

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

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 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 was to elucidate the role of IL-35 in SLIT-induced immune tolerance.

The clinical and demographic characteristics of the patients 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) inhibited production of IL-17 and IFN-γ dose dependently. A high concentration of rIL-35 (10 µg/mL) inhibited IL-5 production, whereas it stimulated IL-10 production (Figure S1 A–D).

A previous study showed that IL-35 has a different effect on INF-γ production in a collagen-induced arthritis mouse model.⁵ IL-35 expanded IFN-γ production of CD4⁺CD25[−] T cells from the spleen and lymph node with anti-CD3/CD28. In contrast, IL-35 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 hours (for mRNA) or 3 days (for protein). GATA3 mRNA expression was inhibited at high concentrations of rIL-35 (more than 1 µg/mL) (Figure S2 A). IL-5 and IL-13 production were also inhibited by rIL-35 (10 µg/mL) (Figure S2 B–C). In contrast, IFN-γ production was increased (Figure S2 D). 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 (Figure S2 E–F). IFN-γ production was also decreased (Figure S2 G).

Previous studies suggested that IL-35 is expressed in Treg⁶ or Breg⁷ 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 S3 A). CD4⁺Foxp3⁺ cells and CD4⁺Foxp3⁺IL-10⁺ cells (Tr1 cells) were double positive for both p35 and EBI3 subunits, representing IL-35. Notably, a portion of CD4⁺Foxp3⁺IL-10[−] cells were also double positive for p35 and EBI3. The isotype control did not show staining for IL-35-positive cells (Figure S3 A). Representative scattergrams showed the expression of CD19, IL-10, EBI3, and p35 (Figure S3 B). IL-35 was expressed in CD19⁺IL-10⁺ cells (Bregs), shown as double positive staining for p35 and EBI3. A portion of CD19⁺IL-10[−] cells also expressed IL-35 (Figure S3 B).

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 the completion of 12 months of SLIT (duration: 15 ± 2.1 months). All JCP patients exhibited clinical symptom improvement after SLIT (n = 10). PBMC 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-35⁺/CD4⁺ cells) and IL-35 expressing B cells (CD19⁺IL-35⁺/CD19⁺ cells) increased after SLIT (Figure 1A,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 was 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 was 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 compared to patients with JCP (Figure 2A). Finally, we examined the relationship between serum IL-35 and clinical symptoms after SLIT during the peak pollen season. There was an inverse correlation between serum IL-35 and symptom medication score after SLIT during the peak pollen season (Figure 2B).

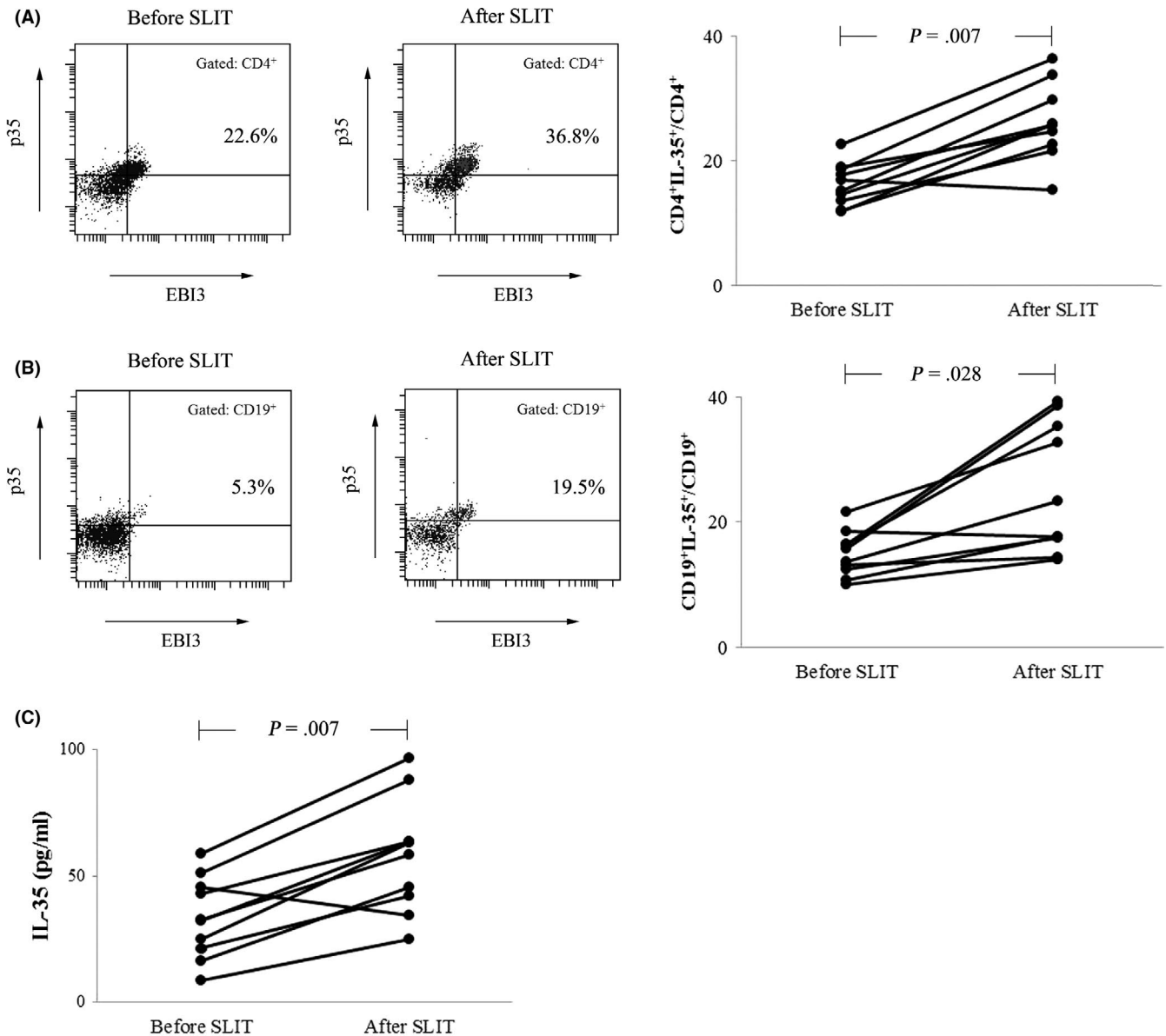


FIGURE 1 Effects of SLIT on IL-35 expression in T cells or B cells. A, PBMC before and after SLIT were incubated with Japanese cedar pollen extract for 2 d. PBMC from patients with JCP before and after SLIT were stained for CD4, EBI3, and p35 and gated for CD4⁺ cells. B, PBMC from patients with JCP before 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

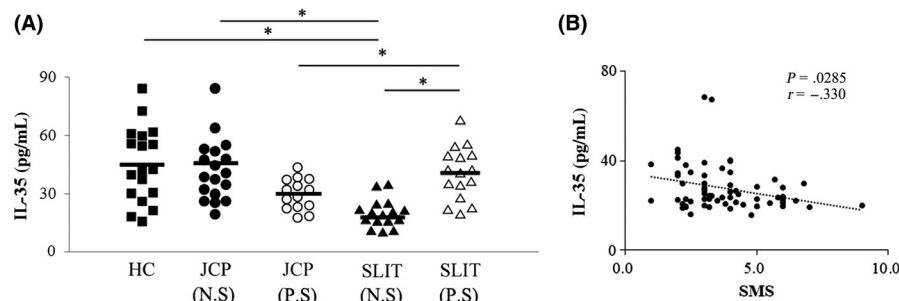


FIGURE 2 Effect of SLIT on serum IL-35 concentrations. A, Serum IL-35 levels in patients with HC, JCP, and SLIT for JCP during and outside of the pollen season were measured by ELISA. B, The correlation between serum IL-35 and symptom medication score after SLIT during the pollen season. The correlation was assessed using Spearman's rank correlation test. N.S, no pollen season; P.S, pollen season; SMS, symptom medication score

A recent study supported that IL-35 suppressed antigen-induced production of IL-5 and IL-13 by CD4⁺CD25⁻ effector T cells from patients with grass pollen AR. IL-35 also inhibited IL-5 and IL-13 production by ILC2 in the presence of IL-25 or IL-33, and CD40 ligand-, IL-4- and IL-21-mediated IgE production by B cells.⁸ In this study, the direct inhibitory effect of IL-35 on Th2 cytokines was limited in PBMC because high concentrations of rIL-35 protein were required to suppress Th2 cytokines. Previous study showed that IL-35 blocks Th2 development by suppressing GATA3 and IL-4 expression and limiting Th2 proliferation and can also mediate conversion of Th2 cells to Treg, although this can be blocked by IFN- γ .⁹ Therefore, IL-35 does not exert its anti-Th2 type inflammation effect via a single pathway, rather it may simultaneously affect a number of different inflammatory pathways.

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

CONFLICTS OF INTEREST

All authors declare that they have no conflicts of interest.

KEYWORDS

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

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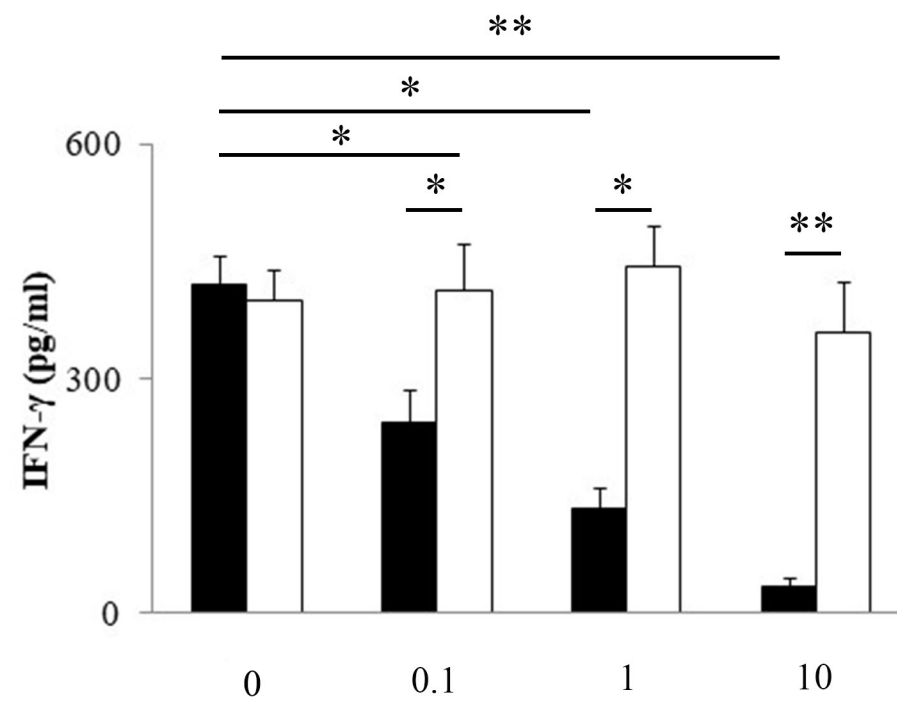
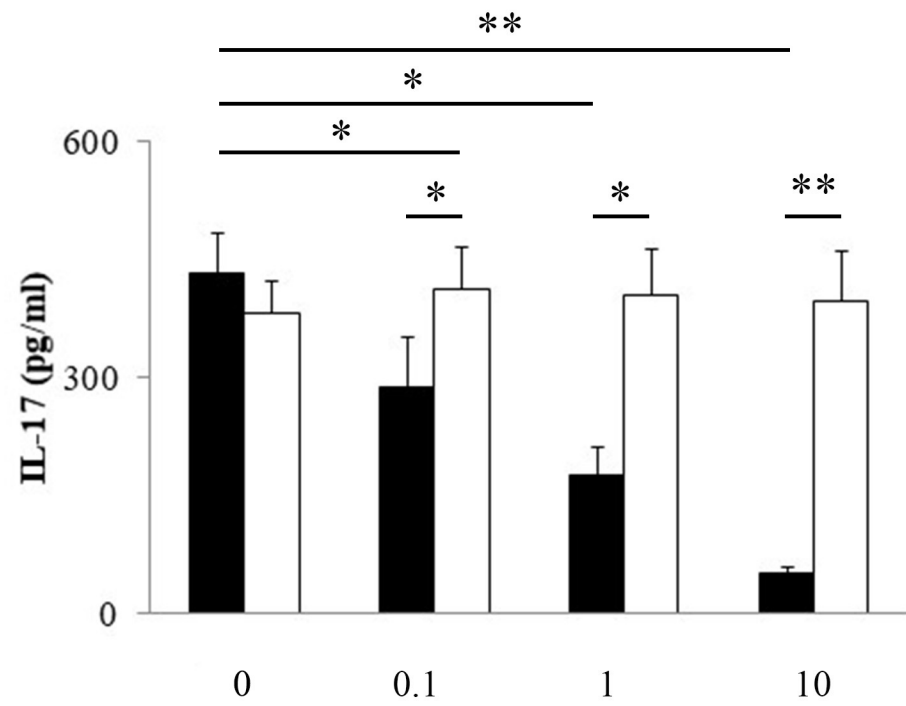
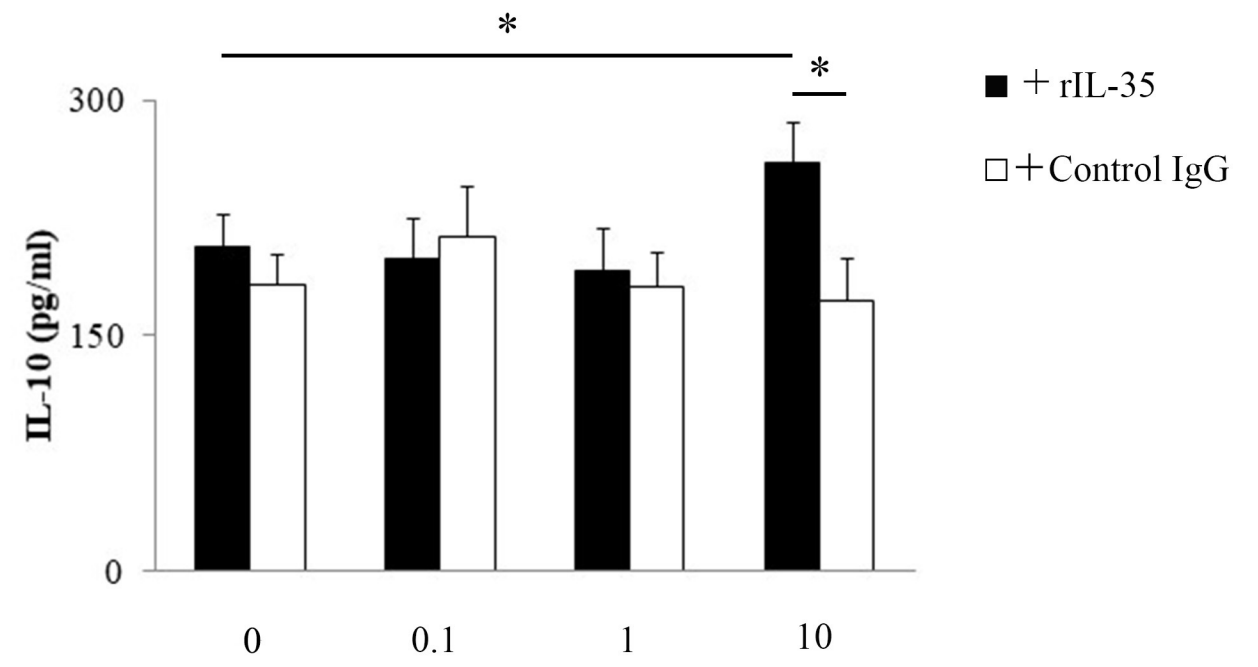
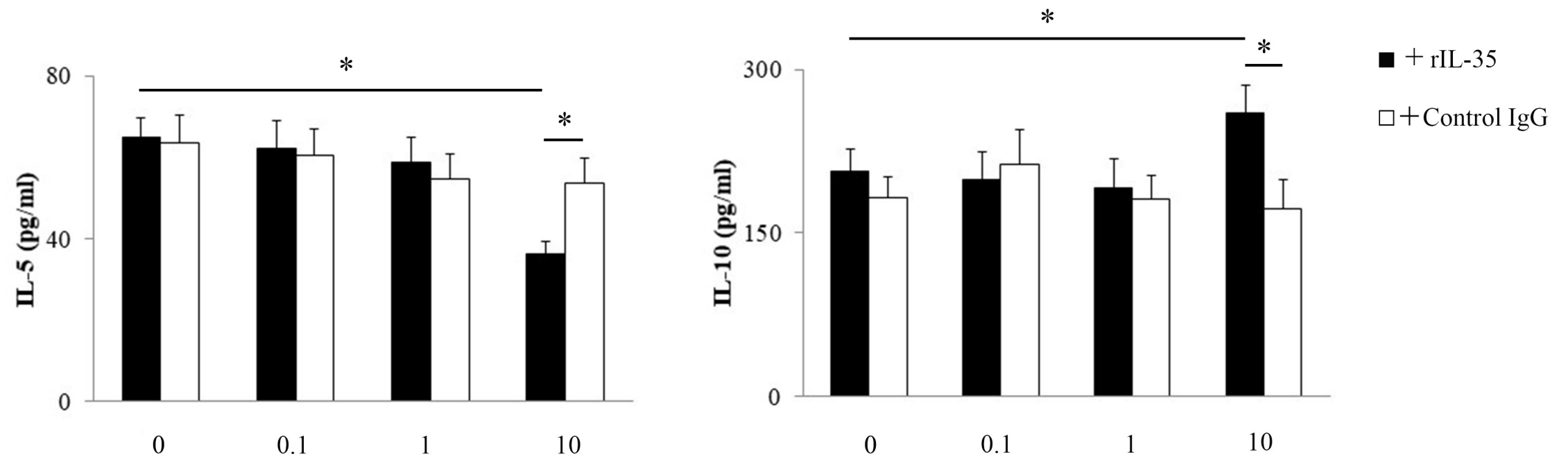
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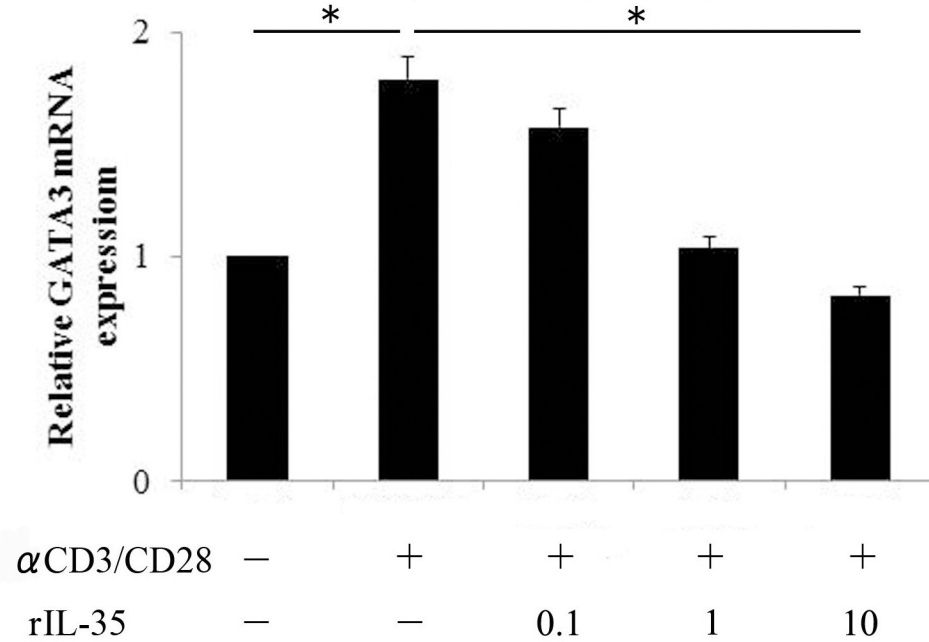
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SUPPORTING INFORMATION

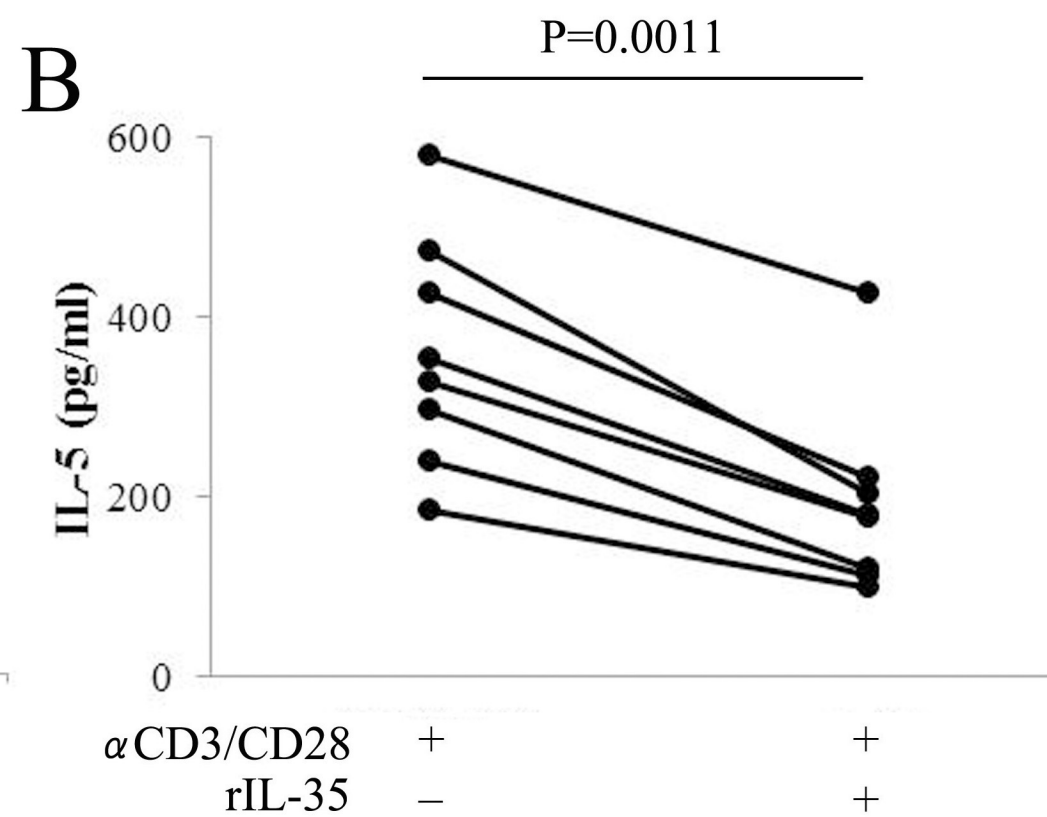
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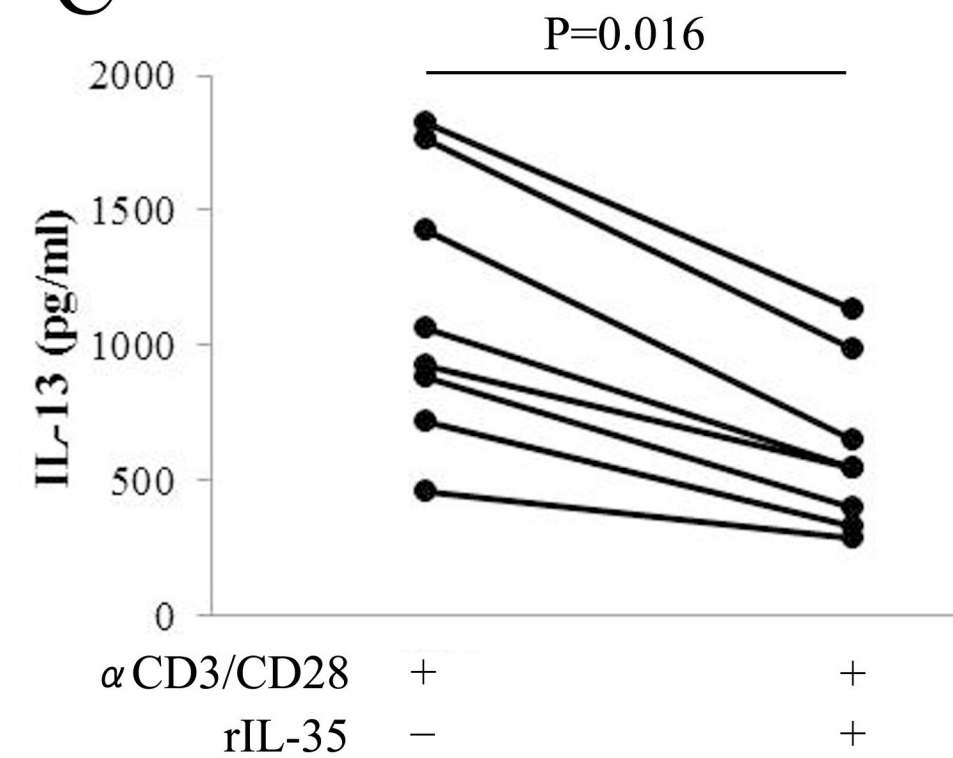
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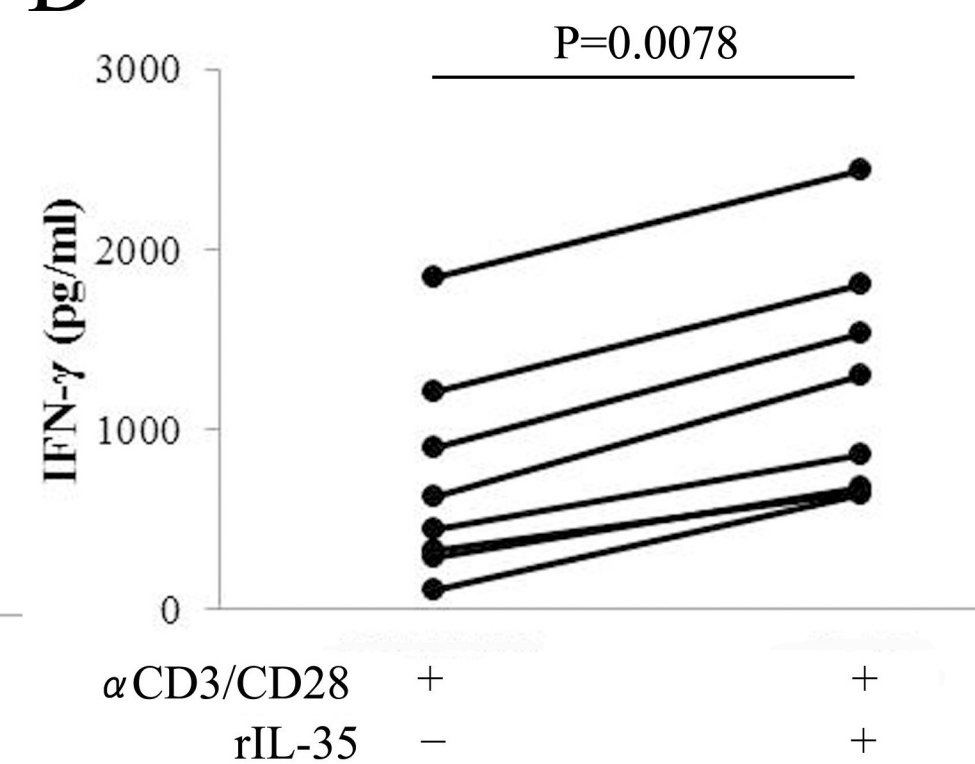
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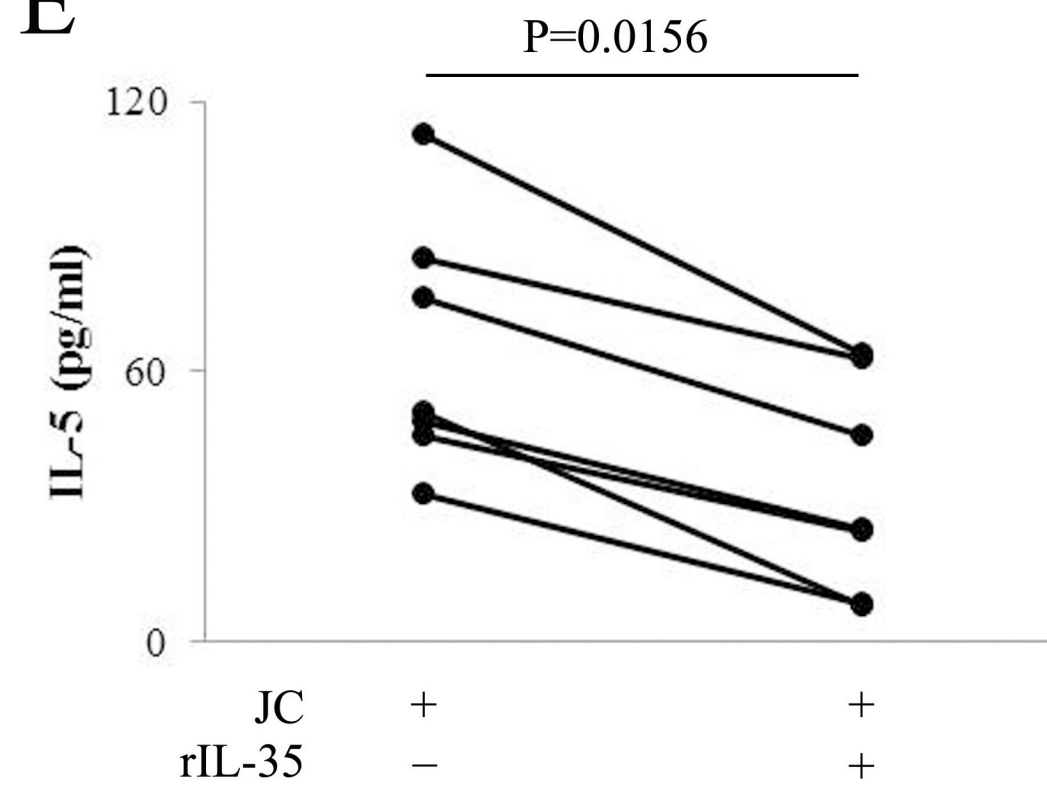
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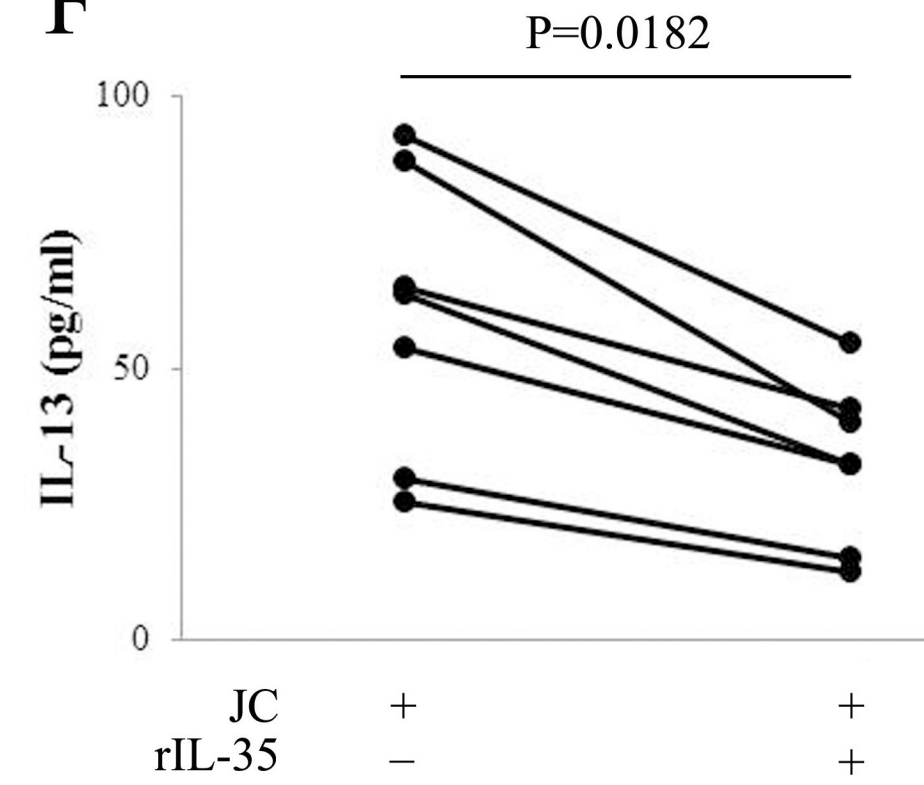
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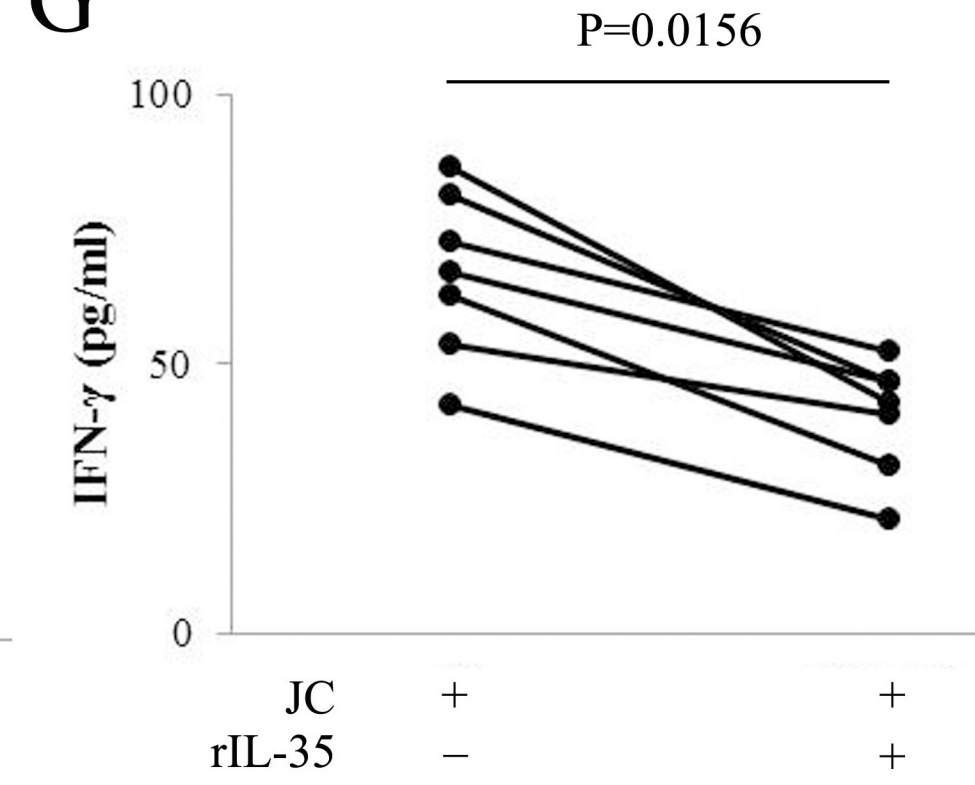
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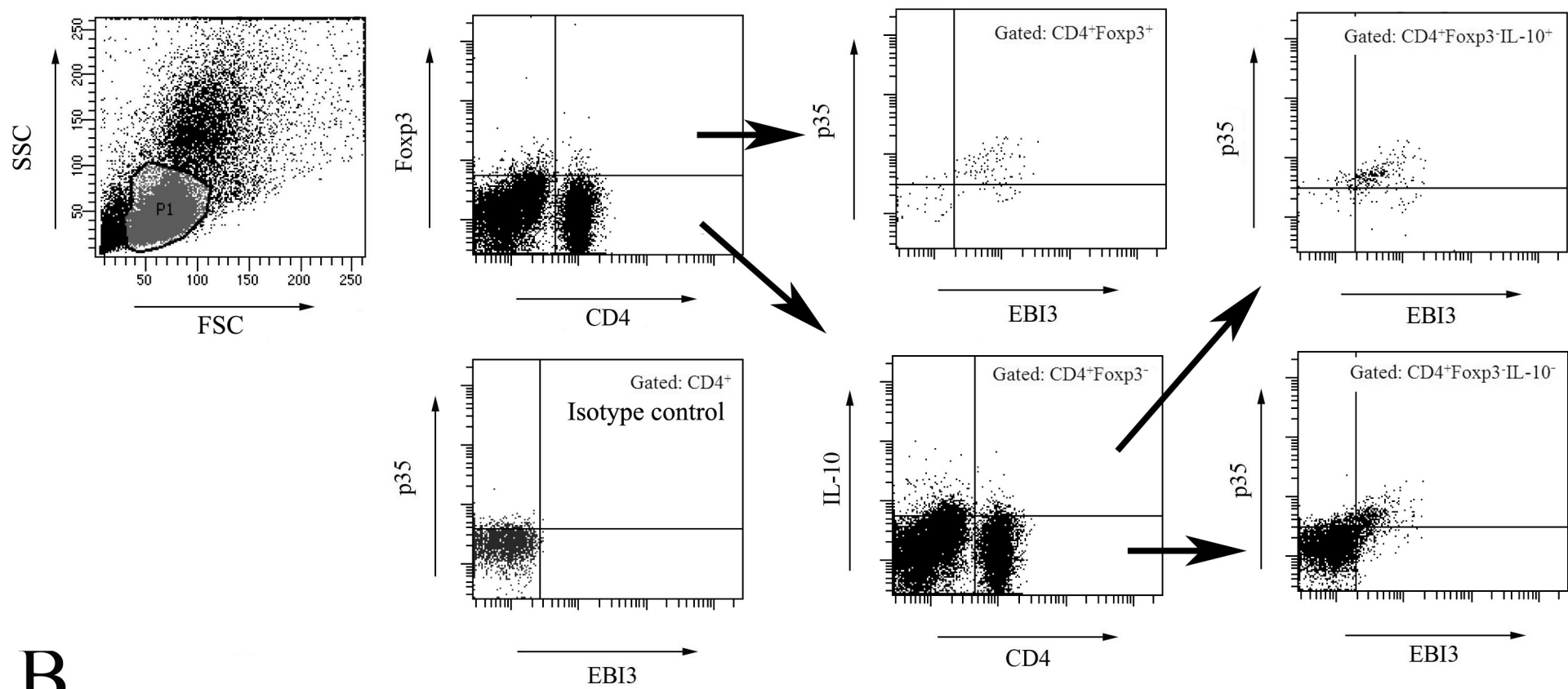
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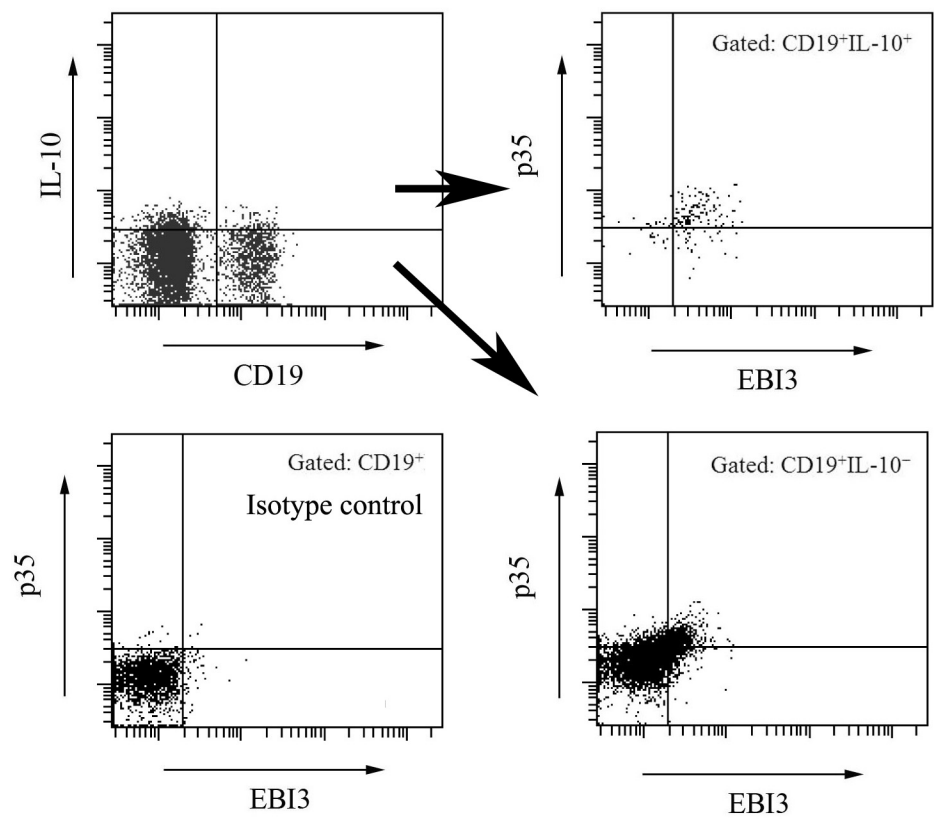
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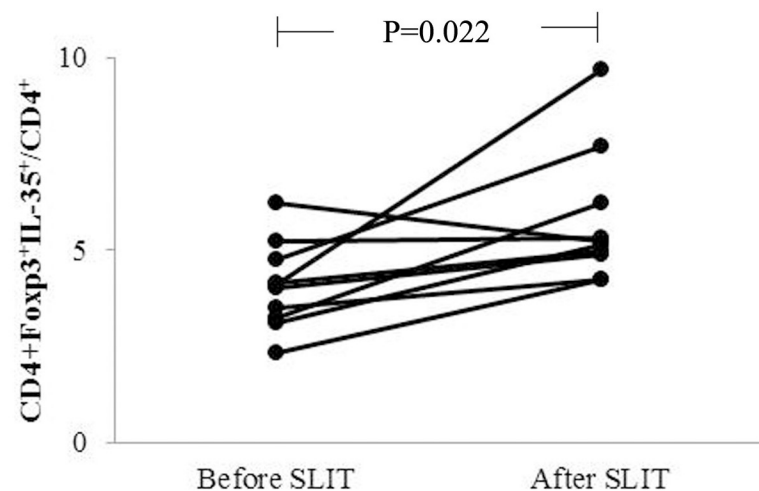
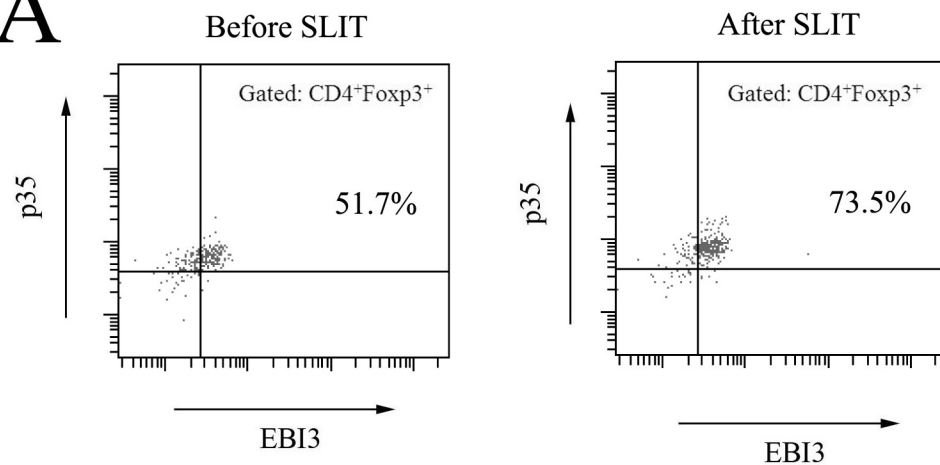
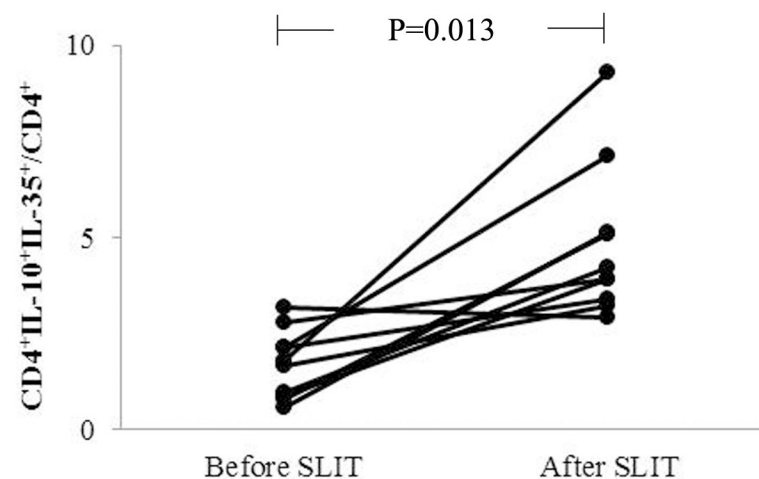
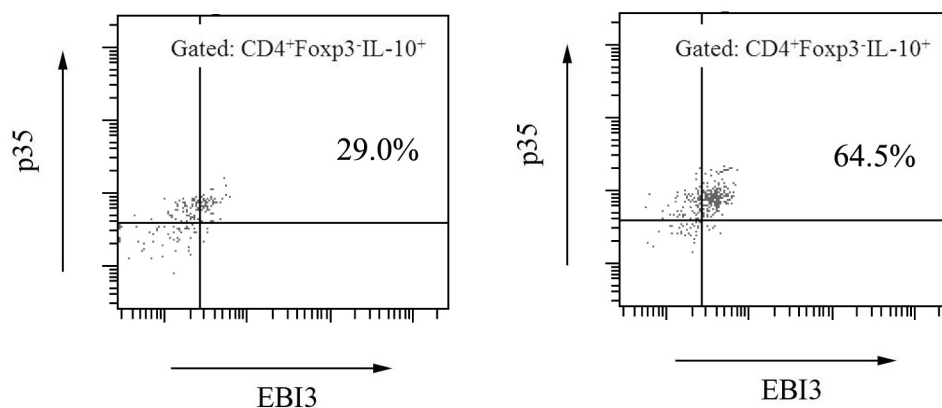
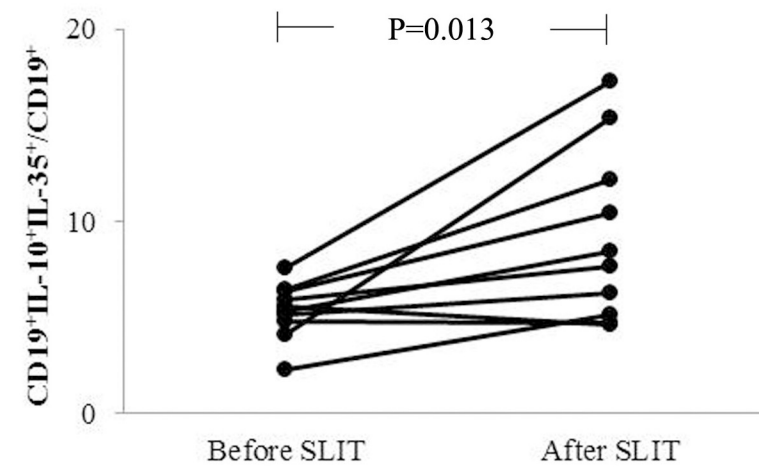
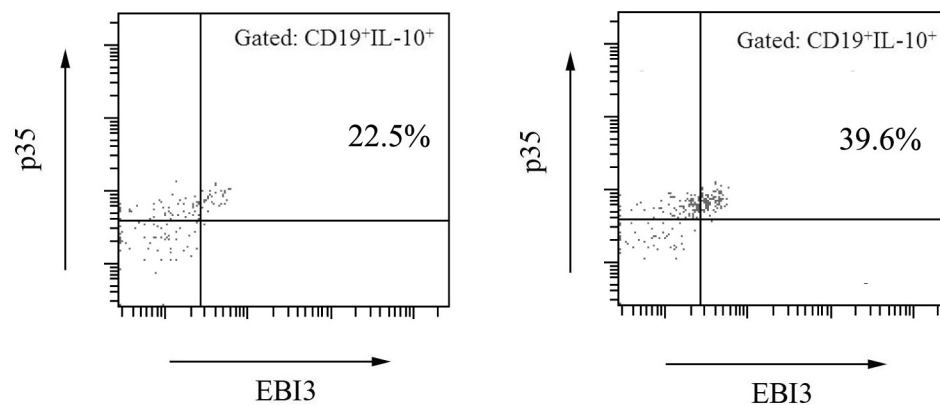


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Dynamic change of anti-inflammatory cytokine IL-35 in allergen immune therapy for
Japanese cedar pollinosis

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On line Data Supplement

Methods

Study participants

This prospective study comprised 21 non-allergic healthy volunteers (age, 36.4 ± 5.7 years; male/female, 10/11), 22 patients (age, 32.3 ± 7.1 years; male/female, 9/13) with allergy to Japanese cedar pollen and 45 patients (age, 35.7 ± 7.2 years; male/female, 17/28) sensitive to Japanese cedar referred to the Department of Otorhinolaryngology of Shiga University Hospital and Yuta Clinic. AR is the presence of typical nasal symptoms during the cedar pollen season with a serum Immuno-CAP score for Japanese cedar (JC) of ≥ 0.70 UA/mL (i.e., IgE immunoCAP class ≥ 2) and positive skin prick test for JC. The exclusion criteria were the presence or history of asthma or asthma-like symptoms. No patient included in this study had been treated with systemic corticosteroids for at least 4 weeks before sample collection. Blood samples were collected before (October to November), during (at the fifth week, February to March), and after SLIT (October to November). The control study participants had no nasal symptoms, no history of atopic disorders and a negative allergen-specific IgE against JC. All patients gave written informed consent before sample collection. The study was approved by the institutional review board of Shiga University of Medical Science (Otsu, Japan).

Evaluation of symptoms

Symptom medication scores (SMS) were evaluated during the JC pollen season between February and March in 2015. According to the Japanese guidelines for AR, symptom scores were evaluated for sneezing, rhinorrhea and nasal congestion each using a 5-point scale (0 = none, 1 = mild, 2 = moderate, 3 = severe and 4 = very severe) (16). Ocular symptoms (itchiness and watery eyes) were scored using a 4-point scale (0 = none, 1 = mild, 2 = moderate and 3 = severe). Patients were allowed to use a medical treatment such as antihistamines. The use of any medication was scored as 0 (not used) or 3 (used).

Reagents and antibodies

We purchased the following materials: Recombinant Human IL-35 (Shenandoah Biotechnology, PA, USA), Dynabeads Human T-Activator CD3/CD28 (Life Technologies, CA, USA), Human transcription factor forkhead box P3 (Foxp3) Buffer Set, Human BD Fc block (BD Biosciences, NJ, USA), EasySep Human CD4⁺ T cell enrichment kit, EasySep Human Myeloid DC enrichment kit, EasySep human memory CD4⁺ T cell enrichment kit (Stem Cell Technologies, Vancouver, Canada), HEK-293 cell IgG isotype control (antibody-online.com, Aachen, Germany). For flow cytometry, we purchased the following anti-human mAbs: Pacific blue anti-human CD4, Pacific blue anti-human CD14, PE/Cy7 anti-human IL-10, Pacific blue anti-human CD19, APC

anti-human IL-10, PE/Cy7 anti-human CD25, PE anti-human IL-27/IL-35 EBI3 (BioLegend, CA, USA), APC anti-human Foxp3 (eBioscience, CA, USA) Human/Mouse IL-12/IL-35 p35 Fluorescein-conjugated antibody (R&D Systems, Minneapolis, MN, USA). Isotype-matched control IgG was purchased from BioLegend.

PBMC culture

PBMC from JCP patients were isolated from 20 mL of heparinized venous blood by density gradient centrifugation using Ficoll-Paque PREMIUM (GE Health Care, Uppsala, Sweden). PBMC were cultured in RPMI 1640 medium (Wako Pure Chemical Industries, Osaka, Japan) containing L-glutamine supplemented with 100 U/mL penicillin, 100 U/mL streptomycin (Invitrogen, Carlsbad, CA, USA), and 10% autologous serum, incubated in 96-well round bottom plates (Falcon; Corning, Inc., NY, USA) at 37°C and 5% CO₂ for 2 or 3 days and then incubated with Japanese Cedar pollen extract (10 µg/mL) (Biostir Inc., Osaka, Japan). In some experiments, memory CD4⁺ T cells were isolated with an EasySep memory CD4⁺ Cell Isolation Kit, seeded at 1×10⁶ cells/mL in round-bottom 96-well tissue culture plates and then cultured in the presence of anti-CD3/anti-CD28 Dynabeads with or without recombinant human IL-35 (0.1-10 µg/mL) or isotype control IgG for 6 h (for mRNA) and 3 days (for protein). In some experiments, CD4⁺ T cells and dendritic cells (DC) were isolated with an EasySep

CD4⁺ or DC Isolation Kit and then cultured at a 1:10 DC:T-cell ratio with Japanese cedar pollen extract (10 µg/mL) with or without recombinant human IL-35 (0.1-10 µg/mL) or isotype control IgG for 3 days. Cell-free supernatants were collected and analyzed for IL-5, IL-13, IFN-γ (R&D Systems) or IL-35 (Cloud-Clone Corp, TX, USA) by ELISA, according to the manufacturer's recommendations.

Flow cytometry

PBMC were stimulated for 2 days with Japanese cedar pollen extract (10 µg/mL) in RPMI 1640 medium and 10% autologous serum and incubated with GolgiPlug (BD Biosciences) for the final 4 hours. For staining, cells were preincubated with Fc receptor blockers for 10 minutes at 4°C. For intracellular staining, the cells were fixed and permeabilized using a Foxp3/Transcription Factor Staining Buffer Kit (eBioscience) and stained with APC-conjugated anti-human Foxp3 antibody, followed by Pacific blue-conjugated anti-human CD4, PE/Cy7-conjugated anti-human IL-10, PE-conjugated anti-human IL-27/IL-35 EBI3, Fluorescein-conjugated IL-12/IL-35 p35. In some experiments, cells were stained with Pacific blue-conjugated anti-human CD19, PE/Cy7-conjugated anti-human IL-10, PE-conjugated anti-human IL-27/IL-35 EBI3 and Fluorescein-conjugated IL-12/IL-35 p35. In other experiments, cells were stained with Pacific blue-conjugated anti-human CD14, PE-conjugated anti-human IL-27/IL-35

EBI3 and Fluorescein-conjugated IL-12/IL-35 p35. As a control, cells were stained with individual isotype antibodies. After washing, the cells were resuspended in PBS containing 1% BSA and analyzed with FACSCanto II (BD Biosciences) using FlowJo software (version 9.8; TreeStar, Ashland, OR; BD Biosciences Immunocytometry Systems).

mRNA isolation and real time RT-PCR

cDNAs were synthesized from 1 µg of a purified RNA sample from cultured memory CD4⁺ T cells using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland). The reaction was incubated at 45°C for 60 min and was stopped by heating to 85°C for 5 min. The real-time RT-PCR reaction contained 1 µL of cDNA, 10.0 µL of LightCycler480 Probe Master (Roche), 0.2 µL of probes, and 0.1 µL of two primer sets: GATA binding protein-3 (GATA-3) (forward primer: CGTGCCCGAGTACAGCTC; reverse primer: ACACACTCCCTGCCTTCTGT) and GAPDH as an endogenous control (forward primer: AGCCACATCGCTCAGACAC; reverse primer: GCCCAATACGACCAAATCC). All primers were purchased from Nihon Gene Research Laboratories (Miyagi, Japan). Reactions were all brought to a final volume of 20 µL with sterile water. Amplification and detection of specific products were performed using a LightCycler® 480 Real-Time PCR System (Roche). The

real-time RT-PCR protocol was as follows: denaturation by a hot start at 95°C for 10 min, followed by 40 cycles of a two-step program (denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min). Transcription was normalized to GAPDH transcription in each sample, and the data were expressed relative to cultured memory CD4⁺ cells.

Cytokine production by PBMC

Cell-free supernatants were collected and analyzed for IL-5, IL-13, IFN- γ (R&D Systems) or IL-35 (Cloud-Clone corp, TX, USA) by ELISA following procedure recommended by the manufacturers. Serum cedar-specific IgE and IgG4 was measured using ImmunoCAP specific IgE (0.1-100 UA/mL) and specific IgG4 (0.07-30 mg_A/L), respectively.

Statistical analysis

The nonparametric Mann-Whitney U test was used to compare data between groups, and the Wilcoxon signed rank test was used to analyze data within each group. Correlations were assessed using Spearman's rank correlation coefficient. All data are reported as the mean $\pm$ SEM from the indicated number of samples. * $P < 0.05$, ** $P < 0.01$.

Table S1: Patient characteristics

Patient information	HC group	JCP group	SLIT group
N (Male/Female)	21 (10/11)	22 (9/13)	45 (17/28)
Age, mean $\pm$ SD	36.4 $\pm$ 5.7	32.3 $\pm$ 7.1	35.7 $\pm$ 7.2
JC-specific IgE, class	0	3.42 $\pm$ 0.25	3.86 $\pm$ 0.15
JC-specific IgE, UA/mL	0	25.11 $\pm$ 5.21	33.42 $\pm$ 4.35
JC-specific IgG4, mg/L	0.02 $\pm$ 0.03	0.04 $\pm$ 0.07	0.75 $\pm$ 0.81*
Polysensitization	0	22/22	43/45
Cypress	0	22/22	43/45
Mite	0	4/22	9/45
Cat	0	2/22	4/45
Ragweed	0	2/22	4/45
SMS	N.A.	4.67 $\pm$ 1.78	3.70 $\pm$ 1.59*

HC: healthy controls, JCP: Japanese cedar pollinosis, SLIT: sublingual immunotherapy,

JC: Japanese cedar, SMS: Symptom medication score

Polysensitization indicates sensitization to other allergens (cypress, mite, cat, or ragweed)

* $P < 0.01$, comparison between the JCP and SLIT groups.

Figure Legends

Figure S1. Effects of recombinant IL-35 (rIL-35) on Japanese cedar pollen extract-induced cytokines in PBMC.

PBMC were incubated with Japanese cedar pollen extract (10 $\mu\text{g/mL}$) in the presence of rIL-35 (0-10 $\mu\text{g/mL}$) for 3 days. IL-5, -10, -17 and IFN- γ concentrations in the cell-free supernatants were measured by ELISA (A-D). * $P < 0.05$ and ** $P < 0.01$, compared to cells with medium alone, by the Mann-Whitney U test, respectively. $n=8$.

Figure S2. The effect of rIL-35 on Th2 cytokine production.

Isolated memory CD4^+ T cells were incubated with anti-CD3/anti-CD28 in the presence or absence of rIL-35 (0.1-10 $\mu\text{g/mL}$) for 6 h (for mRNA) and 3 days. GATA3 mRNA was analyzed by real-time RT-PCR. GATA3 transcription was normalized to GAPDH mRNA transcription in each sample and is expressed as a ratio to PBMC cultured in the absence of rIL-35. *, $P < 0.05$, compared to cells with medium alone by the Mann-Whitney U test (A). IL-5, -13 and IFN- γ concentrations in the cell-free supernatants were measured by ELISA (B-D). P values were determined by the

Wilcoxon signed-rank test. n=8.

Co-cultured DCs/CD4⁺ T cells were incubated with Japanese cedar pollen extract (10 µg/mL) in the presence or absence of 10 µg /mL rIL-35 for 3 days. IL-5, -13 and IFN-γ concentrations in the cell-free supernatants were measured by ELISA (E-G).

P values were determined by the Wilcoxon signed-rank test. n=8.

Figure S3. IL-35 expression in T cells and B cells.

IL-35 expressing CD4⁺T cells or CD19⁺B cells were examined in PBMC collected from JCP patients after completion of SLIT for 12 months. IL-35 expression was defined by double positive staining for EBI3 and p35. PMBC were incubated with JC extract, and representative scattergrams showed the expression of CD4, Foxp3, IL-10, EBI3 and p35 (A). Representative scattergrams showed the expression of CD19, IL-10, EBI3 and p35 (B). FSC: forward scatter, SSC: side scatter.

Figure S4. The effect of SLIT on IL-35 expression in T cells or B cells

PBMC before and after SLIT were incubated with Japanese cedar pollen extract (10 µg/mL) for 2 days. The ratio of Foxp3⁺IL-35⁺ (CD4⁺Foxp3⁺IL-35⁺ /CD4⁺) T cells (A) and IL-10⁺IL-35⁺ (CD4⁺IL-10⁺IL-35⁺ /CD4⁺) T cells (B) were increased after

SLIT. The ratio of IL-10⁺IL-35⁺ (CD19⁺IL-10⁺IL-35⁺ /CD19⁺) B cells (*C*) was also

increased after SLIT. *P* values were determined by the Wilcoxon signed-rank test. n=10.