of TNF.

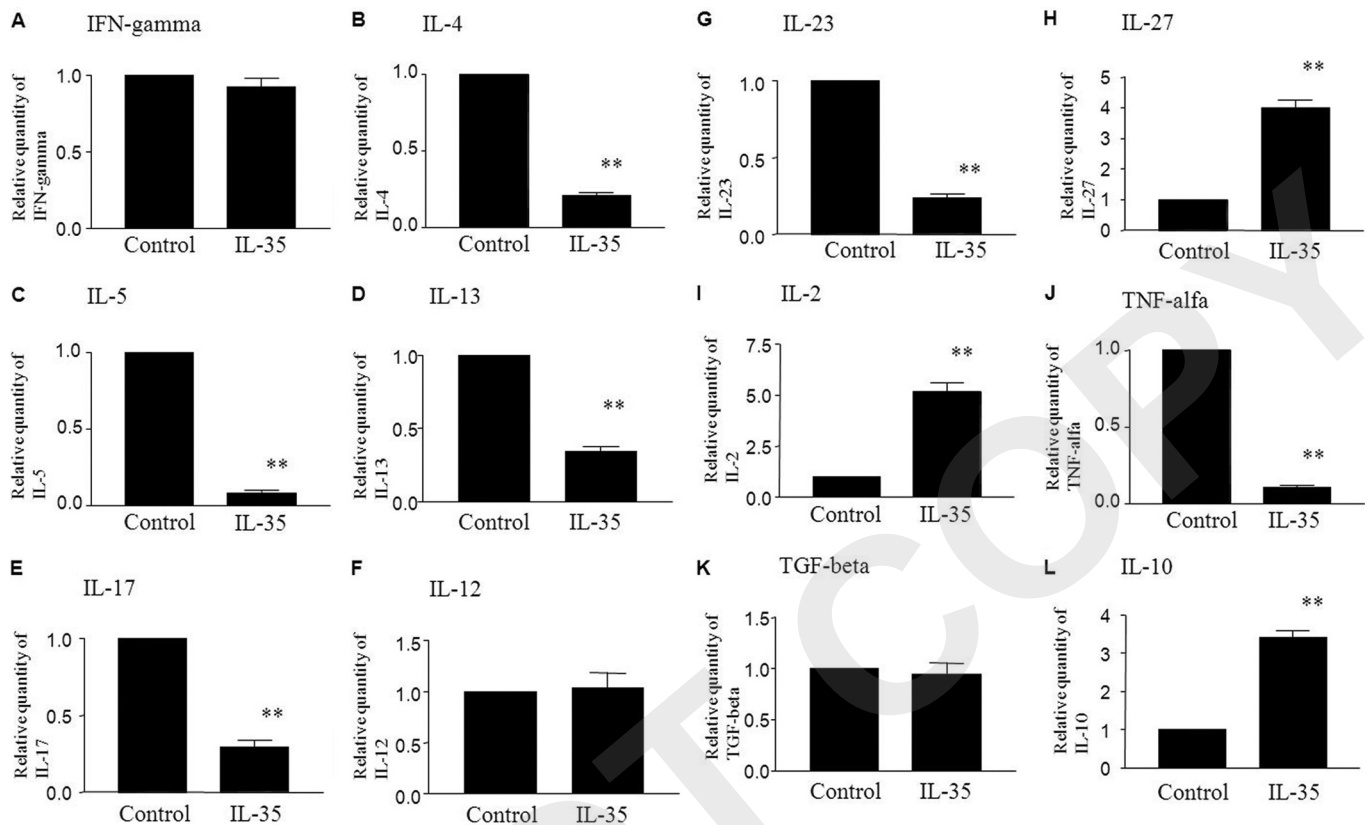


Figure 6. The change of transcript levels for cytokines by IL-35. Mice were injected with OVA and alum, and intranasally challenged with OVA. Splenic CD4⁺CD25⁻ T cells and DCs were cultured in the presence of OVA with or without IL-35. The effects of IL-35 on (A) IFN- γ , (B) IL-4, (C) IL-5, (D) IL-13, (E) IL-17, (F) IL-12, (G) IL-23, (H) IL-27, (I) IL-2, (J) TNF- α , (K) TGF- β , and (L) IL-10 were detected at the messenger RNA level by real-time PCR. ** p < 0.01, paired t -test.

IL-2 is indispensable for the peripheral maintenance of natural CD25⁺ CD4⁺ Treg cells. IL-2 plays a crucial role in the maintenance of natural immunologic self-tolerance.²⁰ In this study, IL-35 significantly increased IL-2 production, which indicates that IL-35 may induce natural immunologic self-tolerance through IL-2 production in allergy. IL-10 also has a direct effect on CD4⁺ Foxp3⁺ Treg *in vivo* by promoting their survival.²¹ Also, intranasal administration of IL-10 efficiently inhibited antigen-induced airway hyperresponsiveness and significantly reduced the number of eosinophils and neutrophils in BALF.²² Therefore, we measured the production of IL-10 with and without IL-35 stimulation. IL-35 was found to significantly increase IL-10 production. Baumann *et al.*¹³ showed that IL-10 may alleviate nasal mucosal allergy. When considering this, IL-35 may inhibit allergic responses through IL-10 production. Although IL-35 induces the transformation of CD4⁺ effector T cells into Treg cells,² it also may promote Treg cell survival through increased IL-10 production.

In this study, IL-35 significantly reduced IL-4, IL-5, IL-13, IL-17, IL-23, and TNF- α production, and significantly increased IL-2, IL-10, and IL-27 production. IL-35 may have the ability to inhibit allergic responses through these cytokine modulations. However, it is not uncertain which cytokine is the most-effective target, although Th2 cytokines (IL-4, IL-5, IL-10, IL-13) and IL-10 pathway may be more effective targets. These findings should provide some insights into the mechanism of the effect of IL-35 and Treg cells, and contribute to the development of a new therapeutic approach to treat allergic rhinitis. Further study is expected to develop IL-35 therapy.

CONCLUSION

This study showed that IL-35 inhibited allergic T-cell response and that IL-35 significantly reduced IL-4, IL-5, IL-13, IL-17, IL-23, and

TNF- α production, and significantly increased IL-2, IL-10, and IL-27 production. This study also indicated the possibility of a novel therapy with IL-35 for the control of allergic rhinitis through these cytokine modulations.

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