Dynamic change of anti-inflammatory cytokine IL-35 in allergen immune therapy for Japanese cedar pollinosis

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Abstract

Background: The immunologic tolerant state following allergen immunotherapy is associated with the induction of distinct phenotypes of regulatory T-cells or B-cells. IL-35 was recently identified as an anti-inflammatory cytokine. However, knowledge about the roles of IL-35 in patients with allergic diseases is limited.

Objective: The purpose of this study is to elucidate the role of IL-35 in sublingual immunotherapy (SLIT)-induced immune tolerance in Japanese cedar pollinosis patients (JCP).

Methods: The inhibitory effects of recombinant IL-35 (rIL-35) on antigen-induced cytokine production were examined using peripheral blood mononuclear cells (PBMC) from JCP patients. IL-35-expressing cells in antigen-stimulated PBMC and the effects of SLIT on the ratio of IL-35 expressing cells and serum IL-35 concentrations were examined.

Results: rIL-35 suppressed IL-5, IFN-γ, and IL-17, but not IL-10 production in PBMC after exposure to Japanese cedar extract. rIL-35 directly suppressed IL-5 and IL-13 production in memory $CD4^+$ T-cells or co-culture of DCs/$CD4^+$ T cells. The ratio of IL-35-expressing T cells and B cells was increased after SLIT. Serum concentration of IL-

35 was elevated during the pollen season in patients treated with SLIT, and it was negatively correlated with symptom medication scores (SMS) during the peak JCP season.

Conclusions: This study demonstrated that SLIT induced the allergen-specific production of IL-35 from Tregs or Bregs during the pollen season; furthermore, SLIT-induced IL-35 production may be critical for the induction of immune tolerance.

Key word

Pollen, immunotherapy and tolerance induction, T cells, B cells, flow cytometry

To the editor:

Japanese cedar pollinosis (JCP) is more than one-third of Japanese population experience severe symptoms during the allergy season (1). The efficacy of sublingual immunotherapy (SLIT) for JCP has been previously appraised (2). The immunologic tolerant state following allergen immunotherapy is associated with the induction of distinct phenotypes of regulatory T-cells or B-cells (3). IL-35 was recently identified as an anti-inflammatory cytokine (4). However, knowledge about the roles of IL-35 in patients with allergic diseases is limited. The purpose of this study is to elucidate the dynamic change of SLIT-induced IL-35 in JCP patients.

The clinical and demographic characteristics of the patients included in this study are shown in Table S1. We examined the bioactivity of human recombinant IL-35 (rIL-35) on antigen-induced cytokines in PBMC. PBMC of patients with JCP were incubated with Japanese cedar pollen (JC) extract in the presence or absence of rIL-35 or isotype control IgG for 3 days. rIL-35 (0.1-10 μg/mL) significantly inhibited JC extract-induced production of IL-17 and IFN-γ dose-dependently. A high concentration of rIL-35 (10 μg/mL) significantly inhibited JC extract-induced IL-5 production, whereas it significantly stimulated IL-10 production (Figure S1A-D).

A previous study showed that IL-35 has a different effect on INF-γ production in a collagen-induced arthritis mice model (5). IL-35 markedly expanded the IFN-γ

production of $CD4^{+}CD25^{-}$ T cells from the spleen and lymph node with anti-CD3/CD28. In contrast, IL-35 markedly inhibited the proliferation and IFN-γ production by $CD4^{+}CD25^{–}$ T cells activated with anti-CD3 antibody and APC. Therefore, we examined the effect of IL-35 on Th2 cytokine production under various conditions. First, we examined the effect of rIL-35 on memory CD4+ T cells. Isolated memory $CD4^{+}$ T cells were incubated with anti-CD3/CD28 in the presence or absence of rIL-35 for 6 h (for mRNA) or 3 days (for protein). GATA3 mRNA expression was inhibited at high concentrations of rIL-35 (more than 1 μg/mL) (Figure S2A). IL-5 and IL-13 production were also significantly inhibited by rIL-35 (10 μg/mL) (Figure S2B-C). In contrast, IFN-γ production was increased (Figure S2D). Next, we examined the effect of rIL-35 on co-cultured DCs/$CD4^{+}$ T cells. Dendritic cells and $CD4^{+}$ T cells at a ratio of 1:10 were co-cultured with JC extract in the presence or absence of rIL-35 for 3 days. rIL-35 (10 μg/mL) inhibited IL-5 and IL-13 production under the co-culture condition (Figure S2E-F). IFN-γ production was also decreased (Figure S2G).

Previous studies suggested that IL-35 is expressed in Treg (6) or Breg (7) in murine models. Therefore, we analyzed IL-35-expressing $CD4^{+}$ T cells and $CD19^{+}$ B cells in PBMC collected from JCP patients after completing 12 months of SLIT using flow cytometry. In addition, we analyzed IL-35 expression in T cells or B cells, focusing

on the relationship between IL-35 and IL-10. PBMC from patients after SLIT were incubated with JC extract. Representative scattergrams show the expression of CD4, Foxp3, IL-10, EBI3, and p35 (Figure S3A). $CD4^{+}Foxp3^{+}$ cells and $CD4^{+}Foxp3^{-}IL\text{-}10^{+}$ cells (Tr1 cells) were double positive for both p35 and EBI3 subunits, representing IL-35. Notably, a portion of $CD4^{+}Foxp3^{-}IL\text{-}10^{-}$ cells were also double positive for p35 and EBI3. The isotype control did not show staining for IL-35 positive cells (Figure S3A). IL-35-expressing $CD19^{+}$B cells were examined in PBMC incubated with JC extract. Representative scattergrams showed the expression of CD19, IL-10, EBI3, and p35 (Figure S3B). IL-35 was expressed in $CD19^{+}IL\text{-}10^{+}$ cells (Bregs), shown as double positive staining for p35 and EBI3. A portion of $CD19^{+}IL\text{-}10^{-}$ cells also expressed IL-35 (Figure S3B).

We next examined differences in IL-35-overexpressing cells before and after SLIT for JCP. Blood samples were collected from JCP patients before the start of SLIT and after completion of 12 months of SLIT (Duration: 15± 2.1 months). All JCP patients exhibited clinical symptom improvement after SLIT (n=10). PBMC in JCP patients before and after SLIT were incubated with JC extract for 2 days and the IL-35-expressing T and B cells were examined by flow cytometry. The ratio of IL-35 expressing T cells ($CD4^{+}IL\text{-}35^{+}/CD4^{+}$ cells) and IL-35 expressing B cells ($CD19^{+}$IL-

35^+/$CD19^+$ cells) significantly increased after SLIT (Figure 1A and B). Next, we examined the phenotype of IL-35 expressing T or B cells after SLIT. The ratio of $Foxp3^+$IL-35^+ ($CD4^+$$Foxp3^+$IL-$35^+$ /$CD4^+$) T cells and IL-10^+IL-35^+ ($CD4^+$IL-10^+IL-35^+ /$CD4^+$) T cells were increased after SLIT (Figure S4A and B), although the ratio of $Foxp3^+$ ($CD4^+$$Foxp3^+$ /$CD4^+$) T cells did not change after SLIT (data not shown). The ratio of IL-10^+IL-35^+ ($CD19^+$IL-10^+IL-35^+ /$CD19^+$) B cells was also increased after SLIT (Figure S4C). Moreover, we measured IL-35 protein levels in PBMC before and after SLIT. JC extract-induced IL-35 production in PBMC from JCP patients was significantly increased after SLIT (Figure 1C).

Serum IL-35 was then examined in patients with HC, JCP, and SLIT for JCP during and outside of the pollen season. Interestingly, serum IL-35 in patients with SLIT for JCP was lower compared to HC and JCP outside the pollen season. In addition, serum IL-35 in patients with SLIT for JCP was elevated during the peak Japanese cedar pollen season compare to patients with JCP (Figure 2A). Finally, we examined the relationship between serum IL-35 and clinical symptoms after SLIT during the peak Japanese cedar pollen season. There was an inverse correlation between serum IL-35 and symptom medication score after SLIT during the peak Japanese cedar pollen season (Figure 2B).

A recent study supported that IL-35 inhibited IL-5 and IL-13 production by ILC2_ in the presence of IL-25 or IL-33, and IL-35 inhibited CD40 ligand-, IL-4- and IL-21-mediated IgE production by B cells. IL-35 therapy has also been studied as a new therapeutic agent for the treatment of allergic diseases (9). Our study showed that rIL-35 has direct inhibitory effects on Th2 and Th17 cytokine production in memory T cells. However, the direct inhibitory effect of IL-35 on Th2 is limited because high concentrations of rIL-35 protein are required to suppress Th2 cytokines. Previous studies support our findings that IL-35 blocks Th2 development by suppressing GATA3 and IL-4 expression and limiting Th2 proliferation (9). IL-35 can also mediate conversion of Th2 cells to Treg, although this can be blocked by IFN-γ (9). Therefore, it is likely that IL-35 dose not exert its anti-Th2 type inflammation effect via a single pathway, rather, it is expected to simultaneously affect a number of different inflammatory pathways.

This study demonstrated that SLIT induced allergen-specific production of IL-35 from allergen-specific Tregs or Bregs during the pollen season, and that SLIT-induced IL-35 production may be critical for the induction of immune tolerance.

Refference

1, Okubo K, Kurono Y, Ichimura K, Enomoto T, Okamoto Y, Kawauchi H, Suzaki H, Fujieda S, Masuyama K. Japanese guidelines for allergic rhinitis 2017. Allergol Int 2017;66:205-219.

2, Masuyama K, Matsuoka T, Kamijo A. Current status of sublingual immunotherapy for allergic rhinitis in Japan. Current status of sublingual immunotherapy for allergic rhinitis in Japan. Allergol Int 2018;67:320-325.

3, Shamji MH, Durham SR. Mechanisms of allergen immunotherapy for inhaled allergens and predictive biomarkers. J Allergy Clin Immunol 2017;140:1485-1498.

4, Egwuagu CE, Yu CR, Sun L, Wang R. Interleukin 35: Critical regulator of immunity and lymphocyte-mediated diseases. Cytokine Growth Factor Rev 2015;26:587-593.

5, Niedbala W, Wei XQ, Cai B, Hueber AJ, Leung BP, McInnes IB, Liew FY. IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. Eur J Immunol 2007;37:3021-3029.

6, Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, Cross R, Sehy D, Blumberg RS, Vignali DA. The inhibitory cytokine IL-35 contributes to regulatory T-cell

function. Nature 2007;450:566-569.

7, Wang RX, Yu CR, Dambuza IM, Mahdi RM, Dolinska MB, Sergeev YV, Wingfield PT, Kim SH, Egwuagu CE. Interleukin-35 induces regulatory B cells that suppress autoimmune disease. Nat Med 2014;20:633-641

8, Shamji MH, Layhadi JA, Achkova D, Kouser L, Perera-Webb A, Couto-Francisco NC, Parkin RV, Matsuoka T, Scadding G, Ashton-Rickardt PG, Durham SR. Role of IL-35 in sublingual allergen immunotherapy. J Allergy Clin Immunol 2018 Jul 25.

9, Suzuki M, Yokota M, Nakamura Y, Ozaki S, Murakami S. Intranasal administration of IL-35 inhibits allergic responses and symptoms in mice with allergic rhinitis. Allergol Int 2017;66:351-356.

Figure Legends

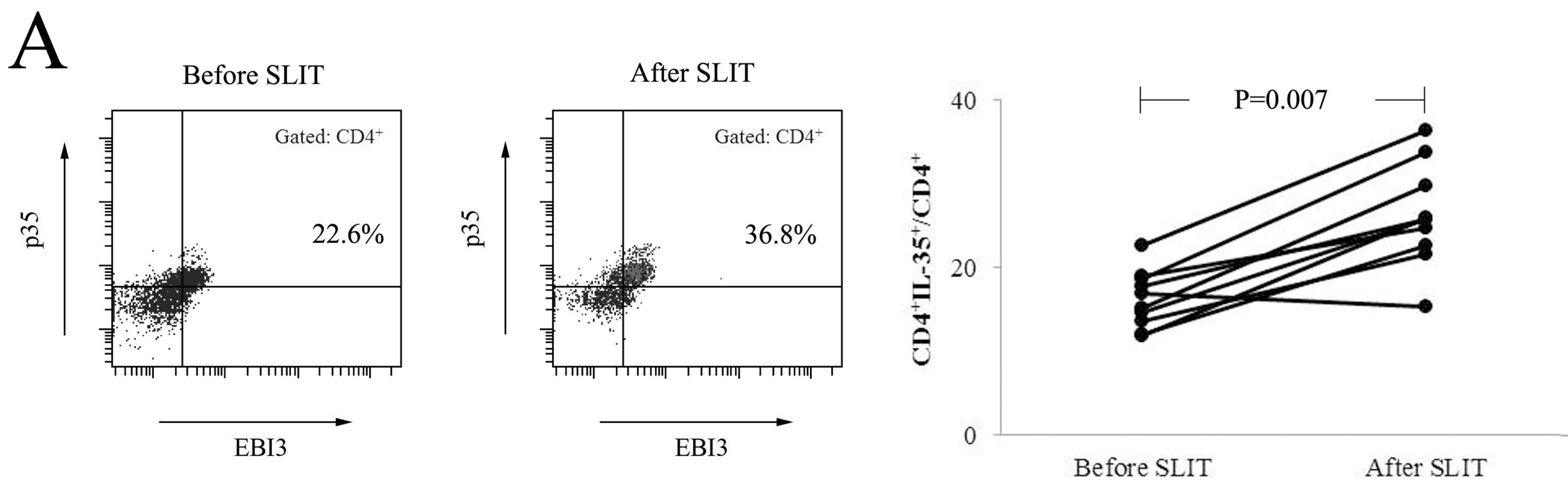
Figure 1. Effects of SLIT on IL-35 expression in T cells or B cells

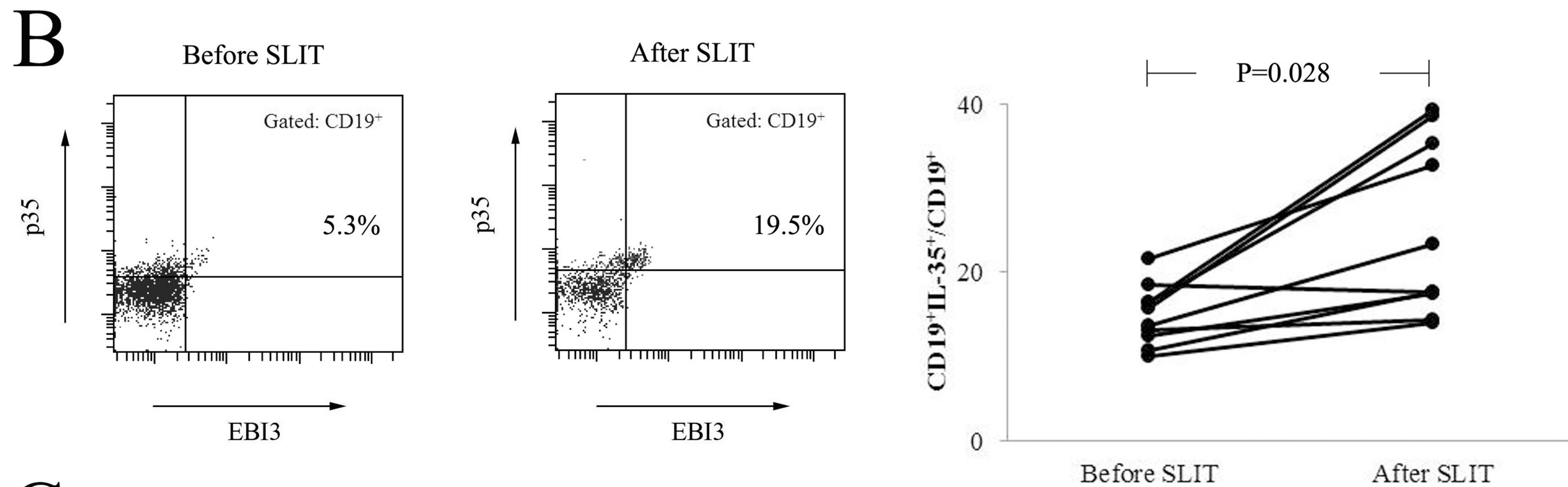
(A) PBMC before and after SLIT were incubated with Japanese cedar pollen extract for 2 days. PBMC from patients with JCP before and after SLIT were stained for CD4, EBI3, and p35 and gated for $CD4^+$ cells. *(B)* PBMC from patients with JCP before and after SLIT were stained for CD19, EBI3, and p35 and gated for $CD19^+$ cells. *(C)*

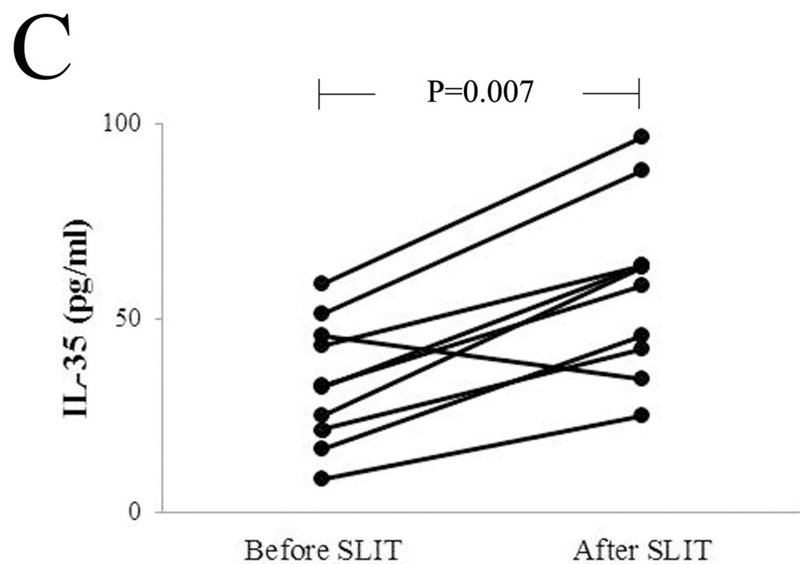
IL-35 concentrations in cell-free supernatants were measured by ELISA. *P* values were determined by the Wilcoxon signed-rank test. n=10.

Figure.2 Effect of SLIT on serum IL-35 concentrations

(A) Serum IL-35 levels in patients with HC, JCP, and SLIT for JCP during and outside of the pollen season were measured by ELISA. *(B)* The correlation between serum IL-35 and symptom medication score after SLIT during the pollen season. The correlation was assessed using Spearman’s rank correlation test. N.S; no pollen season, P.S; pollen season, SMS; symptom medication score.


A
Before SLIT
After SLIT
Gated: CD4+
22.6%
36.8%
p35
EBI3
P=0.007
CD4+IL-35+/CD4+
40
20
0
Before SLIT
After SLIT


B
Before SLIT
After SLIT
Gated: CD19+
5.3%
19.5%
p35
EBI3
P=0.028
CD19+IL-35+/CD19+
40
20
0
Before SLIT
After SLIT


C
P=0.007
IL-35 (pg/ml)
100
50
0
Before SLIT
After SLIT

A

*

*

*

*

90

60

30

0

IL-35 (pg/ml)

HC

JCP (N.S)

JCP (P.S)

SLIT (N.S)

SLIT (P.S)