Role of IL-35 in sublingual allergen immunotherapy

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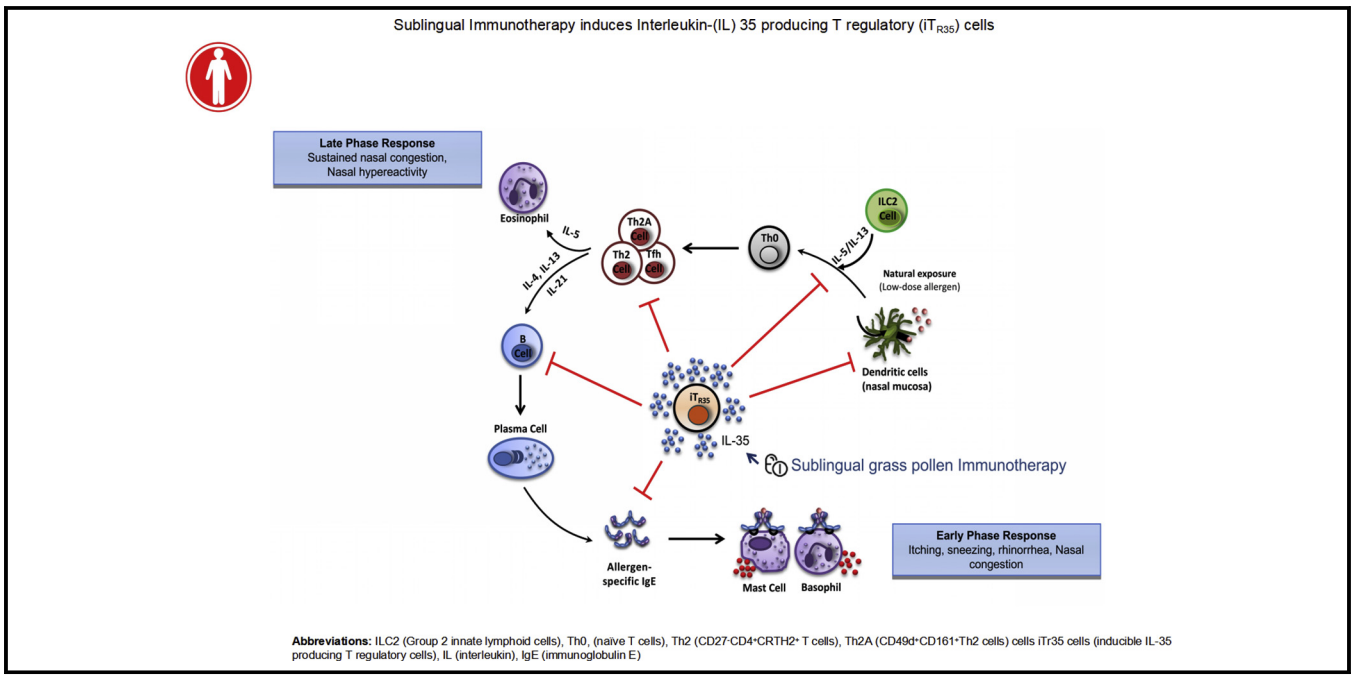
GRAPHICAL ABSTRACT



Background: Grass pollen–specific immunotherapy involves immunomodulation of allergen-specific T_H2 responses and induction of IL-10$^+$ and/or TGF-β$^+$CD4$^+$CD25$^+$ regulatory T cells (induced Treg cells). IL-35$^+$CD4$^+$CD25$^+$ forkhead box protein 3–negative T (IL-35–inducible regulatory T [iT_R35]) cells have been reported as a novel subset of induced Treg cells with modulatory characteristics.

Objective: We sought to investigate mechanisms underlying the induction and maintenance of immunologic tolerance induced by IL-35 and iT_R35 cells.

Methods: The biological effects of IL-35 were assessed on group 2 innate lymphoid cells (ILC2s); dendritic cells primed with thymic stromal lymphopoietin, IL-25, and IL-33; and B and T_H2 cells by using flow cytometry and quantitative RT-PCR. Grass pollen–driven T_H2 cell proliferation and cytokine production were measured by using tritiated thymidine and Luminex MagPix, respectively. iT_R35 cells were quantified in patients with grass pollen allergy (seasonal allergic rhinitis [SAR] group, n = 16), sublingual immunotherapy (SLIT)–treated patients (SLIT group, n = 16), and nonatopic control subjects (NACs; NAC group, n = 16).

Results: The SAR group had increased proportions of ILC2s (P = .002) and IL-5$^+$ cells (P = .042), IL-13$^+$ cells (P = .042), and IL-5$^+$IL-13$^+$ ILC2s (P = .003) compared with NACs. IL-35

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inhibited IL-5 and IL-13 production by ILC2s in the presence of IL-25 or IL-33 ($P = .031$) and allergen-driven T_H2 cytokines by effector T cells. IL-35 inhibited CD40 ligand–, IL-4–, and IL-21–mediated IgE production by B cells ($P = .015$), allergen-driven T-cell proliferation ($P = .001$), and T_H2 cytokine production mediated by primed dendritic cells. iT_R35 cells suppressed T_H2 cell proliferation and cytokine production. In addition, allergen-driven IL-35 levels and iT_R35 cell counts were increased in patients receiving SLIT (all, $P < .001$) and NACs (all, $P < .001$) compared with patients with SAR.
Conclusion: IL-35 and iT_R35 cells are potential novel immune regulators induced by SLIT. The clinical relevance of SLIT can be underscored by restoration of protective iT_R35 cells. (J Allergy Clin Immunol 2019;143:1131-42.)

Key words: *Seasonal allergic rhinitis, sublingual immunotherapy, regulatory T cells, IL-35, IL-35–inducible regulatory T cells*

Abbreviations used

AIT:	Allergen immunotherapy
APC:	Antigen-presenting cell
CD40L:	CD40 ligand
DC:	Dendritic cell
EBI3:	EBV-induced 3
FoxP3:	Forkhead box protein 3
ILC2:	Group 2 innate lymphoid cell
iT_R35:	IL-35–inducible regulatory T
NAC:	Nonatopic control subject
RTSS:	Rhinoconjunctivitis Total Symptom Score
SAR:	Seasonal allergic rhinitis
SLIT:	Sublingual Immunotherapy
STAT:	Signal transducer and activator of transcription
Teff:	Effector T
TSLP:	Thymic stromal lymphopoietin

Allergic rhinitis is a major health epidemic affecting up to 40% of the global population worldwide.[1,2] It is characterized by inflammation of the lining of the nasal mucosa and clinical symptoms, such as runny and itchy nose, sneezing, nasal congestion, and red and watery eyes.[3-5] Symptomatic treatment, such as antihistamines and corticosteroids, are effective in most but not all patients.[6] Allergen immunotherapy (AIT), a disease-modifying treatment, administered as either the sublingual immunotherapy (SLIT) or subcutaneous immunotherapy, is effective in patients who are unresponsive to conventional pharmacotherapy.[6] AIT confers long-term clinical benefits through induction of immune tolerance to the sensitized allergen.[7-9] It has been well established that $CD4^+CD25^-$ effector T (Teff) cells grouped into T_H2 and T_H17 cells contribute toward the pathophysiology of allergic response to the sensitizing allergen.[10] Numbers of regulatory T (Treg) cells, such as forkhead box protein 3 $(FoxP3)^+$ Treg cells, have been shown to be induced after AIT and have the capacity to suppress allergic inflammation by inhibiting the expansion and function of $CD4^+CD25^-$ Teff cells to maintain tolerance to allergens.[11,12]

IL-35 is a newly identified inhibitory cytokine produced by Treg cells that can elicit a suppressive immune response.[13] IL-35 is a member of the IL-12 family of heterodimeric cytokines (IL-12, IL-23, and IL-27) produced mainly by $CD4^+Foxp3^-$ Treg cells, B cells, endothelial cells, smooth muscle cells, and monocytes. The heterodimeric cytokine is composed of an α-chain (p19, p28, or p35) and a β-chain (p40 or EBV-induced 3 [Ebi3]). The p35 subunit pairs with the Ebi3 subunit to form IL-35 and mediate signaling by binding to the IL-12 receptor β2/glycoprotein 130, activating the signal transducer and activator of transcription (STAT) 1 and STAT4 pathway.[14,15] However, in contrast to the IL-12 family involved in the proinflammatory response, IL-35 mediates immunologic functions by suppressing the inflammatory immune response. It has also been reported that IL-35 can significantly reduce airway inflammation induced by allergen-specific T_H2 cells and reverse IL-17–dependent allergic disease in mice.[16,17] Additionally, allergic asthmatic patients have shown reduced IL-35 protein and mRNA levels and reduced frequencies of $CD4^+CD25^+FoxP3$ Treg cells and $CD4^+IL\text{-}12p35^+$ T cells.[18] Additionally, Ebi3-deficient mice have shown an increase in IL-17 production, suggesting that IL-35 expressed by Treg cells regulates the differentiation of $CD4^+$ T cells into T_H17 effector cells.[16,17,19] The biological effect of IL-35 and IL-35–inducible regulatory T (iT_R 35) cells has mostly been investigated in murine models, and limited data are available in human subjects.

Here we demonstrate that production of IL-35 by iT_R35 cells suppresses aberrant type 2 immune responses elicited by group 2 innate lymphoid cells (ILC2s) and T_H2 cells in patients with grass pollen–induced allergic rhinitis. IL-35 inhibited IL-21–induced IgE production from B cells and converted T_H2 cells into suppressive iT_R35 cells. In addition, IL-35, through thymic stromal lymphopoietin (TSLP), also inhibited dendritic cell (DC) priming of T_H2 responses. The clinical relevance is underscored by restoration of protective iT_R35 cells by SLIT. Our findings identify IL-35 and iT_R35 cells as important immune regulators induced by SLIT and provide a rationale for IL-35 therapy for the treatment of respiratory allergic diseases.

METHODS

Subjects

Patients receiving grass pollen SLIT (n = 16; Grazax; ALK-Abelló, Hørsholm, Denmark), untreated allergic subjects with seasonal rhinoconjunctivitis (SAR; n = 16), and nonatopic control subjects (NACs; n = 16) were recruited and completed symptom questionnaires (Table I and see Fig E1 in this article's Online Repository at www.jacionline.org). SLIT-treated patients had been receiving immunotherapy (Grazax tablet, 75,000 SQ-U, once daily) for 12 months to 3 years. Inclusion and exclusion criteria are described in the Methods section in this article's Online Repository at www.jacionline.org). Blood samples from participants (n = 48) were collected during the grass pollen season. The study complied with Good Clinical Practice and was approved by the South West London REC3 Research Ethics Committee and the Research Office of Royal Brompton and Harefield NHS Foundation Trust. Written informed consent was obtained from all participants.

Rhinoconjunctivitis symptom scores

Participants were asked to complete a retrospective symptom questionnaire to assess the severity of their allergic rhinitis symptoms for the relevant pollen season. This included 6 categories of runny nose, blocked nose, itchy nose, sneezing, watery eyes, and itchy eyes. Each category was rated between 0 (no problem) and 3 (severe problems), resulting in a possible total score of 0 to 18 (Rhinoconjunctivitis Total Symptom Score [RTSS]). Participants were asked to evaluate their symptoms when their allergic rhinitis was at its most severe during the pollen season.

TABLE I. Subjects' characteristics

	SLIT group (n = 16)	SAR group (n = 16)	NAC group (n = 16)
Sex (male/female)	6/10	7/9	7/9
Age (y), mean (range)	38 (20-56)	37 (21-51)	36 (20-55)
Grass pollen–specific IgE (kU/L), mean ± SD	42.2 ± 14.2	36.5 ± 30.2	0.0 ± 0.0*
Total IgE (kU/L), mean ± SD	352.7 ± 421.0	489.2 ± 479.5	29.8.0 ± 16.22*
Monosensitized	2/16	0/16	0/16
Polysensitized	14/16	16/16	0/16
HDM	5/16	3/16	0/16
Cat	3/16	2/16	0/16
Birch	9/16	9/16	0/16

Distribution of age, sex, specific IgE levels, and sensitization profile.
*$P < .01$, comparison between the NAC and SAR groups. There were no significant differences for any other parameters among the 3 groups.

Methods for PBMC isolation, mRNA extraction, cDNA, and quantitative RT-PCR are described in the Methods section in this article's Online Repository.

Phenotyping of ILC2s and effect of IL-35 on ILC2s

IL-5$^+$, IL-13$^+$, and dual IL-5$^+$IL-13$^+$ ILC2s were quantified from PBMCs obtained from patients with SAR and NAC participants. Lineage-negative cells comprised of ILC2s were enriched from PBMCs by using a lineage cell depletion kit (Miltenyi Biotec, Surrey, United Kingdom). ILC2s (Lin$^-$CD127$^+$CRTH2$^+$) were enriched by using the BD FACSAria III (Becton Dickinson, Oxford, United Kingdom) maintained in medium containing rhIL-2 (10 ng/mL; PeproTech, London, United Kingdom) and rhIL-7 (40 ng/mL; R&D Systems, Abingdon, United Kingdom). The effect of rhIL-35 (Enzo Life Sciences, Farmingdale, NY) on sorted ILC2s was studied over 72 hours when stimulated with rhIL-25 (50 ng/mL) or rhIL-33 (50 ng/mL; both from R&D Systems). Supernatants were then collected to assess IL-5 and IL-13 production. Further details can be found in the Methods section in this article's Online Repository.

Effect of IL-35 on Teff cells

CD4$^+$CD25$^-$ Teff cells were negatively selected and purified from PBMCs by using magnetic separation (STEMCELL Technologies, Cambridge, United Kingdom) and cultured in a 1:1 ratio with irradiated antigen-presenting cells (APCs) in the presence of *Phleum pratense* allergen (5 μg/mL) and varying concentrations of IL-35 or C-peptide linker (Enzo Life Sciences, Exeter, United Kingdom). Monoclonal antibodies against IL-35 (B-P35; 2BScientific, Hayford, United Kingdom) were used to neutralize suppression. Cells were cultured for 6 days at 37°C in a 5% CO_2 atmosphere, and rate of proliferation was assessed through incorporation of tritiated thymidine, whereas supernatants were collected to measure production of T_H1 and T_H2 cytokines. Additionally, the effect of IL-35 was assessed on T_H1 and T_H2 clones. Further details can be found in the Methods section in this article's Online Repository.

Generation of iT$_R$35 cells

Naive T cells were isolated from PBMCs by using EasySep magnetic separation (STEMCELL Technologies) and stimulated with anti-CD3 and anti-CD28 (both at 1 μg/mL; R&D Systems) in the presence of 10 ng/mL IL-35 for 9 days at 37°C in a 5% CO_2 atmosphere to generate the iT$_R$35 cell lineage.[20] iT$_R$35 cells were cocultured at a 1:1:1 ratio with CD4$^+$ (or memory) T cells and irradiated APCs in the presence of 3 μg/mL P pratense for 7 days at 37°C in a 5% CO_2 atmosphere. Where indicated, the neutralizing antibodies anti–IL-10 (10 μg/mL; BioLegend, London, United Kingdom), anti–TGF-β (10 μg/mL; R&D Systems), and anti–IL-35 (10 μg/mL) were added to the culture. Proliferation of memory T cells was assessed through incorporation of tritiated thymidine. T_H2 cytokine levels were measured in cell-culture supernatants by using the MagPix Milliplex Kit (EMD Millipore, Watford, United Kingdom) based on the manufacturer's protocol.

Expression of STATs induced by IL-35

CD4$^+$CD25$^-$ T cells were stimulated with IL-35. Cells were harvested, fixed in 2% formaldehyde, and permeabilized in 90% methanol for 30 minutes. Cells were then stained and labeled with anti−phospho-STAT1 and STAT4,[21] according to the manufacturer's protocol (BioLegend).

Suppression of IgE by IL-35 in PBMCs of allergic patients

B cells were isolated from PBMCs by using negative isolation (Dynal Biotech, Oslo, Norway). Cells (0.5-50 × 10^3/0.2 mL) were cultured with CD40 ligand (CD40L; 100 ng/mL) alone or with IL-4 (100 U/mL; both from R&D Systems) or IL-4 plus IL-21 (100 ng/mL; BioLegend). Cells were harvested after 14 days and cultured again (10-20 × 10^3/0.2 mL) for 2 days with CD40L or CD40L plus IL-4. IgE was detected with ImmunoCAP, according to the manufacturer's protocol. IL-35 (10 ng/mL) was used to determine suppression of IgE production.

DCs: Naive T-cell coculture assays

DCs (5 × 10^3 cells/well) were primed with TSLP (15 ng/mL), IL-25 (10 ng/mL), and IL-33 (10 ng/mL) for 24 hours. Primed DCs were then washed and cocultured with naive T cells (5 × 10^4 cells/well) and *P pratense* (5 μg/mL) in the presence or absence of IL-35 (10 ng/mL) at 37°C in a 5% CO_2 atmosphere. Cell-culture supernatants were collected on day 9, and grass pollen−driven T-cell proliferation was measured by determining tritiated thymidine incorporation (MP Biomedicals, Costa Mesa, Calif).

iT$_R$35 cell and IL-35 production in patients receiving SLIT, patients with SAR, and NACs

PBMCs from the SLIT, SAR, and NAC groups were stimulated with phorbol 12-myristate 13-acetate/ionomycin for 6 hours, permeabilized, and subjected to intracellular staining with anti-human IL-12p35 and anti-human EBI3 antibodies. Dual IL-12p35$^+$ and EBI3$^+$ (IL-35) CD4$^+$ T cells were quantified by means of flow cytometry to allow quantification of iT$_R$35 cells (IL-35$^+$CD4$^+$CD25$^+$). Furthermore, PBMCs from patients receiving SLIT, patients with SAR, and NACs were stimulated with grass pollen allergen for 6 days. IL-35 levels in cell-culture supernatants were measured by using ELISA (Cusabio Biotech, Wuhan, China), according to the manufacturer's instructions.[22,23]

Total and specific IgE

Total and timothy grass−specific IgE levels were quantified by using the ImmunoCAP FEIA system, according to the manufacturer's recommendations (Pharmacia, Uppsala, Sweden).

Statistics

Between-group comparisons were performed by using the Mann-Whitney *U* test, and within-group comparisons were performed by using the Wilcoxon

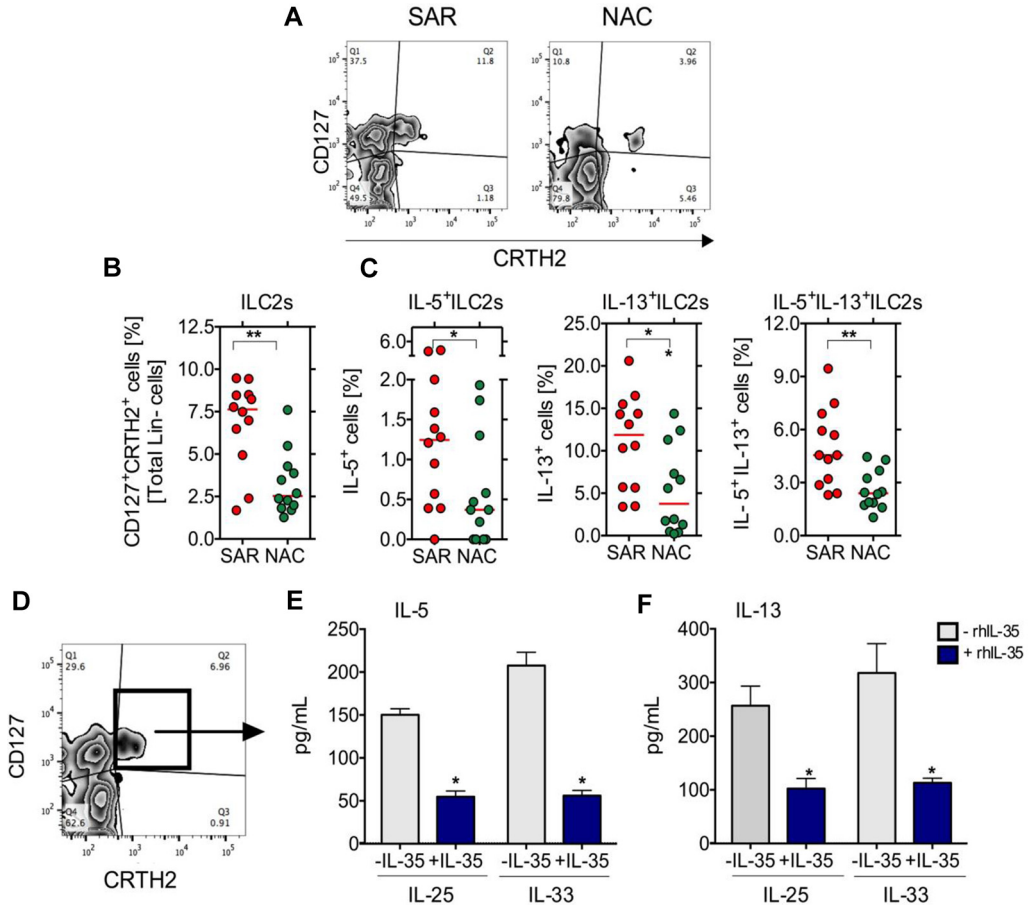

FIG 1. IL-35 suppresses proallergic cytokine (IL-5 and IL-13) production by ILC2s after IL-25 and IL-33 *ex vivo* stimulation. **A** and **B,** Proportions of $Lin^-CD127^+CRTH2^+$ cells (ILC2s) were quantified in PBMCs obtained from patients with grass pollen allergy (SAR group, n = 12) and NACs (n = 12). **C,** $IL\text{-}5^+$, $IL\text{-}13^+$, and $IL\text{-}5^+IL\text{-}13^+$ ILC2s were also quantified in both groups. **D-F,** ILC2s were sorted by means of fluorescence-activated cell sorting and stimulated with IL-25 and IL-33 in the presence/absence of IL-35. IL-5 and IL-13 levels are shown. *P* values were determined by using the Mann-Whitney *U* test or Wilcoxon matched-pairs signed-rank test: **P* < .05 and ***P* < .001.

matched-pairs signed-rank test. Correlation analysis was determined by using the Spearman rank method. The statistical software package used was GraphPad Prism (version 5; GraphPad Software, La Jolla, Calif). A *P* value of less than .05 was considered significant.

RESULTS

Enumeration of ILC2s in patients with grass pollen allergy and NACs

To examine the biological effect of IL-35[13,15,20] on ILC2s ($Lin^-CD127^+CD294^+$), which have previously been shown to have a role in type II allergic inflammation,[24-26] we enumerated ILC2s in peripheral blood of patients with moderate-to-severe SAR. During the pollen season, proportions of ILC2s were greater in patients with SAR compared with those in NACs ($P = .002$; Fig 1, *A* and *B*). Similarly, $IL\text{-}5^+$, $IL\text{-}13^+$, and dual $IL\text{-}5^+IL\text{-}13^+$ ILC2 proportions were increased in patients with SAR compared with NACs ($P = .042$, $P = .042$, and $P = .003$; Fig 1, *C*), supporting a pathophysiologic role for ILC2s in patients with SAR during natural grass pollen allergen exposure. We then assessed whether IL-35 would inhibit IL-5 and IL-13 production by ILC2s. IL-5 and IL-13 production by ILC2s was significantly reduced by IL-35 (all $P = .031$; Fig 1, *E* and *F*).

IL-35 suppressed, allergen-driven Teff cell proliferation

The effect of IL-35 on allergen-specific memory/effector T_H2 cells in allergic patients with SAR was investigated. Teff cells ($CD4^+CD25^-$ cells) were cocultured with APCs and stimulated with grass pollen allergen *(P pratense)* in the presence of IL-35. Allergen-driven Teff cell proliferation was suppressed in an IL-35 dose-dependent manner (optimal at 10 ng/mL, $P = .0001$) but not when exposed to blocking anti–IL-35 mAbs (Fig 2, *A*). Allergen-specific T cells produce IL-4, IL-5, IL-13 (T_H2 cytokines), and IL-17 (T_H17 cytokine), which contribute to allergic rhinitis.[27] IL-35 suppressed allergen-driven production of IL-4 ($P < .0001$), IL-5 ($P < .0001$), IL-9 ($P < .0001$), IL-13 ($P < .009$), and IL-17 ($P < .0001$) by Teff cells (Fig 2, *B*). Interestingly, IL-35 induced expression of IFN-γ ($P = .004$) and IL-10 ($P < .0002$) by Teff cells (Fig 2, *C*). The effects of IL-35 were specific because the C-peptide linker used for its

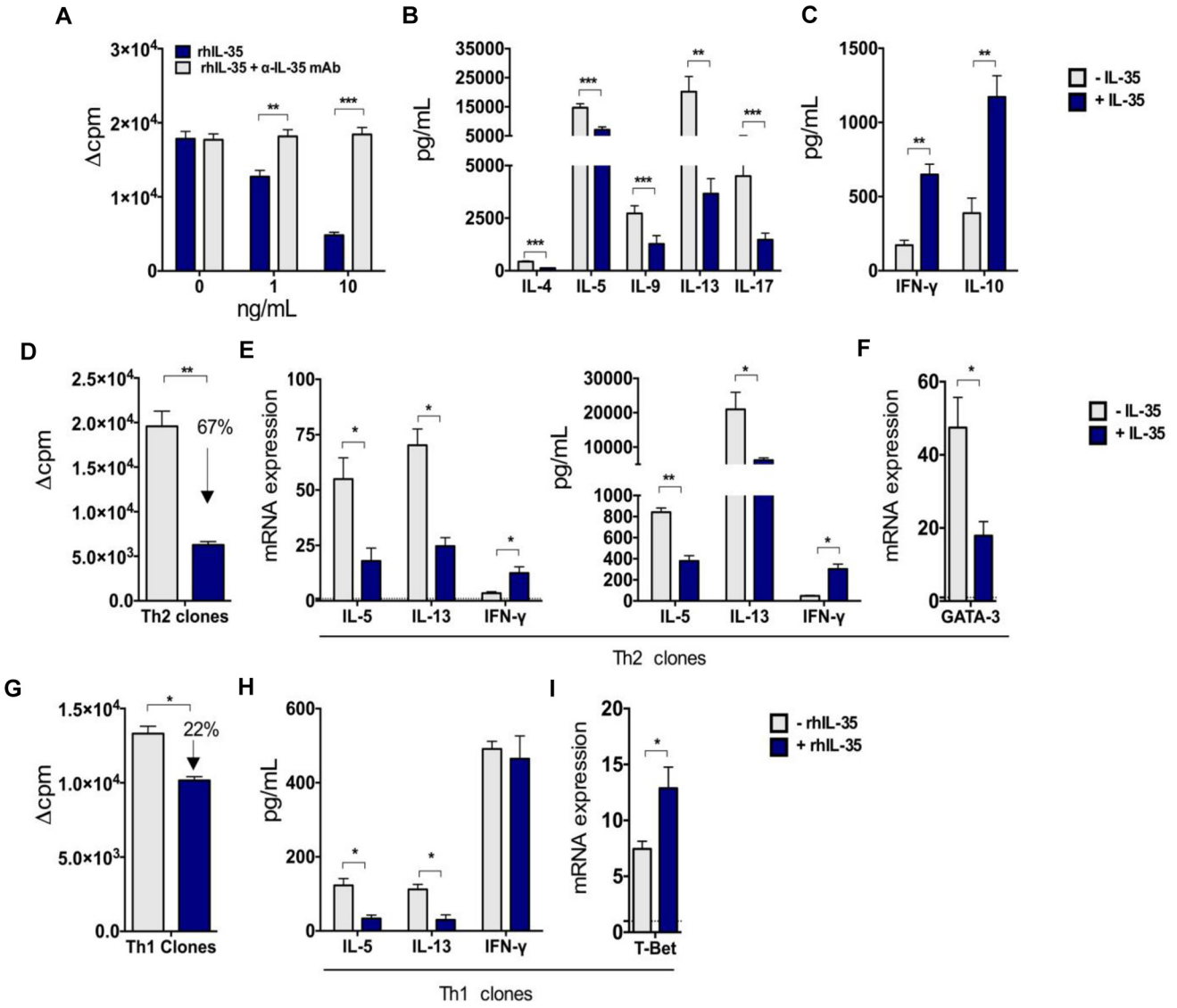

FIG 2. IL-35 suppresses grass pollen allergen–driven T_H2 effector cell proliferation and cytokine responses. **A,** $CD4^+CD25^-$ T cells were cocultured with APCs and stimulated with *P pratense* allergen in the presence of IL-35. Proliferation was assessed by using tritiated thymidine incorporation. **B** and **C,** Effect of IL-35 on allergen-driven production of T_H2 cytokines (Fig 2, *B*) and T_H1 cytokines and IL-10 (Fig 2, *C*). **D-F,** Effects of IL-35 were also assessed on T_H2 clone proliferation (Fig 2, *D*); *IL5, IL13,* and *IFNG* mRNA expression (Fig 2, *E*), and *GATA3* expression (Fig 2, *F*). **G-I,** Effects of IL-35 on T_H1 clone proliferation (Fig 2, *G*), cytokine production (Fig 2, *H*), and *T-bet* mRNA expression (Fig 2, *I*) were assessed. *P* values were determined by using the Wilcoxon matched-pairs signed-rank test: $*P < .05$, $**P < .001$, and $***P < .0001$.

expression had no effect on either Teff cell proliferation or cytokine production (see Fig E2 in this article's Online Repository at www.jacionline.org).

In addition to this, the effect of IL-35 on *in vitro*–generated T_H1 and T_H2 clones was also investigated.[27] IL-35 significantly reduced T_H2 clone proliferation ($P = .0001$; Fig 2, *D*), as well as IL-5 ($P = .0001$) and IL-13 ($P < .02$) gene expression (Fig 2, *E*). T_H2 transcription factor *GATA3* mRNA expression was decreased by IL-35 ($P = .039$; Fig 2, *F*). Proliferation of T_H1 clones was also suppressed, although to a lesser magnitude ($P = .003$; Fig 2, *G*). However, in the presence of IL-35, level of IFN-γ production by T_H1 clones was unaffected, whereas *T-bet* mRNA expression was upregulated ($P = .041$; Fig 2, *G-I*).

IL-35–treated Teff cells inhibit grass pollen–driven T_H2 effector cell responses

Prior treatment of Teff cells with IL-35 imparted a suppressive phenotype in coculture experiments with untreated allergen-specific Teff cells (Fig 3). This suppression was optimal at 2:1 (IL-35–treated Teff cells/untreated Teff cells) and still persisted at 64:1. Anti–IL-35–neutralizing mAb reversed this inhibition in a dose-dependent manner (Fig 3, *A*). Furthermore, IL-35–treated Teff cells suppressed allergen-induced IL-4 ($P = .0004$), IL-5 ($P = .0004$), IL-13 ($P = .0001$), and IL-17 ($P = .0004$) production by Teff cells, which was also reversed by anti–IL-35–neutralizing antibody (Fig 3, *B*).

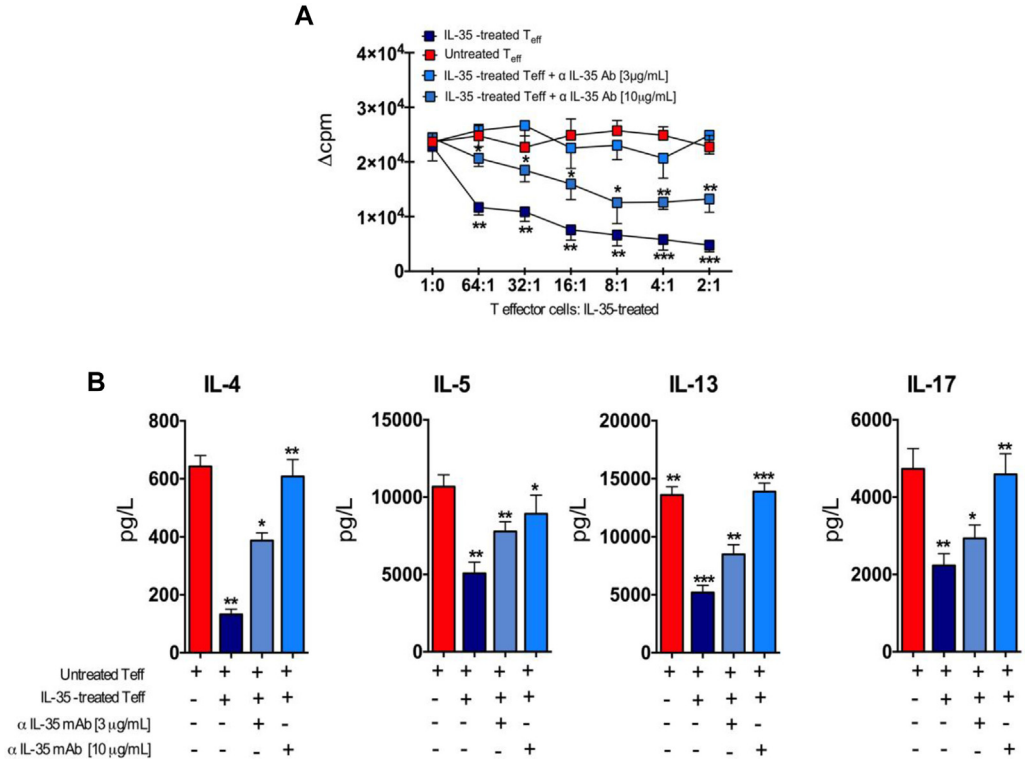

FIG 3. IL-35–treated Teff cells inhibit grass pollen–driven T_H2 effector cell responses, and this is reversed by neutralizing anti–IL-35. $CD4^+CD25^-$ T cells were enriched from PBMCs obtained from patients with grass pollen allergy (n = 12) and treated with IL-35 or control for 9 days. IL-35^- or control-treated T cells were cocultured with autologous $CD4^+CD25^-$ T cells or APCs in the presence of *P pratense* allergen supplemented with neutralizing anti–IL-35 antibody. Allergen-driven T_H2 cell proliferation **(A)** and IL-4, IL-5, IL-13, and IL-17 responses **(B)** were measured. *P* values were determined by using the Wilcoxon matched-pairs signed-rank test: $*P < .05$, $**P < .001$, and $***P < .0001$.

IL-35 induced generation of iT_R35 cells with the ability to suppress T_H2 cytokine production

Murine studies have shown that IL-35 can convert Teff cells into iT_R35 cells.[13,20,27,28] Therefore we assessed whether human IL-35 could polarize human $CD4^+CD45RA^+$ (naive) T cells into iT_R35 cells. Naive T cells obtained from patients with SAR were exposed to IL-35 and stimulated with anti-CD3/CD28 mAbs for 9 days. This resulted in induction of *IL12A* ($P = .0005$) and *Ebi3* ($P = .0005$) gene expression (Fig 4, *A*) and production of iT_R35 cells ($P = .0005$; Fig 4, *B*). These iT_R35 cells suppressed allergen-driven $CD4^+CD45RO^+$ memory T cells in an IL-35– and IL-10–dependent manner (Fig 4, *C* and *D*). The iT_R35 cells also suppressed IL-4 ($P = .004$), IL-5 ($P = .004$), and IL-13 ($P = .001$) production by memory T_H2 cells (Fig 4, *E*). Furthermore, we confirmed that IL-35 exerted its effects on human Teff cells by signaling through STAT1 and STAT4 after T-cell receptor stimulation (Fig 4, *F*).

IL-35 inhibits production of allergen-specific IgE by B cells

T_H2 cytokines drive isotype class-switching of B cells toward IgE antibody production.[29-32] IL-21 and IL-4 act synergistically on B cells to facilitate IgE secretion ($P = .002$; Fig 5, *A* and *B*),[33,34] which binds to FcεRI on mast cells and basophils in the nasal mucosa. Subsequent cross-linking by allergen results in release of the histamine and lipid mediators that are responsible for allergy symptoms.[32]

We investigated whether IL-35 can counteract the effects of IL-4 and IL-21 and inhibit IgE production by B cells. IL-35 suppressed IgE production by allergen-specific PBMCs after costimulation with CD40L, IL-4, and IL-21 ($P = .015$; Fig 5, *C*). This suppression was reversed by neutralizing IL-35 antibodies in a dose-dependent manner ($P = .031$; Fig 5, *C*). Therefore, in addition to exerting effects on T_H2 responses, IL-35 inhibited allergen-specific IgE production.

IL-35 inhibits TSLP-induced naive T-cell activation and differentiation into T_H2 cells

Recent evidence has implicated TSLP in the DC priming of T_H2 responses in asthmatic patients.[35] On stimulation with *P pratense*, TSLP-primed DCs were able to induce significantly greater proliferation of naive T cells when compared with non–TSLP-primed DCs ($P = .001$; Fig 6, *A*). In contrast to this, IL-25– and IL-33–primed DCs did not affect the proliferation of naive T cells (Fig 6, *A*). The increase in TSLP-induced, allergen-driven naive T-cell proliferative response was accompanied by an increase in IL-5 ($P = .004$; Fig 6, *B*) and IL-13 ($P = .004$; Fig 6, *C*) production. In parallel to this, the effect of IL-35 on TSLP priming of specific T_H2 cells by DCs from patients with grass pollen allergy was investigated.

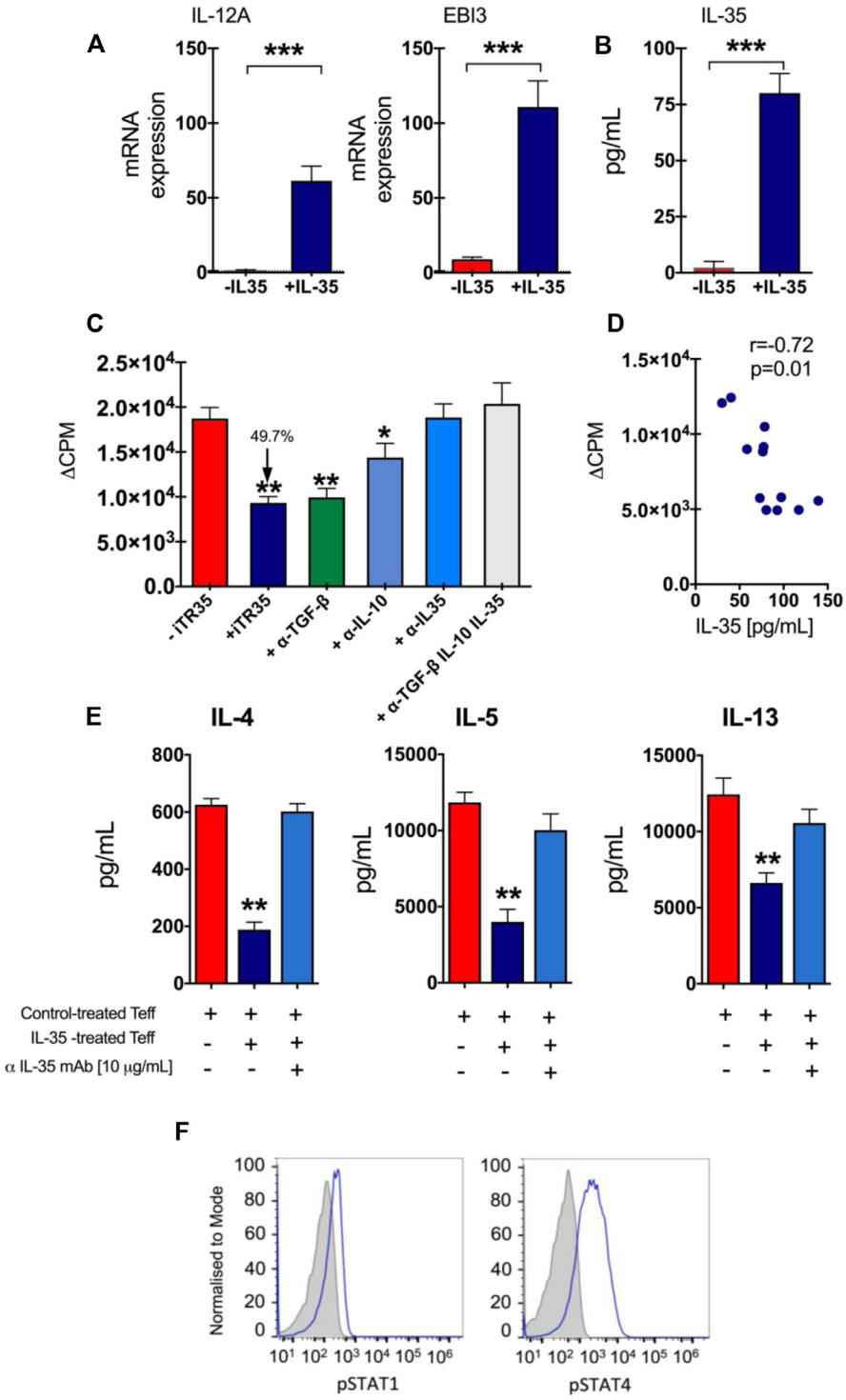

FIG 4. IL-35 polarizes naive T cells into the iT_R35 phenotype. **A,** Naive and memory T cells were enriched from PBMCs of patients with grass pollen allergy (n = 12). Naive T cells were stimulated with CD3/CD28 and treated with IL-35 to allow generation of iT_R35 cells. mRNA expression of *IL12A* and *EBI3* were confirmed in iT_R35 and control cells. **B,** IL-35 protein levels were quantified in iT_R35 and control cells. **C,** Allergen-driven memory T_H2 cell proliferation was assessed. **D,** Relationship between suppression of proliferation and IL-35 levels. **E,** Effects of iT_R35 and control cells on memory T_H2 cytokine responses. **F,** IL-35 exerts its action on Teff cells through STAT1 and STAT4 signaling. *P* values were determined by using the Wilcoxon matched-pairs signed-rank test: $*P < .05$, $**P < .001$, and $***P < .0001$.

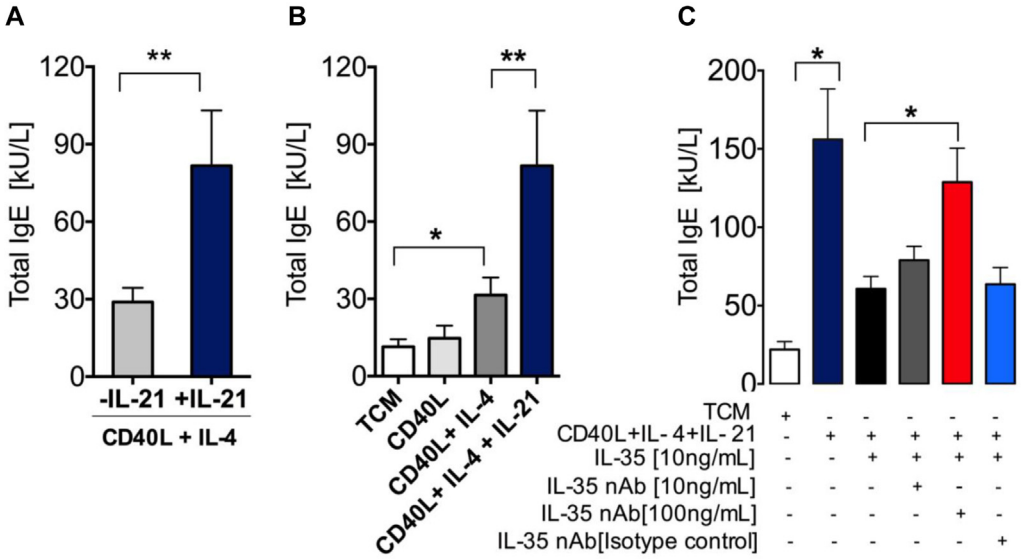

FIG 5. IL-35 suppresses CD40L/IL-4/IL-21–driven IgE production in PBMCs from patients with grass pollen allergy. **A,** Total IgE production from B cells in the presence or absence of IL-4, CD40L, and IL-21 was measured in cell-culture supernatants. **B,** Total IgE production by B cells with or without IL-4, CD40L, and IL-21. **C,** Effect of CD40L, IL-4, and IL-21 on IgE production in the presence of IL-35–neutralizing antibodies. *P* values were determined by using the Wilcoxon matched-pairs signed-rank test: $*P < .05$ and $**P < .001$. *nAb,* Neutralizing antibody; *TCM,* tissue culture medium.

TSLP-primed DCs were cocultured with naive T cells for 6 days in the presence or absence of IL-35. IL-35 inhibited naive T-cell proliferation ($P = .001$; Fig 6, *D*) and T_H2 cytokine production: IL-5 ($P = .008$; Fig 6, *E*) and IL-13 ($P = .003$; Fig 6, *F*). Therefore IL-35 inhibits the TSLP-driven DC priming of naive T cells for differentiation into T_H2 cells.

Enumeration of iT_R35 cells in patients receiving AIT, patients with SAR, and NACs

SLIT-treated subjects had significantly reduced rhinitis symptom scores compared with patients with SAR (Fig 7, *A*). We measured numbers of peripheral blood iT_R35 cells in patients who had received a course of SLIT for severe allergic rhinitis (Fig 7, *B* and *C*). SLIT-treated patients had increased numbers of iT_R35 cells ($P < .001$) compared with patients with untreated SAR, and no difference was observed when compared with NACs (Fig 7, *B* and *C*). *Ex vivo* stimulation revealed increased levels of IL-35 from SLIT-treated patients ($P < .001$) and NACs ($P < .001$) compared with patients with untreated SAR (Fig 7, *D*). A positive correlation between IL-35 levels and proportion of IL-35^+ Treg cells was observed (Fig 7, *E*). For all participants, both the proportion of IL-35^+ Treg cells and the levels of IL-35 in culture supernatants inversely correlated with clinical symptoms (Fig 7, *F* and *G*) and for IL-35; this was also significant within the SLIT-treated group alone.

DISCUSSION

For the first time, we report that IL-35 production by iT_R35 cells can suppress proallergic type 2 immune responses, such as IL-5 and IL-13 production by ILC2s and IL-4, IL-5, IL-9, and IL-13 production by T_H2 cells, in patients with grass pollen allergy. IL-35 induced production of IL-10 and a T_H1 response (IFN-γ). We also observed that IL-35 significantly reduced proliferation of T_H2 clones and downregulated mRNA expression of *IL5* and *IL13*. This reduction of IL-5 and IL-13 was also confirmed at the protein level. T_H1 clone proliferation was also significantly reduced, although to a smaller extent (ie, 22%), when compared with T_H2 clone proliferation. However, IL-35 treatment had no significant effect on *IFNG* expression at the mRNA level. Interestingly, IL-35–treated Teff cells suppressed allergen-induced production of IL-4, IL-5, IL-9, IL-13, and IL-17, which was lost in the presence of anti–IL-35–neutralizing antibody. Most importantly, IL-35 suppressed production of IgE by B cells stimulated with CD40L, IL-4, and IL-21 and converted T_H2 cells into suppressive iT_R35 cells. IL-35 also suppressed differentiation of TSLP-driven DC priming of naive T cells into T_H2 cells and inhibited allergen-induced IL-5 and IL-13 cytokine production. These findings collectively highlight the protective roles of IL-35 and iT_R35 cells.

Several studies have shown that ILC2s primed with epithelium-derived mediators, such as IL-33,[36] IL-25,[37] TSLP,[38] and leukotriene D_4, induce type 2 allergic inflammation and tissue repair.[39] The involvement of ILC2s in the pathogenesis of allergic rhinitis was first demonstrated in subjects undergoing intranasal cat allergen provocation, which led to an increased percentage of ILC2s after 4 hours.[40] Furthermore, numbers of $CD117^+$ and $IL\text{-}13^+$ ILC2s in peripheral blood of patients with grass pollen allergy were increased during the grass pollen season compared with numbers out of the pollen season. Interestingly, out of the grass pollen season, no significant changes were observed between levels of $IL\text{-}13^+$ ILC2s in patients with grass pollen allergy and NACs.[41] These reports further confirm that ILC2s are involved in production of type 2 response in allergic patients.

Consistent with previous findings,[41] we observed that proportions of $IL\text{-}5^+$, $IL\text{-}13^+$, and dual $IL\text{-}5^+IL\text{-}13^+$ ILC2s were increased in patients with SAR during the grass pollen season compared with those in NACs. Although these differences were significant, we observed heterogeneous ILC2 responses

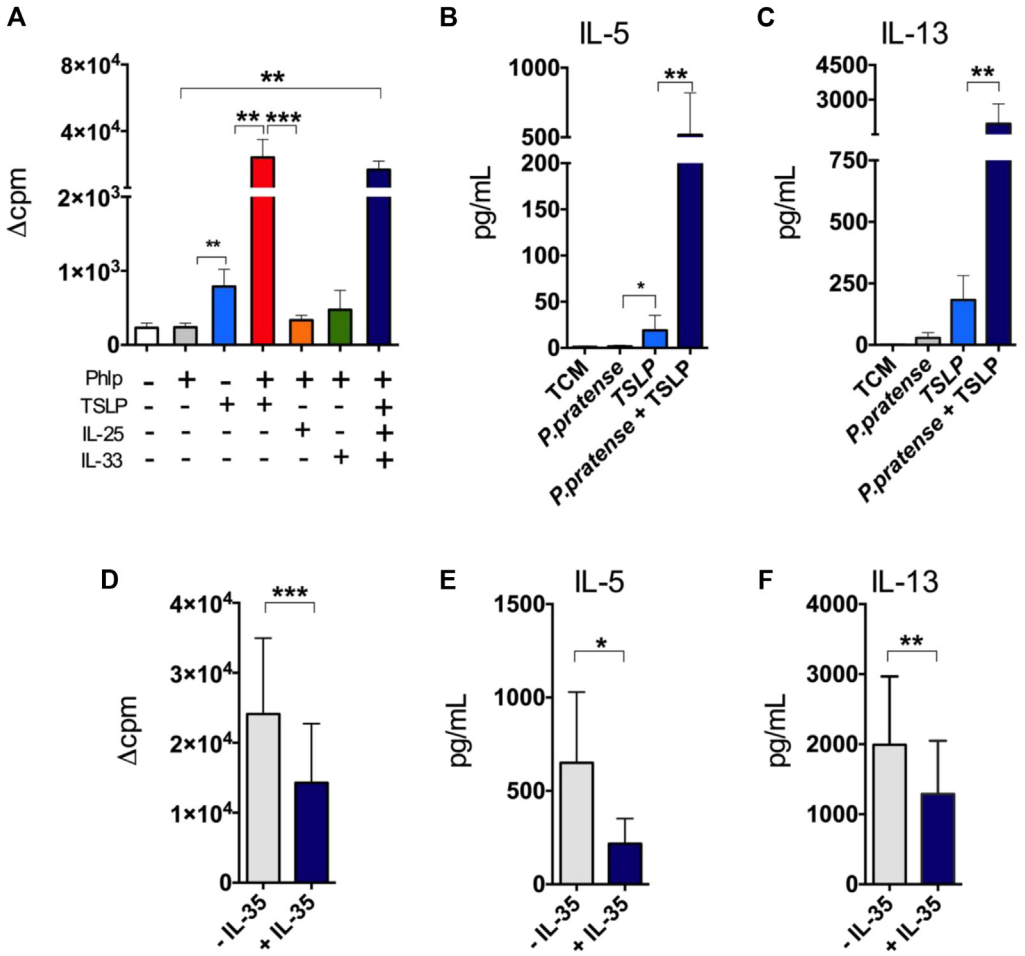

FIG 6. Effect of IL-35 on proliferation and cytokine production. DCs and naive T cells were obtained from PBMCs of patients with allergic rhinitis. **A,** TSLP-, IL-25–, and IL-33–primed DCs were challenged with or without allergen to determine T-cell proliferation. **B** and **C,** Allergen-driven IL-5 (Fig 6, *B*) and IL-13 (Fig 6, *C*) secretion was measured in the presence or absence of TSLP. **D-F,** Suppressive effect of IL-35 on naive T-cell proliferation (Fig 6, *D*) and T_H2-driven cytokine production of IL-5 (Fig 6, *E*) and IL-13 (Fig 6, *F*). *P* values were determined by using the Wilcoxon matched-pairs signed-rank test: $*P < .05$, $**P < .001$, and $***P < .0001$. *TCM*, Tissue culture medium.

caused by donor-to-donor variation. More importantly, to address the factors involved in inhibiting type 2 responses, we show that IL-35 suppressed production of IL-5 and IL-13 cytokines by ILC2s primed with IL-25 or IL-33. This highlights the ability of IL-35 to suppress proallergic type II immune responses mediated by ILC2s. We measured the innate responses in peripheral blood as a surrogate of local mucosal response. Biopsy specimens from patients with SAR and NACs were not collected because this was beyond the scope of this particular study. However, it would be relevant to evaluate the effect IL-35 on ILC2s from nasal biopsy specimens.

This study shows that IL-35 polarizes naive T cells into iT_R35 cells in patients with SAR, which is consistent with a previously described murine study.[20] The iT_R35 cells also significantly suppressed the allergen-driven IL-4, IL-5, and IL-13 production by T_H2 cells. These suppressions were found to be IL-35 and IL-10 dependent. However, another study showed that naive T cells treated with IL-35 produce Treg cells that induce suppression exclusively through IL-35 and not through other inhibitory factors, such as TGF-β and IL-10.[20] This suggests that IL-35 contributes to immunologic tolerance by inducing iT_R35 cells after natural allergen exposure. With these observations, we further investigated the signaling pathways required for polarization of T cells with IL-35. Here we confirmed through flow cytometry that IL-35 signals through STAT1 and STAT4 after T-cell receptor stimulation, which corroborates with a previous study.[28] Further studies showed that a deficiency in one chain of the IL-35 receptor resulted in activation of only one of the STAT proteins.[28] It has been speculated that IL-12 and IFN-γ, as well as IL-35, stimulate STAT1 and STAT4 to drive the T_H1 response.[28]

T_H2 cells produce the conventional cytokines IL-4, IL-5, and IL-13, which are essentially responsible for induction of effector cells and induction of B cells to produce allergen-specific IgE.[42] Interestingly, IL-35 suppressed total IgE production by B cells when stimulated with CD40L, IL-4, and IL-21. However, neutralizing IL-35 antibody was able to reverse the amount of IgE-producing B cells, which can facilitate the classical T_H2 allergic immune response.[42] This study also showed that IL-4 and IL-21 act synergistically to promote production of IgE by

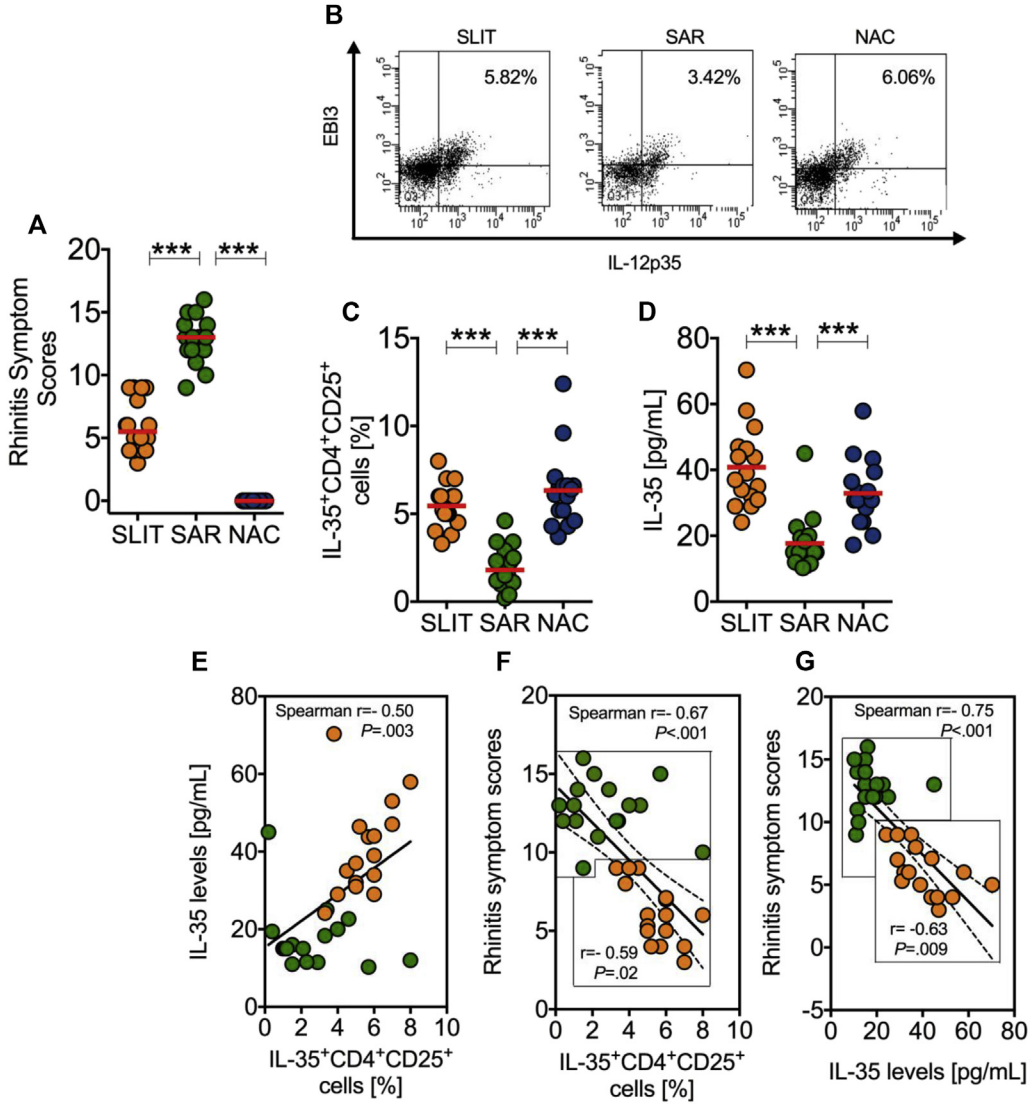

FIG 7. IL-35 levels and iT_R35 cell counts were increased in the SLIT-treated group and NACs but not the SAR group. **A,** RTSSs were evaluated in the SLIT (n = 16), SAR (n = 16), and NAC (n = 16) groups. **B** and **C,** iT_R35 cells ($IL\text{-}35^+CD4^+CD25^+$) were quantified in PBMCs obtained from each group by using flow cytometry. **D,** IL-35 levels were measured in supernatants of $CD4^+$ T cells cocultured with APCs and *P pratense*. **E,** Correlation between IL-35 levels and iT_R35 cell counts. **F** and **G,** RTSSs correlated with IL-35 levels and iT_R35 counts. *P* values were determined by using the Mann-Whitney *U* test: ***$P < .0001$. *Symbols* represent individual patients, and *horizontal bars* represent means. Correlation was determined by using the Spearman rank method.

B cells. Therefore it is likely that IL-35 can attenuate the allergic rhinitis response through the decrease in IgE levels and the aberrant type 2 immune responses by ILC2s and T_H2 cells.

Natural allergen-exposed epithelia release cytokines and alarmins, such as TSLP, IL-25, and IL-33, which promote ILC2s or DCs to polarize toward a T_H2 response.[43,44] However, during AIT, DCs polarize toward an IL-10–producing Treg cell response. In particular, TSLP in patients with allergic inflammation is understood to drive DCs to promote allergen-specific naive T cells to proliferate and differentiate into T_H2 cells.[45,46] Indeed, we have shown that IL-35 suppressed TSLP-driven DC priming of naive T-cell proliferation and differentiation into T_H2 cells, resulting in inhibition of allergen-induced IL-5 and IL-13 production. This suggests that IL-35 might be a potential therapeutic target for seasonal allergic rhinitis immunotherapy.

Furthermore, SLIT-treated patients also had significantly reduced RTSSs. Numbers of IL-35 and iT_R35 ($IL\text{-}35^+CD4^+CD25^+$) cells were greater in the SLIT-treated group compared with those in the SAR and NAC groups. RTSSs and IL-35 levels or iT_R35 cell counts correlated inversely, highlighting their protective role toward allergic rhinitis symptoms and therefore decreasing the need for taking medication and possibly improving quality of life. In this study all subjects who received grass pollen SLIT had reduced RTSSs and were all responders. There were not any nonresponders. However, at the population level, there was a clear correlation between RTSSs and proportions of iT_R35 cells ($r = -0.59$, $P = .02$) or IL-35 levels ($r = -0.63$, $P = .009$) in the grass SLIT–treated group. These findings indicate the potential use of iT_R35 cells and IL-35 levels as an immune-monitoring marker

of the effect of SLIT. However, this warrants validation in a large study, in which there would be a high likelihood of comparing nonresponders and responders in the context of efficacy (RTSS) and improvement of quality of life.

IL-35 has been investigated in murine models of autoimmunity[13,20,47] and allergic airway inflammation.[48] Although these studies provide useful insight into the biological effect of IL-35 and iT_R35, the relevance of mouse models of lung allergic inflammation to human respiratory disease remains to be explored. Here we showed that IL-35 suppresses allergen-specific T_H2 inflammatory responses in human upper airway allergic disease. IL-35 and iT_R35 cells can be immune-monitoring markers of AIT. Furthermore, our data showed that iT_R35 cells were dysregulated in patients with SAR and that treatment with sublingual immunotherapy was associated with recovery of iT_R35 cells to numbers comparable with those observed in NACs. These findings open new avenues for developing adjunct treatment in which IL-35 could be administered either alone for its antiallergic effects or in combination with SLIT to enhance efficacy and promote durable allergen tolerance.

Key messages

- **Production of IL-35 by iT_R35 cells mediates suppression of type II immune response induced by ILC2s and T_H2 cells in patients with grass pollen allergy (SAR).**
- **Levels of IL-35 and numbers of iT_R35 cells were increased in the SLIT-treated group and NACs when compared with those in patients with grass pollen allergy.**

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METHODS

Subjects: Inclusion and exclusion criteria

All allergic participants were skin prick test positive (wheal of >3mm) to *Phleum pratense* (Phl p) and suffered from moderate-severe grass-pollen induced symptoms for at least two years. Anti-histamine tablets and nasal sprays were excluded at least 3 days before sample collection, while topical corticosteroids were excluded for 14 days. Nonatopic control had no history of allergic symptoms and had a negative skin prick test to Phl p and to a panel of eleven common aeroallergens. Participants were excluded if they had a clinical history of other allergic diseases, asthma, chronic-obstructive pulmonary disease (COPD), recurrent acute sinusitis, chronic sinusitis, or other significant illness.

Isolation of human PBMCs

PBMCs were isolated from fresh heparinized venous blood via centrifugation over Ficoll gradients (Histopaque-1077; Sigma-Aldrich, Gillingham, United Kingdom). Heparinised blood diluted 1:1 with RPMI-1640 media (Invitrogen, Paisley, United Kingdom) was layered on 30% Ficoll-Paque Plus (GE Healthcare, Buckinghamshire, United Kingdom) density gradient and centrifuged for 25 minutes at 2200 rpm at room temperature. The PBMC layer was collected, washed and re-suspended in RPMI-1640. The cell viability was greater than 97%, as determined using trypan blue exclusion. Dendritic cells (DCs) and naive T cells were enriched from PBMCs obtained from grass pollen allergic subjects using cell enrichment kits (StemCell Technologies, Cambridge, United Kingdom).

Phenotyping of ILC2s

PBMCs ($1x10^6$ cells) isolated from SAR and NAC participants were stimulated for 4 hours with PMA (50 ng/mL) and Ionomycin (500 ng/mL; both from Sigma-Aldrich), in the presence of Brefeldin A (BD, Oxford, United Kingdom) for the final 3 hours of culture. Cells were surface immunostained with lineage markers-APC, CD127-PerCP-Cy5.5, CRTH2 V450 and anti-FcεRI-APC. ILC2s were defined as $CD127^+CRTH2^+Lin^-Fc\varepsilon RI^-$ population. Intracellular staining with IL-5 PE (BD) and IL-13 FITC (eBioscience, Hatfield, United Kingdom) were performed following fixation and permeabilization of cells. Cells were analysed using the BD FACSCanto II instrument (BD).

Effect of IL-35 on ILC2s

Lineage-negative cells comprising of ILC2s were enriched from PBMCs using lineage cell depletion kit (Miltenyi Biotec, Surrey, United Kingdom). Cells were immunostained for Lineage markers (CD3/CD14/CD16/ CD19/CD20/CD56) (Biolegend, London, United Kingdom), CD127 (eBioscience, Hatfield, United Kingdom) and CRTH2 (Miltenyi Biotec) to allow FACS sorting of ILC2s ($Lin^-CD127^+CRTH2^+$) using BD FACSAria III (Becton Dickinson, Oxford, United Kingdom). FACS sorted ILC2s were then maintained in media containing rhIL-2 (10 ng/mL; Peprotech, London, United Kingdom) and rhIL-7 (40 ng/mL; R&D Systems, Abingdon, United Kingdom). The effect of IL-35 on sorted ILC2s were studied over 72 hours when stimulated with rhIL-25 (50 ng/mL) or rhIL-33 (50 ng/mL; both from R&D Systems). Supernatants were then collected for the assessment of IL-5 and IL-13 production.

Effect of IL-35 on *in vitro* generated T_H1 and T_H2 clones

Naive T ($CD4^+CD45RA^+$) cells were polarized towards T_H1 and T_H2 clones according to previously described protocol.[E1] T_H1 clones were defined as those expressing high IFN-γ and low IL-4, IL-17 and IL-22, while T_H2 clones were defined as those expressing high IL-4 and low IFN-γ, IL-17, IL-22. The effect of IL-35 on CD3/CD28-mediated proliferation of *in vitro* generated T_H1 or T_H2 clones was measured. mRNA expression (IL-5, IL-13, IFN-γ GATA-3 and Tbet) and IL-5, IL-13 and IFN-γ levels were quantified.

mRNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Manchester, United Kingdom) according to the manufacturer's protocol. RNA was quantitated spectrophotometrically, and cDNA was synthesised using RevertAid First Strand cDNA Synthesis Kit (Fermentas, United Kingdom). The cDNA samples were subjected to 40 cycles of amplification on Applied Biosystems 7900HT Real-Time PCR System in an ABI PRISM 384-Well Clear Optical Reaction Plate using SYBR Green PCR master mix (Applied Biosystems, Foster City, Calif) (primer sequences listed in Table E1) as described previously.[E2] Data was analysed on SDS 2.3 Software (Applied Biosystems). Data was normalised to housekeeping gene *GAPDH* and represented as fold change, as quantified using the $2^{-(\triangle\triangle Ct)}$ method.

Intracellular staining, flow cytometry

Isolated naive $CD4^+$ T cells were cultured with IL-35 protein (Enzo Life Sciences, Farmingdale, NY) for either 18h or 9d on a CD3/CD28-coated plate. Cells were stimulated for 1 hour with PMA (20 ng/mL; Invitrogen, Carlsbad, Calif) and ionomycin (1 μg/mL; Sigma-Aldrich), followed by 4 hours with Brefeldin A (BD). Cell surface molecules were stained with a combination of fluorescent-labelled antibodies: CD4 APC (RPA-T4; Fisher Scientific, Loughborough, United Kingdom), CD25 APC/Cy7 (M-A251; BD Biosciences). Cells were then subjected to intracellular staining according to the manufacturer's protocol. The following fluorescent-conjugated antibodies were used for intracellular staining: CD278 PE (DX29; BD), IL-10 PE-Cy7 (JES3-907; BD), IL12p35 PerCP (27537; Fisher Scientific), EBI3 Alexa Fluor 488 (607201; R&D Systems), and Foxp3 V450 (236A/E7; BD). Cells were analysed using the BD FACSCanto II instrument (Becton Dickinson, Oxford, United Kingdom).

Multiplex assay

The secretion of 42 cytokines and chemokines following the induction of iT_R35 and 7d co-culture with or without neutralising antibodies were measured by MagPix Milliplex kit (EMD Millipore, Watford, United Kingdom) based on manufacturer's protocol.

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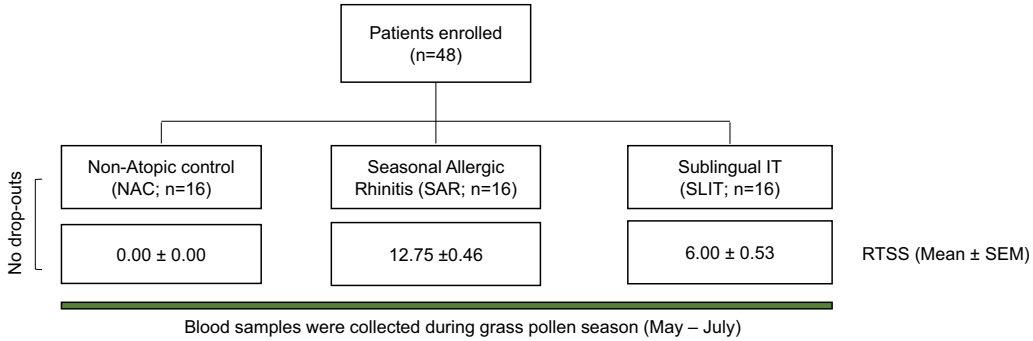

FIG E1. Patient recruitment flow chart. Sixteen NACs, 16 patients with SAR, and 16 SLIT-treated patients were recruited for this study. Blood samples were collected during the grass pollen season (May-July). Averages of RTSSs and T-cell responses are presented as means ± SEMs.

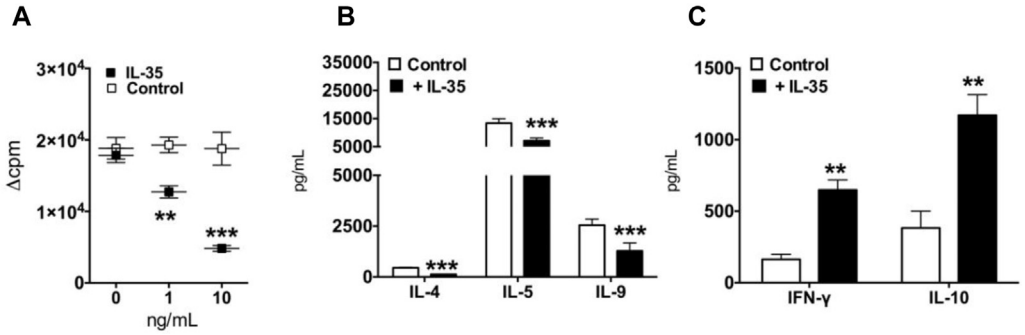

FIG E2. Recombinant IL-35 but not the C-peptide linker used for its expression (control) suppresses grass pollen allergen–driven T_H2 effector cell proliferation and cytokine responses. **A,** $CD4^+CD25^-$ Teff cells were cocultured with irradiated APCs and stimulated with *P pratense* allergen in the presence of IL-35 or control. Proliferation was assessed. **B** and **C,** T_H2 (Fig E2, *B*) and T_H1 and IL-10 (Fig E2, *C*). *P* values were determined by using the Mann-Whitney *U* test: $*P < .05$, $**P < .001$, and $***P < .0001$.

TABLE E1. Forward and reverse primer sequences for quantitative PCR analysis

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
IL5	AGCTGCCTACGTGTATGCCA	GCAGTGCCAAGGTCTCTTTCA
IL13	GATTCCAGGGCTGCACAGTA	GGTCAACATCACCCAGAACC
GAPDH	CTTCGCTCTCTGCTCCTCCT	GCTGTGATCTTGAGGCTGTTG
GATA3	GCGGGCTCTATCACAAAATGA	GCTCTCCTGGCTGCAGACAGC
IFNG	TCTCGGAAACGATGAAATATACAAGTT	GTAACAGCCAAGAGAACCCAAAA
T-bet	GATGCGCCAGGAAGTTTC	GCACAATCATCTGGGTCACATT