



# Inhibited interleukin 35 expression and interleukin 35–induced regulatory T cells promote type II innate lymphoid cell response in allergic rhinitis

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## ARTICLE INFO

### Article history:

Received for publication April 30, 2020.

Received in revised form July 25, 2020.

Accepted for publication August 4, 2020.

## ABSTRACT

**Background:** Interleukin (IL)-35 and IL-35–producing regulatory T cells (iT<sub>H</sub>35) have been reported to inhibit T<sub>H</sub>2 response in allergic rhinitis (AR). However, its effects on type II innate lymphoid cells (ILC2) are not well characterized.

**Objective:** To investigate the effect of IL-35 on ILC2 in AR.

**Methods:** A total of 25 patients with AR and 20 controls were recruited. The expression and regulation of IL-35 receptor in ILC2 were analyzed by real-time polymerase chain reaction. The effect of IL-35 on ILC2 differentiation and cytokine production was analyzed by real-time polymerase chain reaction and enzyme-linked immunosorbent assay. In addition, iT<sub>H</sub>35 were cocultured with ILC2 to explore the effect of iT<sub>H</sub>35 on ILC2. The AR mice models were also established to confirm the role of IL-35 in vivo.

**Results:** The patients with AR had decreased IL-35 expression and iT<sub>H</sub>35 proportion and increased ILC2 and type II cytokines compared with the controls. Notably, IL-35 inhibited ILC2 differentiation and type II cytokine production by regulating IL-12Rβ2 and gp130. IL-35 promoted the inducible costimulatory molecule expression by iT<sub>H</sub>35 and the inducible costimulatory molecule ligand expression by ILC2. IL-35–treated mice with AR presented decreased frequency and function of nasal ILC2.

**Conclusion:** IL-35 inhibited ILC2 responses directly or through mutual contact between iT<sub>H</sub>35 and ILC2 in AR, suggesting that IL-35 may be used as a potential treatment target in AR.

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## Introduction

Interleukin 35 (IL-35), composed of Epstein-Barr virus–induced gene 3 (Ebi3) and IL-12α, is a newly identified cytokine of the IL-12 family.<sup>1,2</sup> The main source of IL-35 is natural regulatory T cells

(Tregs), and in turn, IL-35 induces the generation of a unique class of IL-35–producing regulatory T cells (iT<sub>H</sub>35).<sup>3</sup>

The binding of IL-35 and its receptors, IL-12Rβ2, gp130, or a heterodimer of IL-12Rβ2:gp130, activates the Janus kinase–signal transducer and activator of transcription (JAK-STAT) pathway and induces immunosuppression.<sup>4</sup> IL-35 exerts a potent inhibitive effect on T<sub>H</sub>2 cell–mediated allergic lung inflammation, allergen-specific immunoglobulin E (IgE) and IgG1 production, and total IgE production.<sup>5</sup> Moreover, IL-35 inhibits the differentiation of T<sub>H</sub>17 cells in vitro in collagen-induced arthritis.<sup>6</sup>

Type II innate lymphoid cells (ILC2), located in the lymphoid tissue, intestine, lung, and skin, play important roles in the early phase of immune response. IL-25, IL-33, and thymic stromal lymphopoietin induced by allergens directly activate ILC2 and promote the production of type II cytokines such as IL-13, IL-5, IL-4, and IL-9.<sup>7</sup> Studies have found that the proportion of ILC2 in the peripheral blood of the patients with allergic rhinitis (AR) allergic to cat increased markedly after nasal stimulation.<sup>8</sup> In the pollen

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**Disclosures:** The authors have no conflicts of interest to report.

**Funding:** This study was supported by grants from the National Natural Science Foundation of China (numbers, 81600785, 81700892, and 81970861), the Pearl River S&T Nova Program of Guangzhou (number, 201710010085), the Key Clinical Specialty of Guangzhou Women and Children's Medical Center, grant of the Institute of Pediatrics of Guangzhou Women and Children's Medical Center (YIP-2016-022, Pre-NSFC-2018-005), and the Dongguan Social Science and Technology Development (Key) Project (201950715024194).

<https://doi.org/10.1016/j.anai.2020.08.005>

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season, ILC2 in the peripheral blood of the patients with AR who are sensitized to pollen also increased compared with the control group.<sup>9</sup> Fan et al<sup>10</sup> reported that ILC2 may play different roles in AR subtypes mediated by dust mites and mugwort. However, one study revealed that the level of ILC2 in the peripheral blood of the patients with AR has no significant change compared with the control group.<sup>11</sup> Previous study had provided preliminary evidence that IL-35 had negative effect on the function of ILC2.<sup>12</sup> However, the detailed mechanism was not explored. Therefore, we aimed to explore the possible mechanism and the effect of regulation of IL-35 on the proliferation and cytokine production of ILC2.

In this study, we compared the expression of IL-35 and ILC2-related cytokines between those with AR and controls. We also investigated the effect of IL-35 and iTr35 on ILC2 differentiation and cytokine production using the coculture system and the AR mice models. Our results confirmed IL-35 as an important immune regulator and provided a rationale for IL-35 target therapy in the treatment of AR.

## Methods

### Patients

A total of 25 patients with AR and 20 healthy controls were recruited in this study. The persistent AR was confirmed on the basis of the criteria of the Allergic Rhinitis and its Impact on Asthma guideline.<sup>13</sup> The typical symptoms (nose itching, sneezing, rhinorrhea, nasal block), positive skin prick test, or specific immunoglobulin to *Dermatophagoides pteronyssinus* or *D farinae* were confirmed. The patients with AR who used systemic corticosteroids in the previous 1 month, had smoking history, had immune diseases, and were pregnant or lactating, were excluded. A total of 20 healthy controls with comparable age and sex and who received negative results to allergen test were selected as controls. The study protocols were approved by the local ethical committee boards, and written informed consent was obtained (approval number, 20190208).

### Symptom Scores

The total nasal symptom score (TNSS) was used for the evaluation of disease severity. Symptoms such as sneezing, rhinorrhea, itchy nose, and nasal congestion were scored on a 0 to 3 scale (0, none; 1, mild; 2, moderate; and 3, severe), and the TNSS (0–12) was summed. The nasal symptoms were recorded and averaged in an 8-week observation period.

### Flow Cytometry and Sorting for ILC2 in Peripheral Blood Mononuclear Cells

The peripheral blood mononuclear cells (PBMCs, 15 mL) were prepared by density-gradient centrifugation. The peridinin-chlorophyll-protein complex-conjugated CD45 (BioLegend, San Diego, California), fluorescein isothiocyanate-conjugated lineage markers (CD235a, FcεR1α, TCR γ and δ, CD4 [BioLegend], and CD3, CD14, CD16, CD19, CD20, CD56 [BD lineage cocktail, BD Biosciences, Franklin Lakes, New Jersey]), allophycocyanin (APC)-conjugated CD161 (BioLegend), phycoerythrin-conjugated chemoattractant receptor-homologous molecule expressed on T<sub>H</sub>2 cell (CRTH2) antibody (BM16, BD Biosciences), and PE-Cy7-conjugated CD127 antibody (HIL-7R-M21, BD Biosciences) were used for staining and tested by Beckman flow cytometer (Beckman Coulter, Brea, California). The Lin<sup>−</sup>CRTH2<sup>+</sup>CD127<sup>+</sup> cells (ILC2 cells, 2 × 10<sup>5</sup> cells/mL) were sorted on a MoFlo XDP cell sorter (Beckman Coulter) and cultured in the Roswell Park Memorial Institute (RPMI 1640) medium with 10% fetal bovine serum and 1% penicillin or streptomycin for 3 days. The recombinant IL-25 (10 ng/mL), IL-33 (10 ng/mL), thymic stromal lymphopoietin

(10 ng/mL), and IL-2 (10 U/mL) were used for stimulation. Different concentrations of IL-35 (10–100 ng/mL), anti-IL-35 (100 ng/mL), JAK inhibitor (100 ng/mL), or STAT inhibitor (100 ng/mL) were added. All cytokines and inhibitors were purchased from the Research and Diagnostic Systems, Inc (Minneapolis, Minnesota). The phosphate-buffered saline (PBS) was used as the negative control. The stimulation concentrations of the different stimulants were determined in the pretest.

The ILC2 cells (1 × 10<sup>5</sup> cells/mL) were stimulated for 6 hours using 10 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich, St Louis, Missouri) and 500 nM ionomycin (Merck, Kenilworth, New Jersey) for restimulation. For intracellular cytokine staining of IL-5 and IL-13, Cytofix (BD Biosciences) was used according to the manufacturer's instructions. First, brefeldin A (3 μg/mL) and monensin (1/2000 final dilution; both from eBioscience, Inc, San Diego, California) were added into the PBMCs to block cytokine secretion. Then, the cells were stained with fluorochrome-conjugated anti-IL-5 and anti-IL-13 antibodies or negative control before the flow cytometric analysis. The proliferation of ILC2 was assessed using tritiated thymidine incorporation. The dead cells were gated out through propidium iodide.

### Flow Cytometry and Induction of Human iTr35 In Vitro

The PBMCs from patients with AR and the control group were stimulated with phorbol 12-myristate 13-acetate/ionomycin for 6 hours and permeabilized. The cell surface was stained with CD4 APC (RPA-T4; Fisher Scientific, Loughborough, United Kingdom) and CD25APC Cy7 (M-A251; BD Biosciences). For intracellular staining, IL-12p35 peridinin-chlorophyll-protein complex (Fisher Scientific) and EB13 Alexa Fluor 488 (607201; Research and Diagnostic Systems, Inc) were stained by flow cytometry for the identification of iTr35 cells.

For the induction of iTr35, naive T cells were prepared from PBMCs using EasySep magnetic separation (STEMCELL Technologies, Vancouver, Canada). The naive T cells were incubated with 1 μg/mL anti-CD3, 1 μg/mL anti-CD28, and 10 μg/mL IL-35 for 9 days at 37°C in a 5% CO<sub>2</sub> atmosphere for the induction of iTr35. Flow cytometry confirmed that the cells being analyzed are largely iTr35.

### Coculture of iTr35 and ILC2

The iTr35 and ILC2 were cultured directly or in Transwell (BD Biosciences) system with the ratios of 2:1, 1:1, 1:2, and 1:4 for 3 days before the experiment, and 2:1 was used for further experiment. The recombinant IL-25 (10 ng/mL), IL-33 (10 ng/mL), thymic stromal lymphopoietin (10 ng/mL), and IL-2 (10 U/mL) were used for ILC2 stimulation. The following neutralizing antibodies were also added into the coculture medium: anti-IL-35 (100 ng/mL), anti-inducible costimulatory molecule (ICOS) (100 ng/mL), anti-inducible costimulatory molecule ligand (ICOSL) (100 ng/mL). All cytokines and inhibitors were purchased from the Research and Diagnostic Systems, Inc. The proliferation of ILC2 was assessed by using tritiated thymidine incorporation.

### Western Blot

The ILC2 or iTr35 were lysed with cold radioimmunoprecipitation assay buffer, and 20 μg protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. After blocking with 5% Tris-buffered saline milk for 1 hour, the membranes were incubated with rabbit anti-gp130, anti-IL-12Rβ2, anti-ICOS, and β-actin (Santa Cruz Biotechnology, Inc, Dallas, Texas; 1:1000) overnight at 4°C. After the incubation with horseradish peroxidase-labeled anti-rabbit IgG antibody, the signals were detected by enhanced chemiluminescence kit (Pierce Biotechnology, Inc, Rockford, Illinois).

**Table 1**  
Demographic Characteristic of Patients With AR and Normal Controls

| Groups            | AR group                        | Control         |
|-------------------|---------------------------------|-----------------|
| Number            | 25                              | 20              |
| Sex (male:female) | 13:12                           | 10:10           |
| Age (y)           | 27.5 (18–48)                    | 26.7 (18–51)    |
| TlgE (kU/L)       | 278.1 (58.6–813.4) <sup>a</sup> | 31.5 (6.5–48.3) |
| Sensitization     |                                 |                 |
| D1 (n)            | 34.2 (3.7–98.5)                 | —               |
| D2 (n)            | 45.6 (7.3–86.5)                 | —               |
| TNSS score        | 7.5 (7–12)                      | —               |

Abbreviations: AR, allergic rhinitis; D1, *Dermatophagoides pteronyssinus*; D2, *Dermatophagoides farinae*; TlgE, total IgE; TNSS, total nasal symptom score.

<sup>a</sup>Compared with the control group,  $P < .05$ .

ΔCt method and corrected for the expression of the housekeeping gene. The primers used in the test were listed as follows:

gp130, sense, 5'-TCCCATGGGAGGAATATAG-3', antisense, 5'-CCA TTGGCTTCAGAAAGAGG-3'; IL-12Rβ2 sense, 5'-GGTTGCTGG CTCCTCACCAGG-3', antisense, 5'-ATGCAGCCCTTTGCTCCGG G-3'; ICOS sense, 5'-TTGAACACTGAACGCGAGGA-3', antisense, 5'-GCA-GAACCATTGATTCTCTGT-3'; ICOSL sense, 5'-TCGAGC TGA-TACTCAGGAGAAG-3', antisense, 5'-TTCGACTCACTGGTTT GCCA-3'; GATA3 sense, 5'-GCGGGCTCTATCAGAAATGA-3', antisense, 5'-GCTCTCTGGCTGCAGACAGC-3'; RORα sense, 5'-AAGGAGCCA-GAAGGGATGAAC-3', antisense, 5'-GGAACA ACAGACGCCAGTAAG-3'; β-actin sense, 5'-CGAACTACCT TCAACTCCATC-3', antisense, 5'-AGTGATCTCTCTGCAT CCT-3'.

### Quantitative Real-Time Polymerase Chain Reaction

The total RNA was extracted by TRIzol reagent (Life Technologies, Carlsbad, California). The RNA (1 μg) was reverse transcribed using cDNA kit (Qiagen, Hilden, Germany). The polymerase chain reaction was performed using an ABI Prism 7300 Detection System. The expression levels of the tested genes were calculated by comparative

### Enzyme-Linked Immunosorbent Assay

The concentration of cytokines was assayed by enzyme-linked immunosorbent assay kits (Research and Diagnostic Systems, Inc) according to the instructions provided by the manufacturer. The sensitivity for cytokines was as follows: IL-5, 3.9 pg/mL; IL-13, 125 pg/mL; and IL-35, 62.5 pg/mL.

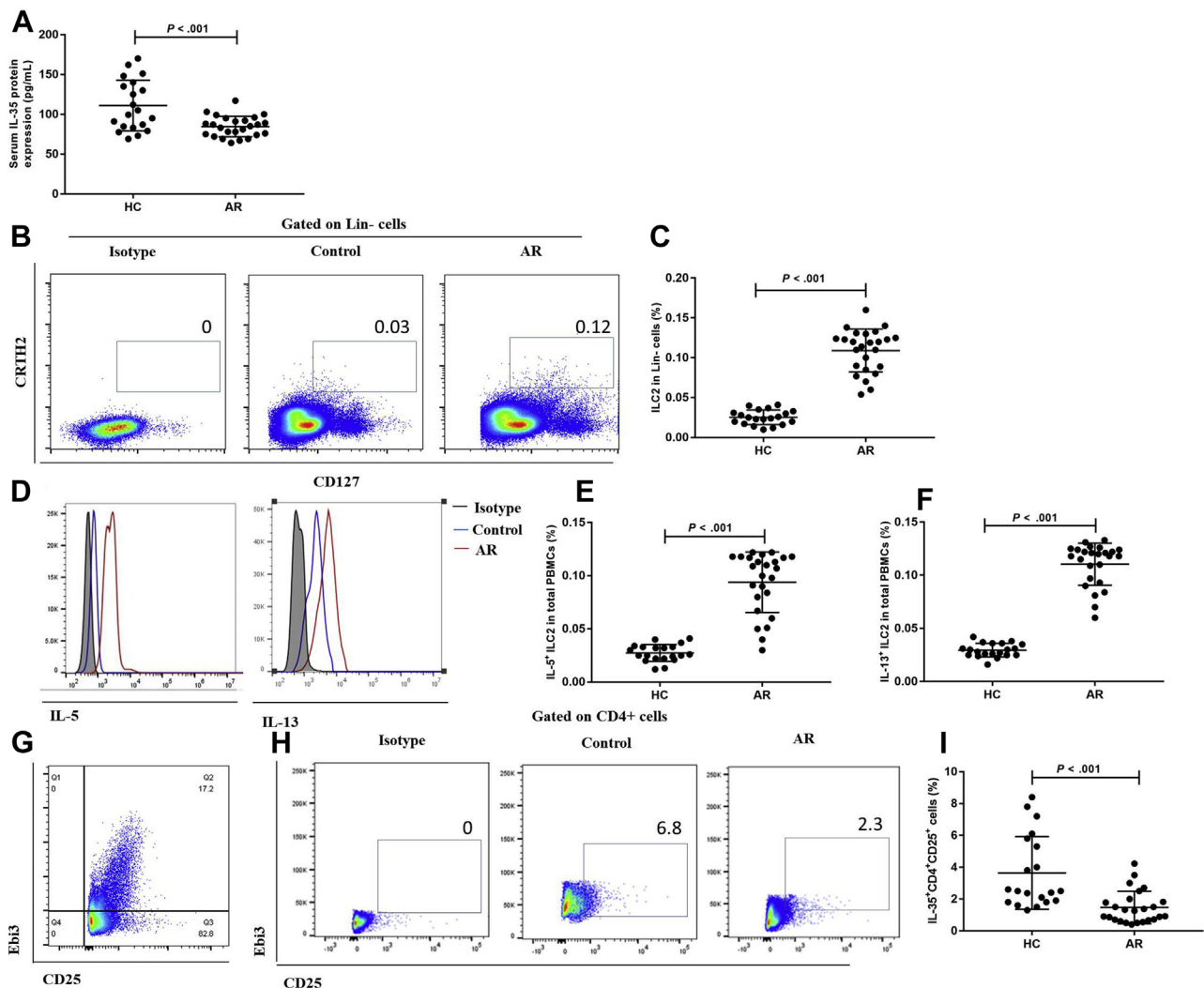


**Figure 1.** The expression of IL-35 and the proportion of ILC2, IL-5<sup>+</sup>ILC2, IL-13<sup>+</sup>ILC2, and IL-35<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells between the patients with AR and the controls. A, The serum IL-35 protein levels between 20 HCs and 25 patients with AR assessed by enzyme-linked immunosorbent assay. B, C, Flow cytometric results of the proportion of ILC2 between HC and AR. D–F, The proportions of IL-5<sup>+</sup>ILC2 and IL-13<sup>+</sup>ILC2 by flow cytometry. G, The IL-35 is primarily produced from CD25<sup>+</sup> cells. H, I, The proportions of IL-35<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells by flow cytometry. AR, allergic rhinitis; HC, healthy control; IL, interleukin; ILC2, type II innate lymphoid cells.

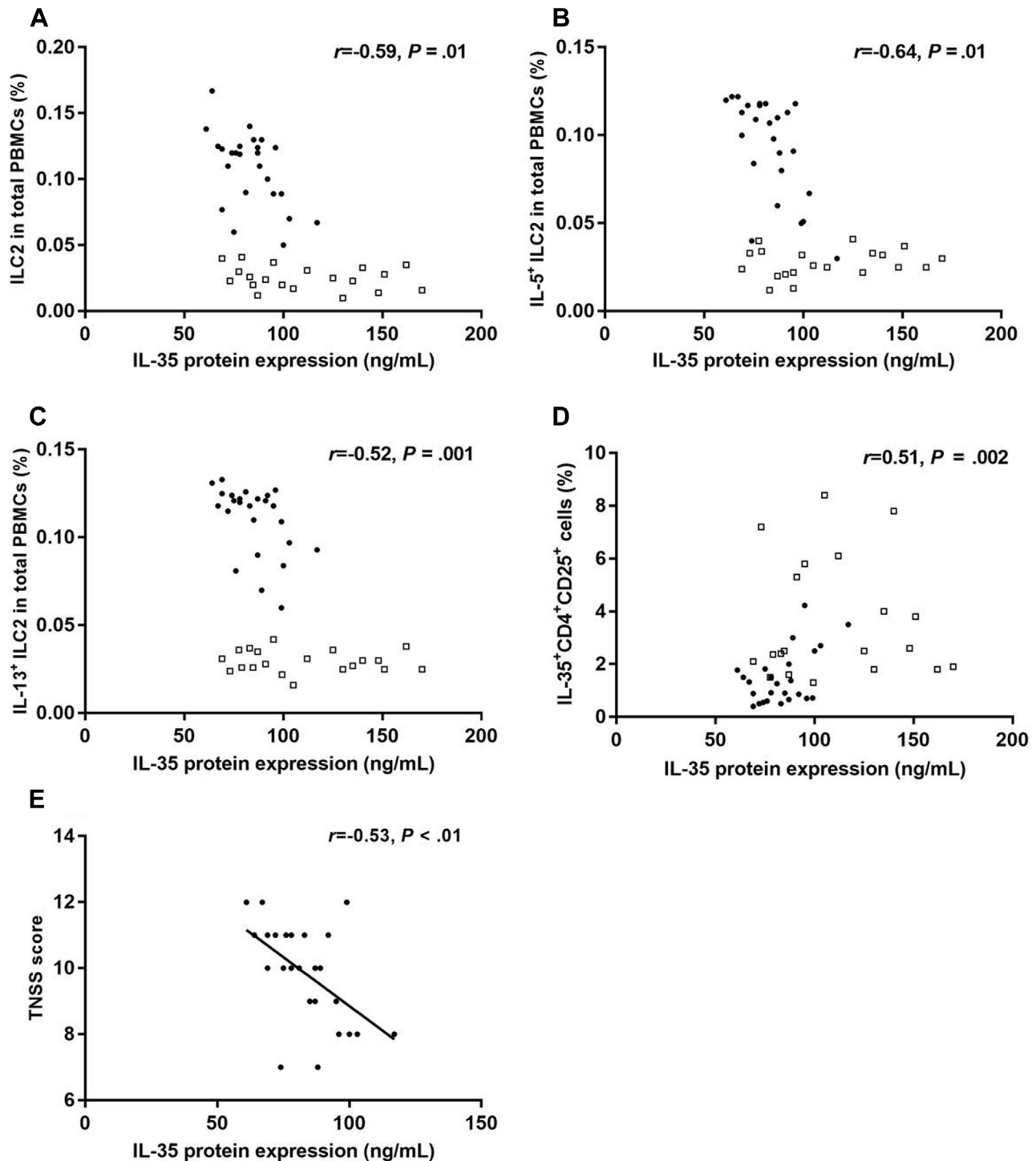


**Figure 2.** Correlation between the IL-35 levels and the proportion of ILC2, IL-5<sup>+</sup>ILC2, IL-13<sup>+</sup>ILC2, IL-35<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells, and total nasal symptom score. A–C, E, Negative correlation between IL-35 protein expression in the patients with AR and the proportions of ILC2, IL-5<sup>+</sup>ILC2, IL-13<sup>+</sup>ILC2, and the total nasal symptom score. D, Negative correlation between IL-35 protein expression in the patients with AR and the proportions of IL-35<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells. The • symbol indicates patients with AR and the □ symbol indicates healthy control. AR, allergic rhinitis; IL, interleukin; ILC2, type II innate lymphoid cells.

#### Animal Model

The 6–8 week-old male BALB/c mice (30 mice) were purchased and housed in the specific pathogen-free animal facility with free access to food and water. On days 0 and 7, the mice were sensitized with 100  $\mu$ g of Der p1 and 1.6 mg of aluminum hydroxide in 100  $\mu$ L of PBS intraperitoneally. These mice were grouped and challenged by 100  $\mu$ g of Der p1, IL-35, and Der p1+IL-35 in 100  $\mu$ L

of PBS nasally on days 15, 16, and 19, as previously described.<sup>14</sup> All cytokines and inhibitors were purchased from the Research and Diagnostic Systems, Inc. The mice were killed on the day after the last nasal stimulation, and the nasal tissue and blood samples were collected. All animal care and experimental protocols were approved by the local ethics committee boards (approval number, 20190106).

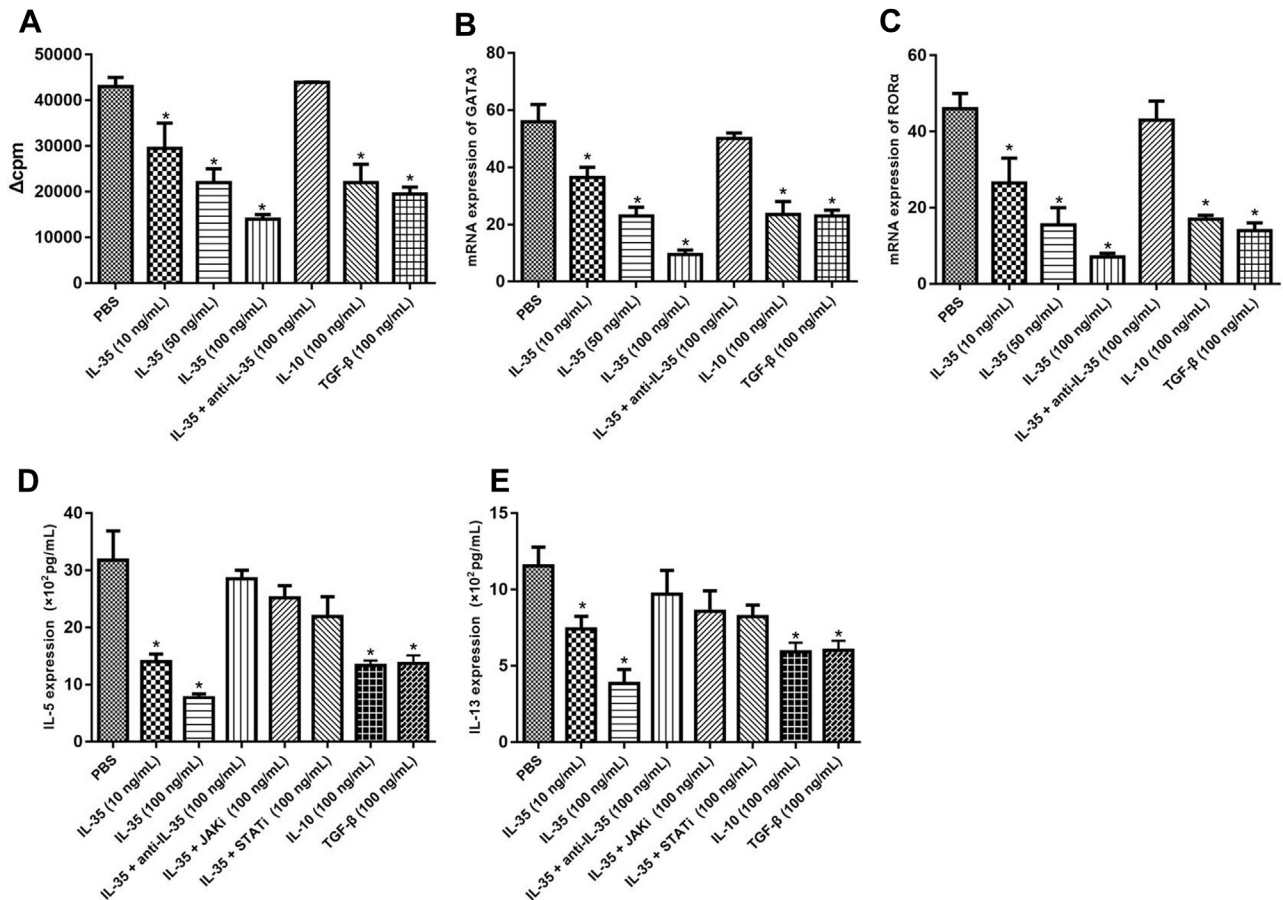


**Figure 3.** The ILC2 cell proliferation and cytokine expression regulated by IL-35. A, Proliferation of ILC2 was assessed by using tritiated thymidine incorporation under IL-35 stimulation. B,C, The expression of GATA3 and ROR $\alpha$  by ILC2 assessed by polymerase chain reaction. D,E, The type II cytokine expression by ILC2 assessed by enzyme-linked immunosorbent assay. A total of 3 independent tests were performed for every experiment. The asterisk indicates the comparison with the PBS group,  $P < .05$ ; IL-35 (10 ng/mL) vs IL-35 (100 ng/mL),  $P < .05$ ; IL-10 (100 ng/mL) vs IL-35 (100 ng/mL),  $P < .05$ ; TGF- $\beta$  (100 ng/mL) vs IL-35 (100 ng/mL),  $P < .05$ . IL, interleukin; ILC2, type II innate lymphoid cells; PBS, phosphate-buffered saline; TGF- $\beta$ , transforming growth factor beta.

#### Flow Cytometry for ILC2 in Nasal Mucosa of Mice Model

The nasal mucosa was obtained from mice and cut into small pieces and digested in RPMI 1640 medium containing collagenase IV (1 mg/mL) at 37°C for 2 hours. After filtered by a cell strainer (70  $\mu$ m and 40  $\mu$ m), the cells underwent Percoll gradient density centrifugation and were cultured in RPMI 1640 medium for further testing. The mice ILC2s in PBMCs were identified as Lin<sup>−</sup> (TCR $\beta$ (H57,597), TCR $\gamma\delta$ (eBioGL3), CD3e(145,2C11), Fc $\epsilon$ R1 $\alpha$ (AER-37), Gr-1(RB6,8C5), CD11b(M1/70), TER-119(TER,119), B220(RA3,6B2), NK1.1(PK136), CD5(53,7.3), and CD11c(N418)), ST2<sup>+</sup> (RMST2,2), CD45<sup>+</sup>(104), CD127<sup>+</sup> (A7R34), KLRG1<sup>+</sup>(2F1), Thy-1<sup>+</sup>(OX7) lymphocytes. The IL-5 (JES1-39D10), IL-4 (3010.211), IL-13 (JES10-5A2), IL-12R $\beta$ 2 (FAB1959A), or gp130 (V49-2176)–positive ILC2 cells in PBMCs were determined by intracellular cytokine staining using Cytofix (BD Biosciences) according to the manufacturer's instructions.

#### Ovalbumin-Specific T-Cell Response

CD4<sup>+</sup>CD25<sup>−</sup> T cells were prepared from cervical lymph nodes using the magnetic-activated cell sorting system as described by the manufacturer's instructions. The CD4 isolation kit and anti-CD25 MACS beads (Miltenyi Biotec, Germany) were used for isolation of CD4<sup>+</sup>CD25<sup>−</sup> T cells. The CD4<sup>+</sup>CD25<sup>−</sup> T cells ( $2 \times 10^5$  cells/mL) were cultured with ovalbumin (OVA) (100 mg/mL) for 72 hours at 37°C in RPMI 1640 medium containing 10% fetal bovine serum, penicillin G/streptomycin (Sigma-Aldrich). The expression

of IL-4, IL-5, and IL-13 by the culture supernatants was determined by enzyme-linked immunosorbent assay (PeproTech, Inc, Rocky Hill, New Jersey).

#### Statistical Analysis

Statistical analysis was performed using the Kruskal-Wallis H test or the nonparametric Mann-Whitney *U* test except for the additional note. Correlations were assessed by the Spearman rank correlation analysis. A *P* value of less than .05 was defined as statistically significant.

#### Results

##### Down-Regulated Expression of IL-35 and iTr35 and Their Correlation With ILC2 Proportion and Type II Cytokines in AR

The demographic characteristics of the participants are presented in Table 1. The protein expression of IL-35 and IL-35<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cell proportion was significantly down-regulated in the patients with AR compared with the controls, whereas the proportion of ILC2, IL-5<sup>+</sup>ILC2, and IL-13<sup>+</sup>ILC2 was significantly elevated in those with AR compared with the controls ( $P < .05$ ) (Fig 1, eFig 1). The down-regulated IL-35 levels in the AR group were significantly correlated with the proportion of ILC2 ( $r = -0.59$ ;  $P = .01$ ), IL-5<sup>+</sup>ILC2 ( $r = -0.64$ ;  $P = .003$ ), IL-13<sup>+</sup>ILC2 ( $r = -0.52$ ;  $P = .002$ ), IL-35<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells ( $r = 0.51$ ;  $P = .004$ ), and TNSS score ( $r = -0.53$ ;  $P = .02$ ) (Fig 2).

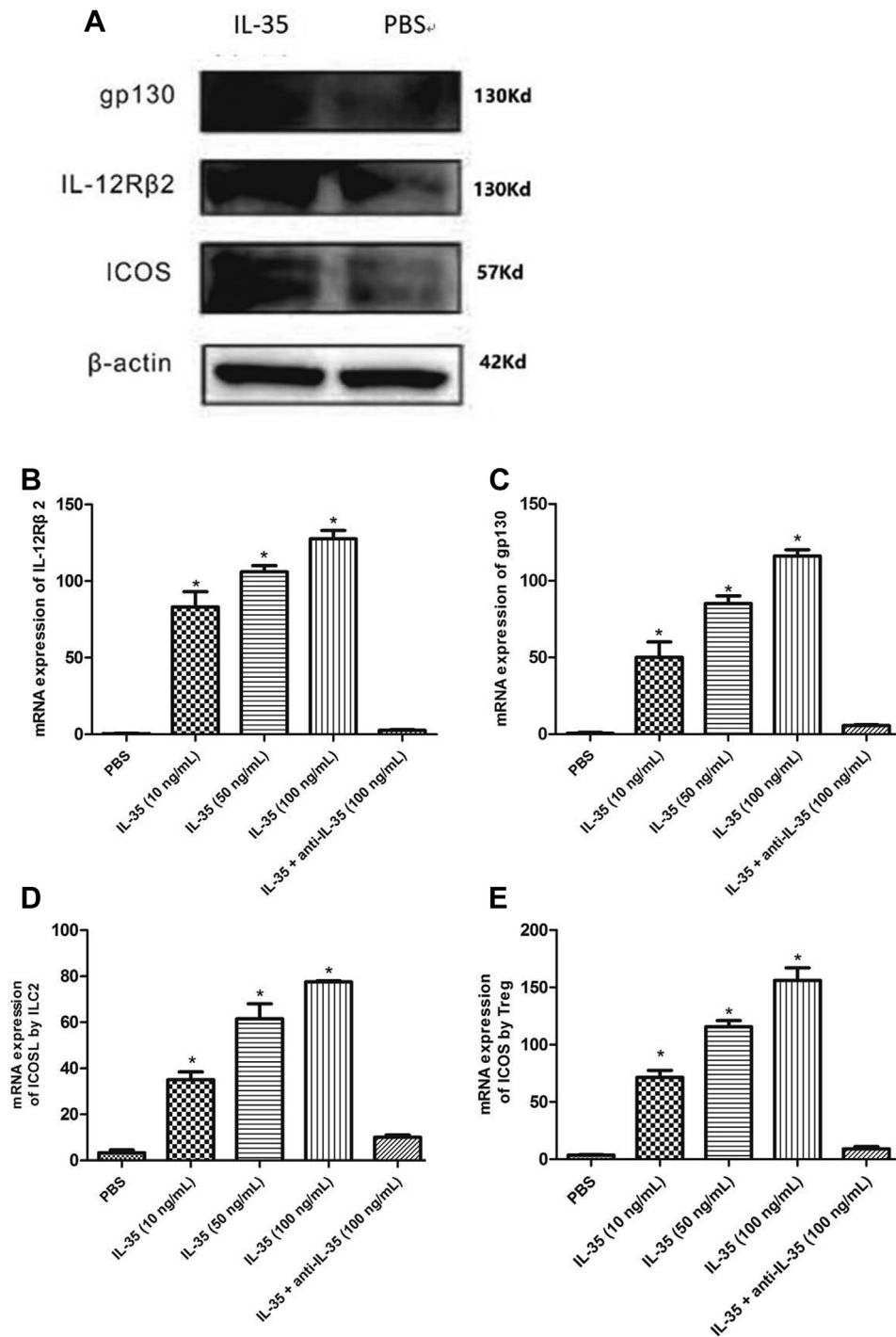


**Figure 4.** IL-35 regulated IL-35 receptor, ICOS, and ICOSL expression in ILC2 and iTreg. A, The protein expression of gp130, IL-12Rβ2, and ICOS by Western Blot. B-E, The mRNA expression of gp130, IL-12Rβ2, and ICOSL by ILC2 after stimulated by IL-35 for 3 days and ICOS by iTreg induced by IL-35. A total of 3 independent tests were performed for every experiment. The asterisk indicated comparison with the PBS group,  $P < .05$ . IL-35 (10 ng/mL) vs IL-35 (50 ng/mL),  $P < .05$ ; IL-35 (50 ng/mL) vs IL-35 (100 ng/mL),  $P < .05$ ; IL-35 (100 ng/mL) vs IL-35 (100 ng/mL) + anti-IL-35 (100 ng/mL),  $P < .05$ . ICOS, inducible costimulatory molecule; ICOSL, inducible costimulatory molecule ligand; IL, interleukin; ILC2, type II innate lymphoid cells; iTreg, IL-35-producing regulatory T cells; PBS, phosphate-buffered saline.

#### The ILC2 Cell Differentiation and Cytokine Expression Stimulated by IL-35

IL-35 inhibited the proliferation of ILC2 and the expression of GATA3 and RORα by ILC2 in a dose-dependent manner (Fig 3A-C). The type II cytokines (IL-5 and IL-13) expression by ILC2 were also down-regulated after IL-35 stimulation (Fig 3D, Fig 3E). However, these effects were alleviated significantly when JAK or STAT

inhibitor was added (Fig 3D, Fig 3E). We also found that IL-35 was more effective in the inhibition of ILC2 proliferation and cytokine production than IL-10 and transforming growth factor beta.

#### The Expression and Regulation of IL-35 Receptor by ILC2

We found that the expression of both protein and mRNA of IL-12Rβ2 and gp130 were significantly up-regulated by ILC2 after

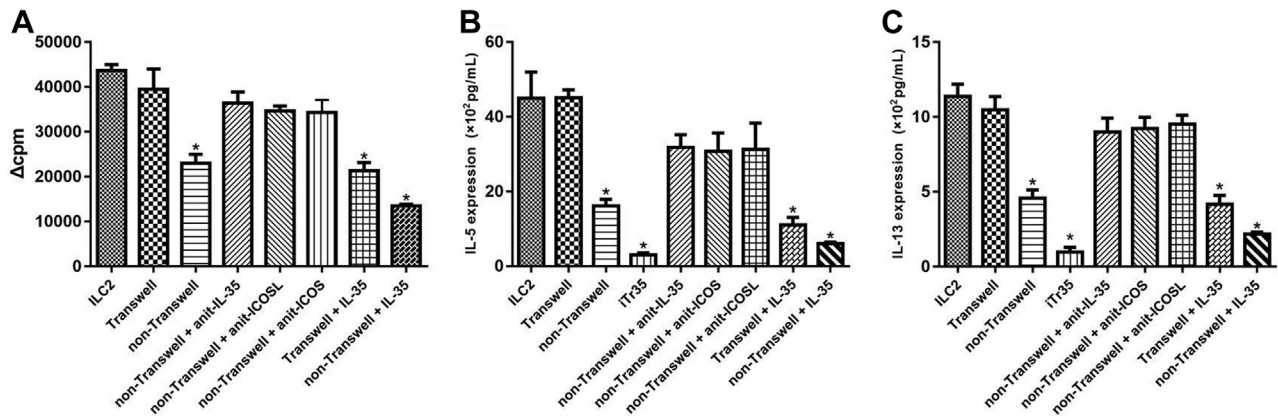


**Figure 5.** The iT35 suppressed the function of ILC2s through direct contact. A, Proliferation of ILC2 was assessed by using tritiated thymidine incorporation. B,C, The iT35 strongly suppressed the secretion of type cytokines by ILC2s in the conventional wells through the interaction of ICOS and ICOSL. A total of 3 independent tests were performed for every experiment. The asterisk indicated the compared with the ILC2 group,  $P < .05$ . ICOS, inducible costimulatory molecule; ICOSL, inducible costimulatory molecule ligand; IL, interleukin; ILC2, type II innate lymphoid cells; iT35, IL-35–producing regulatory T cells.

stimulated by IL-35 (Fig 4A–C), whereas the addition of IL-10 and TGF- $\beta$  had no effect on IL-35 receptor expression (data not found). Moreover, IL-35 induced the expression of ICOSL by ILC2 and the mRNA expression of ICOS by iT35 (Fig 4D, Fig 4E).

#### The iT35 Inhibited ILC2s Through ICOS:ICOSL Cell-Cell Contact and IL-35

To clarify the contact dependency of the iT35 suppressive effect on ILC2s, we cocultured them in a Transwell or conventional well (allowing cell-cell contact) stimulation assay. We found that iT35

strongly suppressed the proliferation and the secretion of type II cytokines by ILC2s in the conventional wells. In the non-Transwell system, the addition of anti-IL-35, anti-ICOS, or anti-ICOSL blocked the inhibitive effect of iT35. In the Transwell system, iT35 suppressive ability was significantly abrogated, whereas the addition of IL-35 partially recovered the inhibitive effect of iT35 (Fig 5). We also found that non-Transwell system and IL-35 produced the maximum inhibitive effect on the proliferation and the secretion of type cytokines by ILC2s.

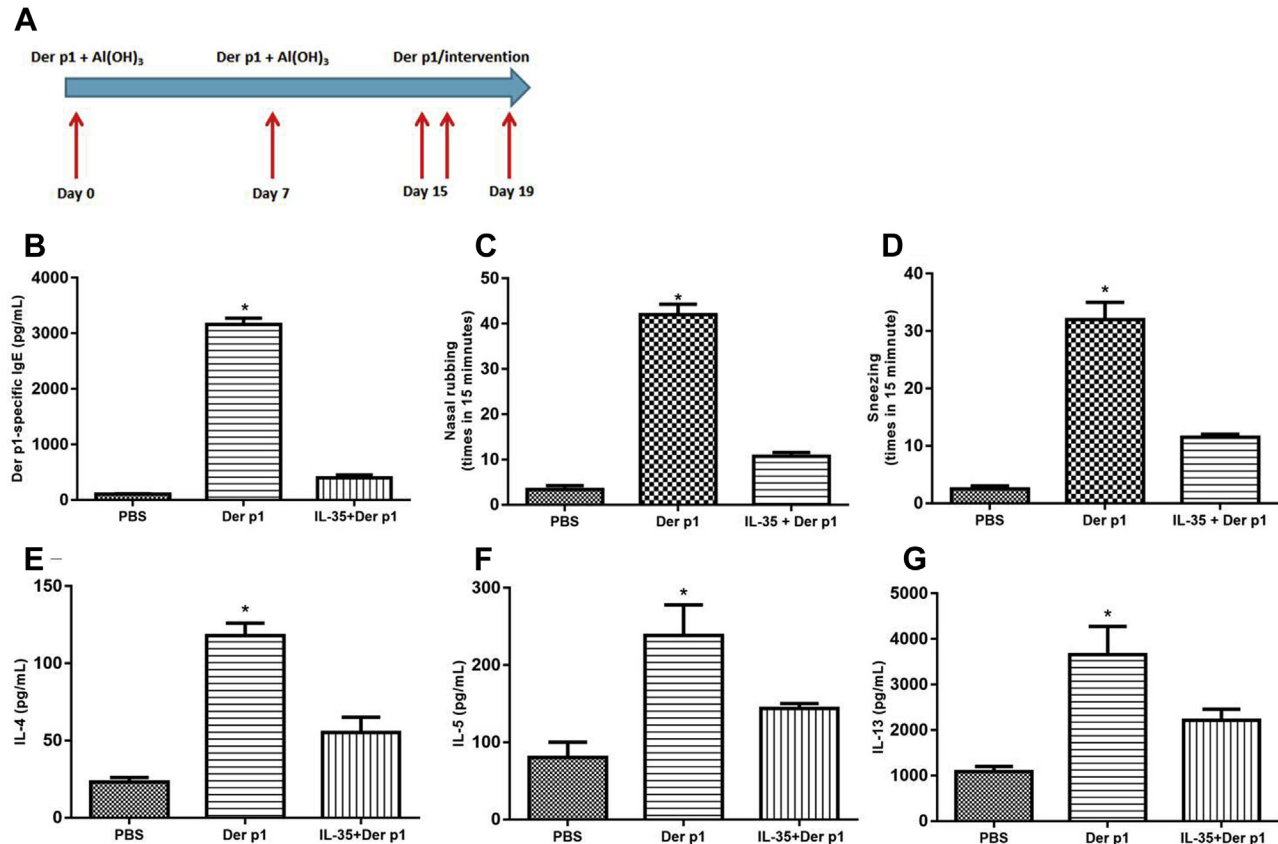


**Figure 6.** The establishment and intervention of mice model with AR. A, The establishment process of mice with AR. B, Levels of Der p1–specific IgE by enzyme-linked immunosorbent assay. C,D, Typical nasal symptoms after IL-35 treatments. E–G, The IL-5, IL-13, and IL-4 levels in OVA-specific T cells after IL-35 treatments. The asterisk indicates the comparison with the PBS group,  $P < .05$ ; Der p1 (100 ng/mL) vs IL-35 (100 ng/mL) + Der p1 (100 ng/mL),  $P < .05$ . AR, allergic rhinitis; Ig, immunoglobulin; IL, interleukin; OVA, ovalbumin; PBS, phosphate-buffered saline.

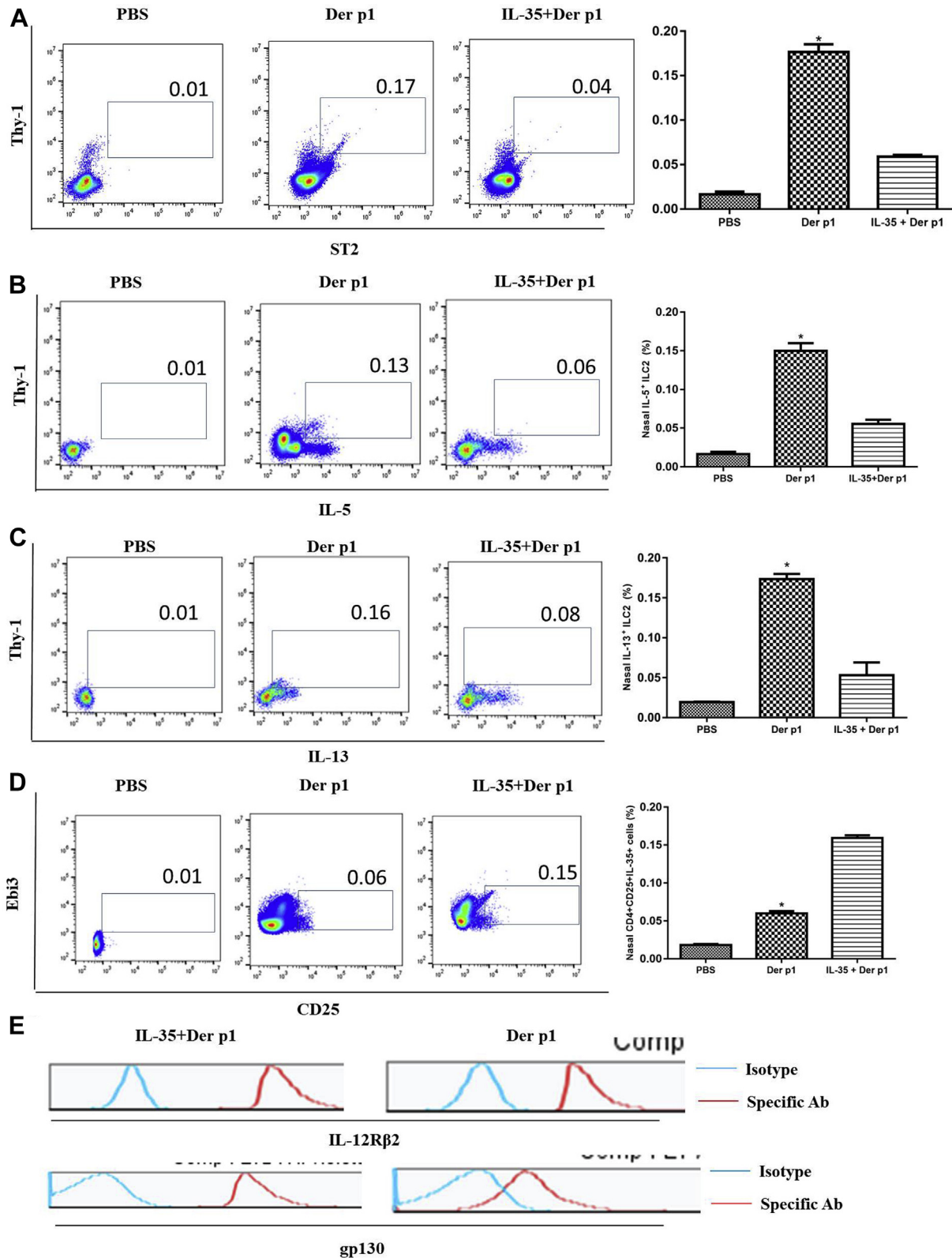


**Figure 7.** IL-35 inhibits ILC2 responses in mice model. A–C, The proportions of nasal ILC2, IL-5<sup>+</sup>ILC2, and IL-13<sup>+</sup>ILC2 in total cells of inferior turbinate tissue after IL-35 treatment by flow cytometry. D, The proportions of nasal iT35 after IL-35 treatment by flow cytometry. E, The expression of IL12R $\beta$ 2 or gp130 by nasal ILC2 by flow cytometry. A total of 30 mice were divided among the PBS group, Der p1 group, and IL-35 + Der p1 group with 10 mice in every group. A total of 3 independent tests were performed for every experiment. The asterisk indicates the comparison with the PBS group,  $P < .05$ ; Der p1 (100 ng/mL) vs IL-35 (100 ng/mL) + Der p1 (100 ng/mL),  $P < .05$ . IL, interleukin; ILC2, type II innate lymphoid cells; iT35, IL-35–producing regulatory T cells; PBS, phosphate-buffered saline.

### IL-35 Inhibits ILC2 Responses in Mice

The establishment of the mice with AR was found in Figure 6A. The treatment of IL-35 in the mice with AR alleviated the allergic inflammation of mice significantly, presented as down-regulated Der p1-specific IgE (Fig 6B) and decreased frequency of nasal rubbing and sneezing (Fig 6C, Fig 6D). Moreover, we also found that IL-5, IL-13, and IL-4 levels are decreased in OVA-specific T cells (Fig 6E–G).

We also found that the treatment of IL-35 in the mice with AR reduced the proportion of nasal ILC2 (Fig 7A) and type II cytokines (Fig 7B, Fig 7C) compared with the OVA-treated mice. Our results also revealed that the addition of IL-35 increased the nasal iTr35 proportion (Fig 7D). The expression of IL12R $\beta$ 2 or gp130 by nasal ILC2 was up-regulated by IL-35 (Fig 7E).

### Discussion

In this study, we provided evidence that IL-35 inhibited the differentiation and type II cytokine expression through the regulation of IL-35 receptors in ILC2 in AR. We also observed that iTr35 inhibited ILC2s through ICOS:ICOSL cell-cell contact and IL-35. Moreover, IL-35-treated mice with AR presented significant inhibition of allergic inflammation.

IL-35, an antiinflammatory cytokine, has been inspected in various pathologic conditions such as different cancers, inflammatory and allergic disorders, and chronic infections.<sup>15</sup> For example, most studies suggested IL-35 as a cytokine promoting tumor growth and metastasis.<sup>16</sup> Functional analysis suggests an immunosuppressive role of IL-35 in the onset and development of inflammatory autoimmune diseases.<sup>17</sup> However, the role of IL-35 in AR was not fully explored.

This study reported that the expression of IL-35 was significantly lower in the group with AR than the controls. The down-regulated IL-35 levels were significantly correlated with the proportion of ILC2 and type II cytokines, suggesting that IL-35 may be involved in the regulation of ILC2. Consistently, Shamji et al<sup>12</sup> reported that IL-35 inhibited IL-5 and IL-13 production by ILC2s. However, the exact mechanisms in IL-35 inhibited type II cytokines were not clarified.

We, for the first time, found that IL-12R $\beta$ 2 and gp130 were significantly up-regulated by ILC2 after stimulated by IL-35, whereas IL-10 and TGF- $\beta$  had no effect on IL-35 receptor expression, suggesting that IL-35 receptor expression was IL-35 dependent. Our data also indicated that IL-35 inhibited the differentiation and cytokine expression of ILC2 in a dose-dependent manner, and the effect was mediated by the JAK or STAT pathway. These results suggested that IL-35 had direct regulation on ILC2. We also found that IL-35 was more effective in the inhibition of ILC2 proliferation and cytokine production compared with IL-10 and TGF- $\beta$ . Consistently, the study of Shamji et al<sup>12</sup> reported that IL-35 can induce the production of IL-10 from T-helper clones.

The interaction between ILC2 and T cells had been reported previously. For example, Mirchandani et al<sup>18</sup> had proved that ILC2 promoted the differentiation of naive CD4 T cells to a T<sub>H</sub>2 phenotype in a contact-dependent manner. Because IL-35 was mainly secreted by Tregs, we next explored the effect of iTr35 on ILC2s and the related mechanism. We found that iTr35 strongly suppressed the differentiation and expression of type cytokines by ILC2s in a cell contact manner. In the Transwell system, the iTr35 suppressive ability was significantly abrogated, but the addition of IL-35 partially recovered the inhibitive effect of iTr35. These results suggested that both direct contacts between ILC2 and iTr35 and IL-35 were needed to maximize the inhibitive effect of iTr35. Consistently, Rigas et al<sup>19</sup> have previously found that ILC2s express both ICOS and ICOSL and that Tregs express ICOS, which are also

confirmed in our study. The ICOS:ICOSL interaction is necessary for inhibiting AHR.<sup>19</sup> In this study, we also found that antibody against ICOSL or ICOS contributes to decreased suppression of iTregs on ILC2s, suggesting that the underlying mechanism was ICOS:ICOSL interaction. We also added the transforming growth factor beta and IL-10 in the Transwell system and found that iTr35 suppressive ability was not recovered, suggesting that the inhibitive effect was IL-35 dependent.

We also verified the in vitro results in the AR mice model. We found that IL-35 reversed the allergic inflammation in Der p1-induced mice with AR, presented as decreased frequency of nasal ILC2 and type II cytokines. At the same time, the expression of IL12R $\beta$ 2 or gp130 by nasal ILC2 was up-regulated. Moreover, the T<sub>H</sub>2-mediated responses and serum IgE were also decreased by IL-35. Consistently, previous studies reported that local administration of IL-35 significantly decreased the expression of OVA-specific IgE, the proliferation of T cells, and the production of type cytokines (IL-4 and IL-5) in mice with AR.<sup>20,21</sup> As for the decreased IgE, IL-35 has been proved to suppress the production of IgE by B cells stimulated with CD40L, IL-4, and IL-21. Suzuki et al<sup>20</sup> also suggested that IL-35 can induce the production of IL-10, which plays important roles in alleviating nasal mucosal allergy. However, the comparison of in vivo inhibitory effects between IL-35 and IL-10 needs further exploration.

This study outlined the role and mechanism of IL-35 and iTr35 in the differentiation and function of ILC2s and the interaction between ILC2 and iTr35. Our results suggested that IL-35 may serve as a promising treatment target in AR.

### Supplementary Data

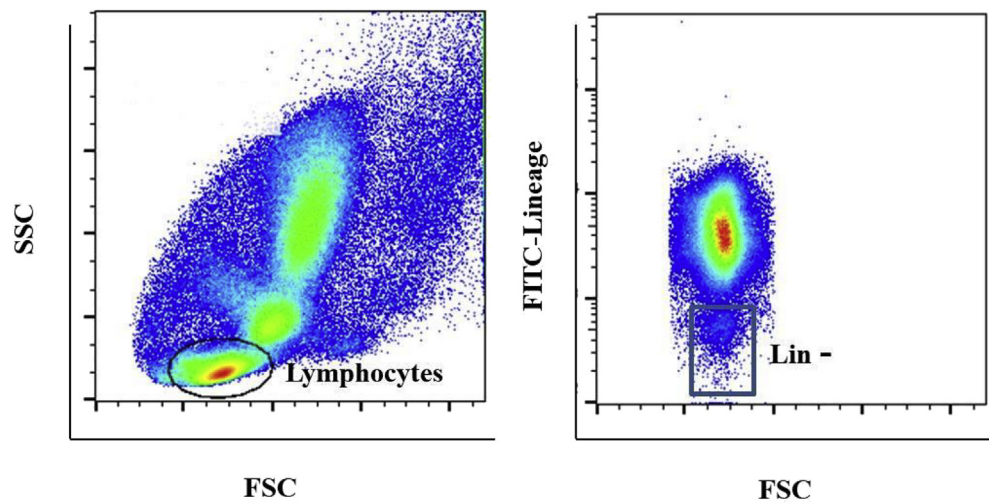
Supplementary data related to this article can be found at <https://doi.org/10.1016/j.anai.2020.08.005>.

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## Supplementary Data



**eFigure 1.** Gating strategy for type II innate lymphoid cells. Lymphocytes were identified from the whole peripheral blood mononuclear cells, and Lin<sup>−</sup> cells were gated. FITC, fluorescein isothiocyanate; FSC, forward scatter; SSC, side scatter.