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Anti-inflammatory roles of interleukin-35 in the pathogenesis of Japanese cedar pollinosis

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ABSTRACT

Background: Interleukin (IL)-35 has been recently identified as an anti-inflammatory cytokine in allergic inflammation. However, its biological role in the pathogenesis of allergic rhinitis has not been fully elucidated.

Objective: The purpose of this study was to investigate the anti-inflammatory activity of IL-35 in the pathogenesis of allergic rhinitis in patients with Japanese cedar pollinosis (JCP).

Methods: Peripheral blood mononuclear cells were collected from healthy controls and JCP patients during the off-season for pollen. Peripheral blood mononuclear cells were incubated with Cry j 1, a major allergen of Japanese cedar pollen and production of IL-5, IL-13, and IL-35 were measured by enzyme-linked immunosorbent assay. Th2 (CD4$^+$ST2$^+$) cells and group 2 innate lymphoid cells were isolated from peripheral blood mononuclear cells of JCP patients, and the inhibitory effects of IL-35 on cell differentiation, proliferation and mRNA expression of IL-5, IL-13, and GATA3 were examined. B cells were also isolated and the effects of IL-35 on total IgE production were examined.

Results: Cry j 1-induced production of IL-5 and IL-13 was significantly increased in peripheral blood mononuclear cells from JCP patients, whereas Cry j 1-induced IL-35 production was significantly decreased compared with healthy controls. IL-35 significantly inhibited Th2 cell differentiation, group 2 innate lymphoid cell proliferation, and mRNA expression of IL-5, IL-13, and GATA3 in Th2 cells and group 2 innate lymphoid cells. IL-35 also inhibited IgE production from B cells.

Conclusion: IL-35 is an important anti-inflammatory cytokine in JCP, and its biological roles include the downregulation of Th2 cells and group 2 innate lymphoid cells, and the inhibition of IgE production from B cells. These findings demonstrate that IL-35 may have the potential to exert anti-allergic effects for the treatment of allergic rhinitis.

Keywords: Japanese cedar pollinosis; IL-35; Th2 cell; Group 2 innate lymphoid cell; B cell

INTRODUCTION

Interleukin (IL)-35 is an IL-12 cytokine family member that forms a heterodimer of 2 subunits, Epstein-Barr virus-induced gene 3 and IL-12p35 [1]. Its family members (IL-12, -23, -27) are similar in structure, receptor binding, and downstream signaling pathways, which

positively or negatively regulate the immune system. IL-35 is produced mainly by CD4+Foxp3+ regulatory T (Treg) cells, B cells, endothelial cells, smooth muscle cells, and monocytes [2]. The receptor for IL-35 consists of regions of gp130 and the IL-12Rβ2 subunit, activating the signal transducer and activator of transcription (STAT) 1 and STAT4 pathways [3-5]. While gp130 is fairly ubiquitously expressed, IL-12Rβ2 is expressed mainly on the surfaces of activated T cells, natural killer cells, B cells, dendritic cells, and group 2 innate lymphoid cells (ILC2s) [6, 7].

IL-35 strongly inhibits immune responses by promoting IL-35-inducible regulatory T (iTr35) cells, but not via other known inhibitory cytokines such as IL-10 or transforming growth factor (TGF)-β, and by inducing regulatory populations of TGF-β and IL-10-producing Treg cells [8-11]. In addition, IL-35 potently inhibits the function of CD4+ effector T cells, including Th1 and Th17 cells, through the expansion of Treg cells and IL-10 production [2]. Several studies have shown the anti-inflammatory function of IL-35 in a number of aspects of the pathogenesis of a mouse allergic rhinitis (AR) model *in vivo* [12-14].

AR was estimated to affect 49.2% of the Japanese population in a 2019 epidemiological study [15]. In particular, the prevalence of Japanese cedar pollinosis (JCP) showed a marked increase from 16.2% in 1998 to 38.8% in 2019 [15]. Th2 cells and ILC2s produce type 2 cytokines and are important inflammatory cells in the pathogenesis of AR [16], and B cells also play crucial roles by producing antigen-specific IgE. Recently, it has been reported that IL-35 may have inhibitory effects on the function of ILC2s, Th2 cells, and IgE-producing B cells [17]. However, the anti-inflammatory roles of IL-35 in the pathogenesis of JCP have not been elucidated.

In the present study, we examined (1) Cry j 1 (a major antigen of Japanese cedar pollen)-induced production of IL-5, IL-13, and IL-35 from peripheral blood mononuclears cells (PBMCs) of JCP patients, and (2) the anti-inflammatory effects of IL-35 on cell differentiation, proliferation and mRNA expression of IL-5, IL-13, and GATA3 in sorted Th2 cells and ILC2s. (3) The inhibitory effects of IL-35 on IgE production from isolated B cells were also examined.

MATERIALS AND METHODS

Study participants
This prospective study is comprised of 15 nonallergic healthy volunteers (age, 30.4 ± 4.6 years; male:female, 7:8), and 17 patients with JCP (age, 32.3 ± 5.9 years; male:female, 7:10) at the Department of Otorhinolaryngology of Shiga University Hospital and Yuta Clinic. JCP is diagnosed by typical nasal symptoms during the cedar pollen season and serum specific-IgE score for Japanese cedar pollen (≥0.70 UA/mL) measured by ImmunoCAP (at the clinical test service of SRL, Tokyo, Japan).

The exclusion criteria include 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 during the off-season for pollen. All patients provided written informed consent before sample collection. The study was approved by the institutional review board of Shiga University of Medical Science (Otsu, Japan).

Reagents and antibodies

We purchased the following materials: recombinant human IL-4, recombinant human IL-21, recombinant human IL-33 (FUJI FILM, Osaka, Japan), recombinant human IL-35 (Chimerigen, San Diego, CA, USA), and recombinant human CD40L (BioLegend, San Diego, CA, USA). For flow cytometry, we purchased the following anti-human mAbs: Pacific blue anti-human CD4, PE anti-ST2/IL-1 R4 (R&D systems, Minneapolis, MN, USA) and anti-CD127 (BioLegend), FITC anti-human CD3, CD11b, CD11c, CD14, CD16, CD19, CD20, CD45, CD56, CD123, CD127, TCRαβ, TCRγδ (BioLegend) and FceR1α (eBioscience, San Diego, CA, USA), PE-Cy7 anti-human CD45 (BioLegend), and isotype-matched control IgG (BioLegend).

Cell culture of peripheral blood mononuclear cells

PBMCs from healthy controls (HC) and JCP patients were isolated from 20 mL of heparinized venous blood by density gradient centrifugation using Ficoll-Paque PREMIUM (GE Health Care, Uppsala, Sweden). PBMCs were cultured in Rosewell Park Memorial Institute 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, then incubated in 96-well round-bottom plates (Falcon; Corning Inc., Corning, NY, USA) at 37°C and 5% CO_2. PBMCs (3×10^6 cells/mL) were incubated with 10 µg/mL of Cry j 1 (Hayashibara Co., Ltd., Okayama, Japan), a major allergen of Japanese cedar pollen for 5 days. Cell-free supernatants were then analyzed for IL-5, IL-13 (R&D Systems, Minneapolis, MN, USA), and IL-35 (Cloud-Clone Corp., Katy, TX, USA) using commercial enzyme-linked immunosorbent assay kits. Cell viability was examined using the trypan blue assay.

CD4+ T cells were isolated from PBMCs of JCP patients using an EasySep Human CD4+ T Cell Isolation Kit (Stem Cell Technologies, Vancouver, Canada), seeded at 1×10^6 cells/mL in round-bottom 96-well tissue culture plates, then cultured with recombinant human IL-33 (100 ng/mL) in the presence or absence of recombinant human IL-35 (10–1,000 ng/mL) for 5 days. Cells were incubated with GolgiPlug (BD Biosciences, San Jose, CA, USA) for the final 4 hours prior to flow cytometry analysis.

For the isolation of Th2 cells, PBMCs were incubated with PMA (50 ng/mL) and ionomycin (1 µg/mL) for 24 hours, and Th2 (CD4+ST2+) cells were sorted using FACSAria (BD Biosciences). For experiments involving mRNA analysis, sorted Th2 cells were cultured with recombinant human IL-33 (100 ng/mL) in the presence or absence of recombinant human IL-35 (10–1,000 ng/mL) for 6 hours.

ILC2s in PBMCs were sorted from JCP patients. Briefly, Lineage (CD3, CD11b, CD11c, CD14, CD16, CD19, CD20, CD56, CD123, TCRαβ, TCRγδ, FceR1α)-negative CD45+ CD127+ CRTH2+ cells were sorted and cultured (1,000 cells/well) in 96-well round-bottom plates for 2 weeks at 37°C with IL-2 (50 ng/mL) in the presence or absence of IL-35 (100 ng/mL). Half of the medium was refreshed every 2 or 3 days. The number of viable ILC2s was then determined using a cell counting kit-8 (Dojindo, Kumamoto, Japan); 10 µL of cell counting kit-8 solution were added to each well, incubated with the ILC2s for 4 hours, and absorbance was measured at 450 nm. Proliferated ILC2s (1×10^4 cells/ mL) were stimulated with IL-33 (100 ng/mL) in the presence or absence of IL-35 (100 ng/mL) for 6 hours prior to mRNA analysis.

B cells were isolated from JCP patients using an EasySep Human B Cell Isolation Kit (Stem Cell Technologies). Isolated B cells (5×10^5 cells/ mL) were cultured with CD40 ligand

(CD40L; 100 ng/mL) alone or with IL-4 (100 ng/mL) or IL-4 plus IL-21 (100 ng/mL) in the presence or absence of recombinant human IL-35 (100 ng/mL) for 72 hours.

Flow cytometry

Cells were preincubated with Fc receptor blockers for 10 minutes at 4℃ before staining with zombie Aqua Fixable Viability Kit (BioLegend) to assess cell viability and stained with Pacific blue-conjugated anti-human CD4 and PE-conjugated anti-ST2/IL-1 R4. As a control, cells were stained with individual isotype antibodies. After washing, the cells were resuspended in phosphate-buffered saline containing 1% bovine serum albumin and analyzed with FACSCanto II (BD Biosciences) using FlowJo software (ver. 9.8; TreeStar, Ashland, OR, USA; BD Biosciences Immunocytometry Systems).

mRNA isolation and real-time RT-PCR

cDNAs were synthesized from 1 μg of a purified RNA from cultured Th2 (CD4$^+$ST2$^+$) cells and ILC2s cells using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Basel, Switzerland). The reaction was incubated at 45℃ for 60 minutes and was stopped by heating to 85℃ for 5 minutes. The real-time reverse transcription-polymerase chain reaction (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 2 primer sets: GATA binding protein-3 (GATA-3) (forward primer: CGTGCCCGAGTACAGCTC; reverse primer: ACACACTCCCTGCCTTCTGT), IL-5(forward primer: CAGTTTGGAGGAAGGGTCTG; reverse primer: GCCTTGCAACCTTGAGACTT), IL-13 (forward primer: TGAATCTTTCTGGATCTCTCAGTG; reverse primer: CCCACTGTGAGAGGGATTGT), or glyceraldehyde 3-phosphate dehydrogenase (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℃ for 10 minutes, followed by 40 cycles of a 2-step program (denaturation at 95℃ for 15 seconds and annealing/extension at 60℃ for 1 minute). Transcription was normalized to GAPDH transcription in each sample, and the data were expressed relative to cultured CD4$^+$ST2$^+$ cells and ILC2.

Statistical analysis

The nonparametric Mann-Whitney *U* test was used to compare data between groups. All data are reported as the mean ± standard error of the mean from the indicated number of samples. A *p* value of <0.05 was considered significant.

RESULTS

Cry j 1-induced production of IL-5, IL-13, and IL-35 from PBMCs

The clinical and demographic characteristics of the HC and JCP patients are shown in **Table 1**. PBMCs were incubated with Cry j 1, a major allergen of Japanese cedar pollen for 5days. Cry j 1-induced production of IL-5 and IL-13 from PBMCs was significantly stimulated in JCP patients compared with HC. In contrast, Cry j 1-induced IL-35 production was significantly decreased in JCP patients compared with HC (**Fig. 1**).

Table 1. Patient characteristics

Characteristic	Healthy controls (n = 15)	Japanese cedar pollinosis (n = 17)
Sex, male:female	7:8	7:10
Age (yr)	30.4±4.6	32.3±5.9
JC specific IgE (class)	0	3.78±0.51
JC specific IgE (UA/mL)	0	27.91±6.21

Values are presented as number or mean ± standard deviation.
JC, Japanese cedar.
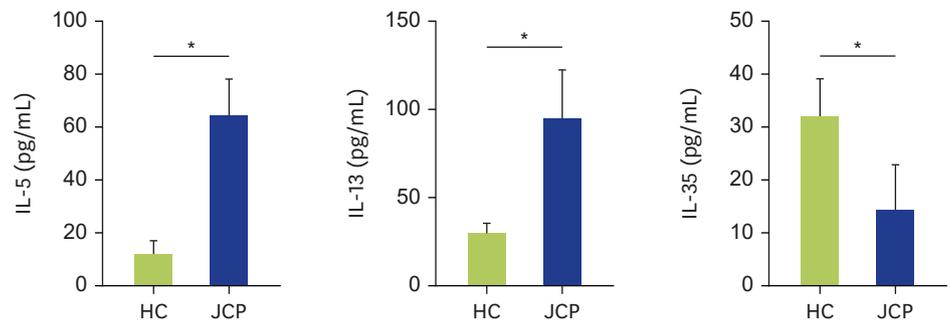
Fig. 1. Cry j 1-induced production of interleukin (IL)-5, IL-13, and IL-35 from peripheral blood mononuclears cells (PBMCs). PBMCs were incubated with Cry j 1, a major allergen of Japanese cedar pollen for 5 days. Concentrations of IL-5, IL-13, and IL-35 in cell-free supernatants were measured by enzyme-linked immunosorbent assay. *$p < 0.05$, by the Mann-Whitney U test. HC (n = 15), JCP patients (n = 17). HC, healthy controls; JCP, Japanese cedar pollinosis.

Effects of IL-35 on IL-33-induced cell differentiation and mRNA expression of IL5, IL-13, and GATA3 in Th2 cells
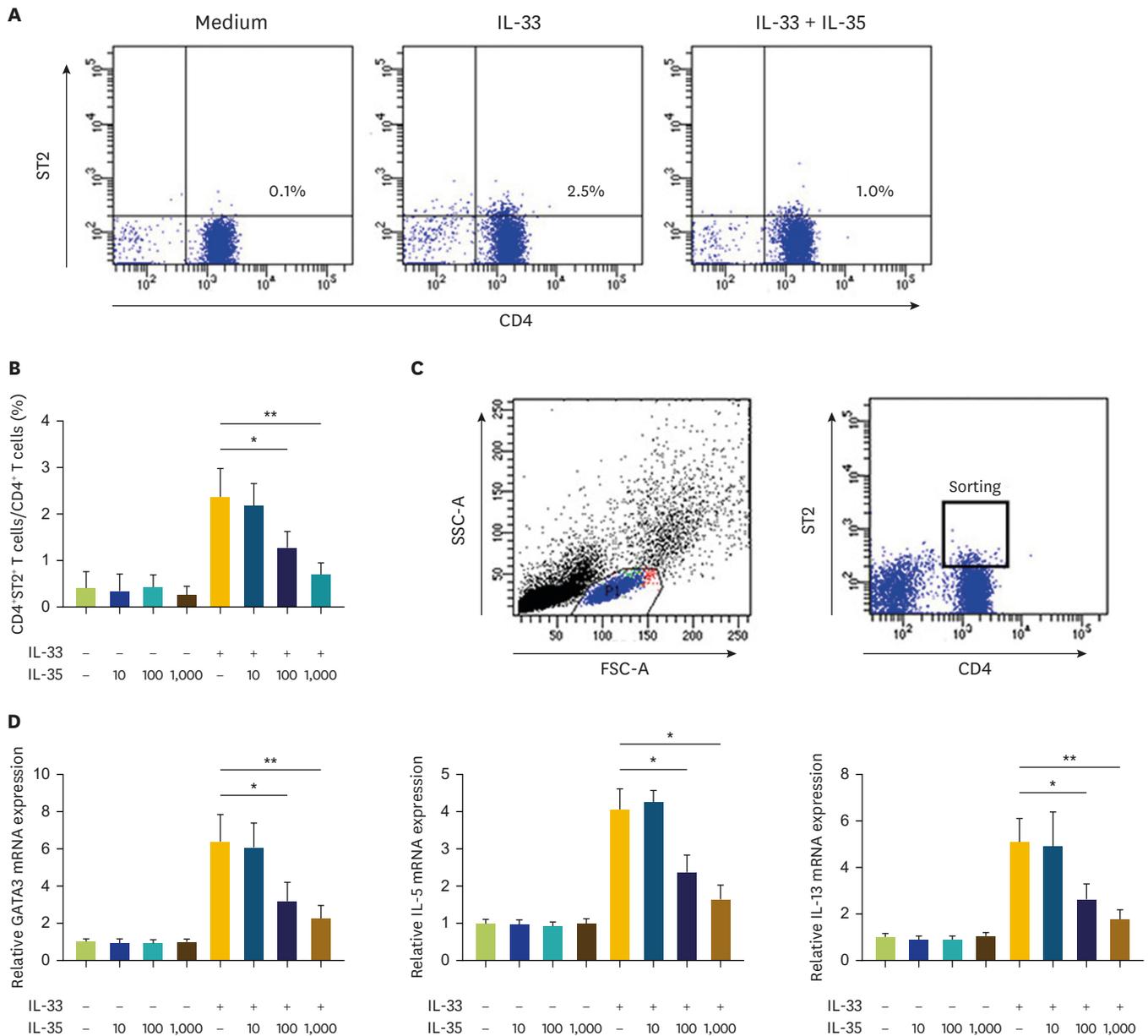
CD4$^+$ T cells were cultured with IL-33 for 5 days and the ratio of Th2 (CD4$^+$ST2$^+$) cells in total CD4$^+$T cells was examined using flow cytometry. IL-33 (100 ng/mL) significantly stimulated the Th2 cell differentiation, and IL-35 (10–1,000 ng/mL) dose-dependently inhibited IL-33-induced Th2 cell differentiation (**Fig. 2A, B**). We then examined the effects of IL-35 on the mRNA expression of Th2 cytokines (IL-5 and IL-13) and transcription factor GATA3 in sorted Th2 cells incubated with IL-33 for 6 h (**Fig. 2C**). IL-33 (100 ng/mL) upregulated the mRNA expression of IL-5, IL-13, and GATA3 in sorted Th2 cells, and IL-35 (10–1,000 ng/mL) significantly inhibited these IL-33-induced changes in a dose-dependent manner (**Fig. 2D**).

Effects of IL-35 on cell proliferation and mRNA expression of IL-5, IL-13, and GATA3 in ILC2s

ILC2s were sorted from PBMCs of JCP patients (**Fig. 3A**) and were incubated with IL-2 (50 ng/mL) for 2 weeks. IL-2 significantly stimulated the proliferation of ILC2s and IL-35 (100 ng/mL) significantly inhibited IL-2-induced proliferation of ILC2s (**Fig. 3B**). IL-33 (100 ng/mL) upregulated the mRNA expression of IL-5, IL-13, and GATA3 in proliferated ILC2s, and IL-35 (100 ng/mL) significantly inhibited these IL-33-induced changes (**Fig. 3C**).

### Effects of IL-35 on IgE production from B cells

Isolated B cells from JCP patients were cultured with CD40L (100 ng/mL) alone or with IL-4 (100 ng/mL) or IL-4 plus IL-21 (100 ng/mL), and IL-4 and IL-21 were observed to synergistically stimulate IgE production (**Fig. 4A**). IL-35 (100 ng/mL) significantly inhibited IgE production from B cells induced by co-stimulation with CD40L, IL-4, and IL-21 (**Fig. 4B**).
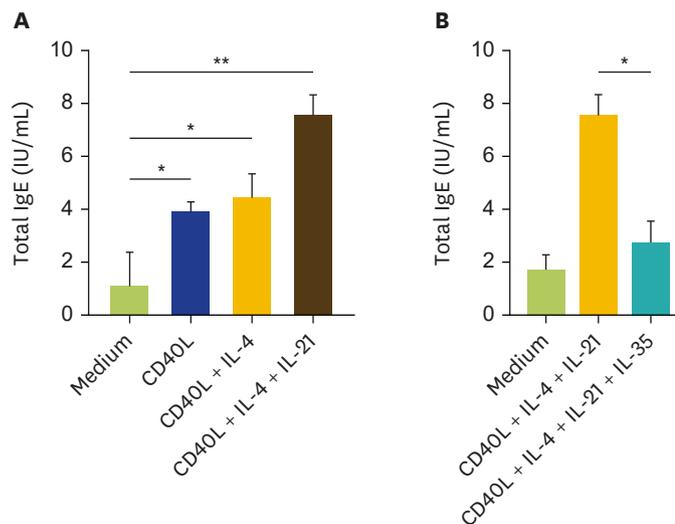
Fig. 2. (A, B) Effects of interleukin (IL)-35 on IL-33-induced cell differentiation and mRNA expression of IL5, IL-13, and GATA3 in Th2 cells. Isolated CD4+T cells were cultured with IL-33 (100 ng/mL) in the presence or absence of IL-35 (10–1,000 ng/mL) for 5 days and the ratio of Th2 (CD4+ST2+) cells in total CD4+T cells was examined using flow cytometry. Sorted Th2 cells (C) were cultured with recombinant human IL-33 (100 ng/mL) in the presence or absence of recombinant human IL-35 (10–1,000 ng/mL) for 6 hours, and mRNA expression of IL-5, IL-13, and GATA3 was measured by reverse transcription-polymerase chain reaction (D). *p < 0.05, and **p < 0.01, by the Mann-Whitney U test. (n = 7).

DISCUSSION

In the present study, Cry j 1-induced production of IL-5 and IL-13 from PBMCs was significantly increased in JCP patients, while Cry j 1-induced production of IL-35 was significantly decreased, compared with HC. We have demonstrated that IL-35 inhibits cell differentiation, proliferation, and mRNA expression of IL-5, IL-13, and GATA3 from sorted Th2 cells and ILC2s, and that IL-35 inhibits IgE production from sorted B cells. These results indicate that IL-35 is an important anti-inflammatory cytokine in allergic inflammation and that decreased production of IL-35 from PBMCs may be involved in the pathogenesis of JCP.
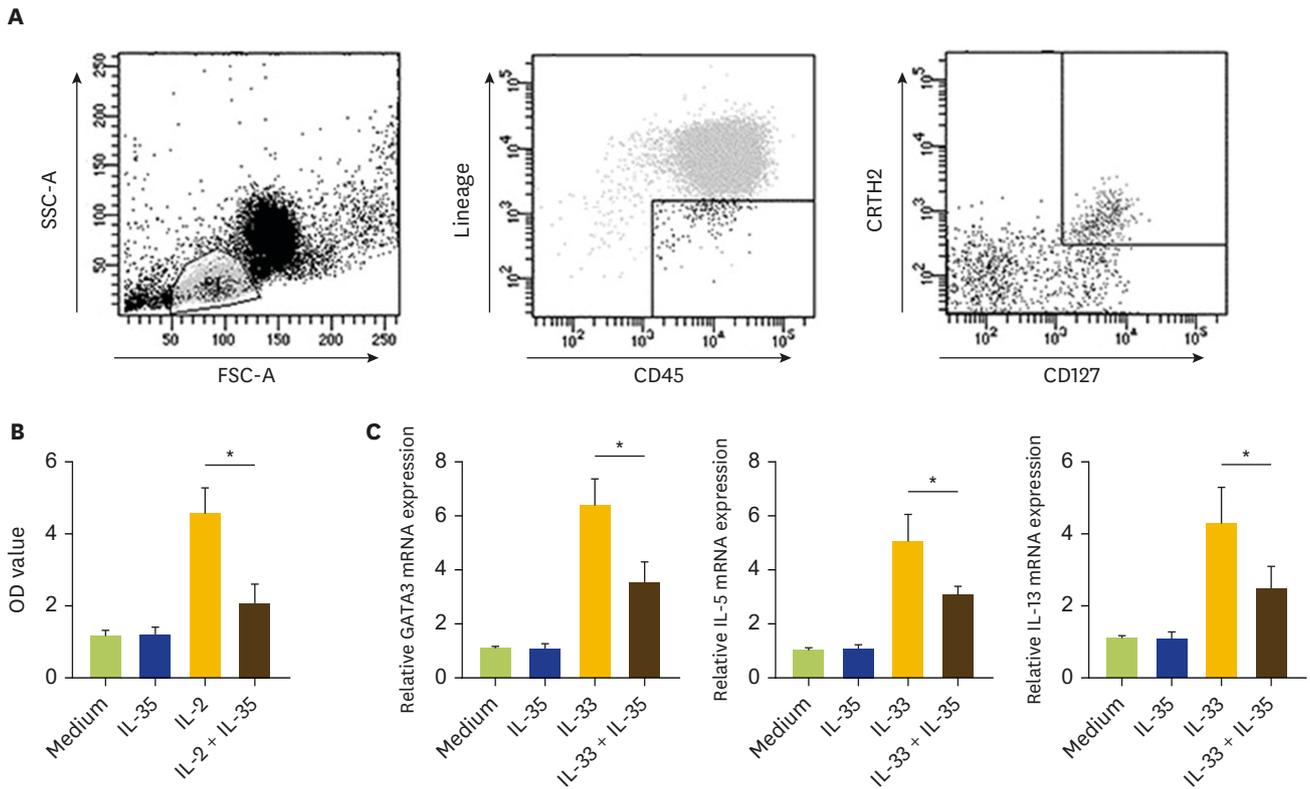
Fig. 3. Effects of interleukin (IL)-35 on cell proliferation and mRNA expression of IL-5, IL-13, and GATA3 in group 2 innate lymphoid cells (ILC2s). (A) Sorted ILC2s were incubated with IL-2 (50 ng/mL) in the presence or absence of IL-35 (100 ng/mL) for 2 weeks. (B) The numbers of viable ILC2s were counted using a cell counting kit-8, and are shown as optical density. (C) Proliferated ILC2s (1×10^4 cells/ mL) were stimulated with IL-33 (100 ng/mL) in the presence or absence of IL-35 (100 ng/mL) for 6 hours, and mRNA expression of IL-5, IL-13, and GATA3 were measured by reverse transcription-polymerase chain reaction. $^*p < 0.05$, by the Mann-Whitney U test (n = 6).

Fig. 4. Effects of interleukin (IL)-35 on IgE production from B cells. Isolated B cells were cultured with CD40L (100 ng/mL) alone or with IL-4 (100 ng/mL) or IL-4 plus IL-21 (100 ng/mL) in the presence or absence of IL-35 (100 ng/mL) for 72 hours. (A and B) Total IgE concentration in cell-free supernatants was measured by enzyme-linked immunosorbent assay. $^*p < 0.05$, and $^*p < 0.01$, by the Mann-Whitney U test. (n = 6).

The inhibitory effects of IL-35 in allergic airway inflammation have been demonstrated using experimental mouse models. In ovalbumin (OVA)-induced asthmatic mice, adenovirus mediated

IL-35 gene transfer significantly suppressed airway hyperresponsiveness, inflammatory cell infiltration, and type 2 cytokine production in lungs [18]. The inflammatory response of lung tissues was strongly inhibited when recombinant IL-35 (rIL-35) was intraperitoneally injected during the sensitization stage in OVA-induced asthmatic mice [14]. Pulmonary delivery of the IL-35 DNA constructs attenuated the house dust mites (HDM) antigen (Blo t 5)-induced eosinophil infiltration and type 2 cytokine production in lungs of mice transferred with a Blo t 5-specific Th2 cell line [13]. Intranasal administration of rIL-35 significantly inhibited OVA-induced allergic inflammation in mouse nasal epithelium [19]. These results suggest that IL-35 has immune suppressing functions in allergic airway inflammation.

IL-33 is involved in the upregulation of ST2 expression and induces Th2 cell differentiation and enhances type 2 cytokine production by CD4$^+$ T cells [20]. A previous study demonstrated that IL-35 effectively inhibits activated T effector cell proliferation [21]. In addition, IL-35 was observed to suppress GATA3 mRNA expression and type 2 cytokine production from naïve CD4$^+$ T cells [22]. Moreover, IL-35 suppresses grass pollen allergen-induced Th2 effector cell proliferation and cytokine responses. In the present study, we found that IL-35 inhibited IL-33-induced differentiation of CD4$^+$ T cells into Th2 cells, and suppressed mRNA expression of GATA-3, IL-5, and IL-13. These findings demonstrated that IL-35 inhibits Th2 cell-immune responses at the differentiation and transcription levels.

Increasing evidence has shown that ILC2s primed with epithelium-derived mediators such as IL-33, IL-25, and leukotriene D$_4$, induce type 2 allergic inflammation [23], and that ILC2s play important roles in the pathogenesis of AR. Doherty et al demonstrated that the ratio of prostaglandin D2 receptor 2 (CRTH2)-expressing ILC2s was increased in PBMCs after a provocation test in patients with cat allergy [24]. Similarly, Zhong et al. [20] demonstrated that ILC2 levels were elevated in patients with HDM sensitivity and the prevalence of peripheral blood ILC2 was correlated with symptom severity. Interestingly, Fan et al. [25] found that the level of ILC2 was elevated in PBMCs of patients with AR and HDM sensitivity compared with HC. These reports further confirm that ILC2s are involved in the induction of type 2 response in allergic patients. In contrast, Shamji et al. [17] showed that IL-35 suppressed the production of IL-5 and IL-13 from ILC2s primed with IL-25 or IL-33. Liu et al. [7] also showed that IL-35 inhibited ILC2 differentiation and type 2 cytokine production by regulating the IL-12Rβ2 and gp130 receptors. In the present study, IL-35 inhibited cell proliferation and mRNA expression of GATA3, IL-5, and IL-13 in ILC2s. These results indicate that IL-35 inhibits Th2 cytokines production from ILC2s by indirectly inhibiting cell proliferation and directly inhibiting transcriptional levels.

Recently, it has been reported that IL-35 inhibits IgE production from B cells induced by CD40L, IL-4, and IL-21 [17]. IL-35 induced the generation and expansion of IL-10-producing B cells and suppressed the proliferation of primary B cells. Furthermore, IL-35 induced the conversion into regulatory B (Breg) cells that secrete IL-35 by activating STAT1 and STAT3 [26]. Thus, IL-35 may inhibit IgE production indirectly through the inhibition of B-cell proliferation. Further studies are needed to investigate the direct effects of IL-35 on IgE production.

In the present study, Cry j 1-induced production of IL-35 was significantly decreased in PBMCs from JCP patients, although Cry j 1-induced production of IL-5 and IL-13 was significantly increased. A previous study showed that the concentration of serum IL-35 was significantly lower in AR patients with HDM than in HC [7]. IL-35 levels in supernatants of CD4$^+$ T cells cocultured with antigen-presenting cells from seasonal AR patients were

decreased compared with those from HC [17]. In addition, AR patients had a decreased proportion of iTr35 (IL-35$^+$CD4$^+$CD25$^+$) cells in PBMCs [7, 17]. In our previous study, serum IL-35 levels and the proportion of iTr35 cells were significantly increased after sublingual immunotherapy (SLIT) in JCP patients, which were positively correlated with the clinical effects of SLIT [27]. These results indicate that serum IL-35 levels represent a potential biomarker for the clinical effects of SLIT in AR patients.

In conclusion, antigen-induced IL-35 production from PBMCs was decreased in JCP patients, and IL-35 suppressed cell differentiation, proliferation, and type 2 cytokine production in Th2 cells and ILC2s from JCP patients. IL-35 also inhibited IgE production from B cells. These findings demonstrate that IL-35 is an important anti-inflammatory cytokine in the pathogenesis of JCP, and that IL-35 may be a potential therapeutic target for the treatment of AR.

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